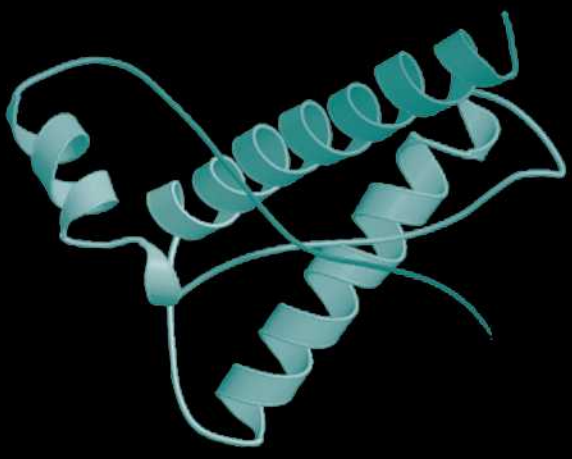
Prions and Mad Cow Disease



edited by
Brian K. Nunnally
Ira S. Krull

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Foreword

In the late 1980s, a short communication in the *Veterinary Record* (1) described a new neurological disease of cattle that was spreading across the United Kingdom. The disease was recognized as being similar to the long-endemic and relatively innocuous sheep disease scrapie, and was given the scientific moniker bovine spongiform encephalopathy, for which scientists wisely substituted the acronym BSE. As news of the disease spread beyond scientific circles, the British press gave it a different name that immediately caught the imagination of the public: mad cow disease. Over the next 15 years, headline after headline proclaimed the dangers of mad cow disease, as almost 1 million cattle contracted BSE. The BSE epidemic peaked in the United Kingdom by 1992, and its subsequent decline there demonstrated the effectiveness of appropriate intervention measures. Despite the dramatic reduction in BSE incidence, however, it continues to alarm many agricultural, medical, and regulatory professionals and the periodic appearance of sensational headlines disquiets the general public.

Is this level of concern warranted? As an agricultural problem, BSE seems less problematic than foot and mouth disease.* It is not a rapid, fulminant infection and it is not readily contagious within herds. As a zoonosis, BSE has caused far less human mortality than many more common viral infections. BSE has been transmitted to humans, probably through contaminated processed meat products, but this appears to be a relatively rare event.

*The recent foot and mouth disease outbreak in Britain induced the destruction of approximately four million cattle, about four times as many as were infected with BSE during the entire epidemic.

There is no evidence of transmission by an insect vector (unlike West Nile virus) and, unlike influenza, the disease is apparently not contagious within the human population. So, why is BSE (or mad cow disease) so frightening?

The answer to that question appears to lie in certain sensational characteristics of such diseases and the agents that cause them. BSE and all known spongiform encephalopathies are invariably fatal in both animals and humans. The human form of BSE, known as variant Creutzfeldt-Jakob disease (vCJD), is an insidious killer of young and otherwise healthy individuals. The life histories of the victims show no apparent pattern, other than residence in a BSE-affected country, and thus there is no obvious way to identify others who might contract the disease. The unrelenting clinical course is equally disturbing, as the evolving dementia tragically robs the victims of their most human qualities.

The agents, called prions, that cause spongiform encephalopathies are strange almost beyond belief. While we have learned much about prions and how they reproduce, almost as much remains unknown. The leading theory holds that prions are the misfolded variant of a common, normal host protein. Replication, it seems, occurs when the infectious prion protein binds to the normal cellular prion protein and induces a change in its conformation. This model for replication brings with it a realization that each of us carries within our cells the essential ingredient for producing these terrible infectious agents and disease. Prions are difficult to detect and destroy, justifying concern that biological products could harbor these silent killers. Long incubation times disconnect the source of infection from the appearance of clinical disease, which further frustrates scientists and clinicians, as well as patients and their families.

A useful analogy, drawn from everyday experience, may help explain fear of BSE infection. It is common knowledge that airline travel is the safest mode of transportation. Statistics tell us that fewer deaths or injuries result per passenger mile flown than from any other means of transport. Yet many people fear flying. Why? People recognize that although airliners rarely crash, when they do crash, many people die. Thus, the anxiety produced by the probable outcome (i.e., dying) of the unlikely event (an airline accident) outweighs the assurance provided by the statistical improbability of its ever happening. With BSE/vCJD, the certain horrible death far outweighs the unknown but apparently small chance of becoming infected.

The enormity of the BSE epidemic must not be minimized, however. The direct economic losses in countries affected by BSE, especially the United Kingdom, have been substantial. Contrast that with the history of sheep scrapie, where the disease and associated economic losses have been tolerated for almost 300 years. Scrapie was known to spread naturally within flocks but prior to the BSE outbreak it had never spread to other farm animals (as far as

was known) and there was no evidence of transmission to humans. Thus, the restricted host range and long incubation times limited the economic losses from scrapie primarily to the death of valuable breeding stock. The perceived risk to humans was negligible. The historical experience with scrapie proved to be misleading with respect to BSE, creating a false assurance that probably slowed official reaction to the developing BSE epidemic.

Indeed, BSE proved to be quite a different beast. Once sheep scrapie was transmitted to cattle, the new agent (i.e., BSE prions) showed an increased capacity to infect other species. Soon after BSE was discovered in cattle, various zoo animals and domestic cats were diagnosed with a similar brain disease, bringing the realization that the species barrier to infection that was thought to protect humans from scrapie might not provide that same protection from mad cow disease. That suspicion was confirmed in 1994 when vCJD, a new variant form of the rare human prion disease Creutzfeldt-Jakob disease, appeared (2). There is little doubt now that vCJD is caused by infection with BSE prions. The appearance of vCJD raised the stakes from the tolerable inconvenience presented by scrapie to the possibility of unknown numbers of human deaths from BSE. One naturally hopes that the regulations eventually put in place in the United Kingdom will limit the human losses caused by this new disease.

BSE has now been identified in a host of countries and the list is growing. In each case, a similar pattern has occurred following its identification. Almost immediately, domestic beef consumption dropped by about 50%, then slowly recovered to nearly normal levels over the following months. Beef exports also dropped as other countries banned or limited importation of beef, beef products such as meat and bone meal (MBM),* and live cattle. In some cases, the import regulations have been extended to include other ruminant animals and products derived from them. The shift in both the supply and the demand for beef pushes prices lower, thus inflicting additional economic damage to the local producers.

Similar economic dynamics undoubtedly contributed to the spread of BSE from the United Kingdom to Europe and Asia early in the epidemic. As the price of beef and beef products dropped in the United Kingdom, new foreign markets were sought. In particular, the price of MBM dropped because its use as a protein supplement for ruminants was restricted in the United Kingdom. Its export was not immediately banned, however, and substantial quantities of MBM were exported. This MBM was repackaged, relabeled, and exported worldwide, in many cases to unsuspecting consumers.

* MBM is a product of rendered carcasses and is used as a protein supplement in animal feed. It is the most likely source of infectious prions that initially transmitted scrapie to cattle and expanded the BSE epidemic by recycling BSE prions to cattle, sheep, and other species.

As low-cost MBM, some of which was contaminated with BSE prions, entered the international markets, many countries lacked import restrictions to keep it out. Once imported, contaminated MBM infected local cattle, many of which would go to market undiagnosed. Recycling those carcasses allowed BSE to spread within the new host country. Surveillance programs, if present, usually were insufficient to detect the incipient epidemics. In a way, BSE spread worldwide because the potential magnitude of the problem was not recognized until it was too late.

The world is now well aware of the dangers of BSE, but other prion diseases still lurk in the shadows. In the United States, chronic wasting disease (CWD) is spreading in both wild and ranch-raised deer and elk populations. The pattern of spread indicates that this disease is naturally contagious in those species. CWD was first identified in 1967 and its origin is unknown (3).* The risk of CWD spreading to domestic sheep and cattle or to humans is also unknown. Other species may harbor natural prion diseases that have not yet been recognized. All mammals are at least theoretically susceptible to prion infection since the prion protein gene is expressed in all mammalian species that have been examined. Existing prion strains (such as scrapie, BSE, CWD, CJD, etc.) might infect new host species, or new prion strains could arise spontaneously.† Under certain circumstances, which we can neither predict nor prevent, a single spontaneous case of prion disease could lead to a new epidemic and a new endemic disease.

Another danger to human health lies in the potential for iatrogenic transmission of vCJD. The current size of the vCJD epidemic is unknown, but epidemiological studies indicate a range of a few hundred cases to many thousands. During the long, silent incubation period, those who are infected will not show any outward signs. They will continue their normal daily activities, which will on occasion bring them into contact with the healthcare system. Some people will require surgery, have a tooth extracted, or undergo root canal work, and others will donate blood or organs. Each of these procedures will carry some risk for transmitting vCJD to others. Since current diagnostic techniques are incapable of identifying the infected individuals, the theoretical chance of transmitting vCJD through various medical procedures must be balanced against the benefits of those procedures to society and the disadvantages inherent in any precautions that might be taken to reduce that risk. Clearly, the risk can never be reduced to zero.

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* It is possible that CWD resulted from a cross-species transmission of sheep scrapie or that it arose spontaneously within deer or elk.

† If sporadic CJD arises in humans due to rare misfolding of the prion protein, then prion diseases might also spontaneously appear in other species.

Better diagnostic tests would certainly reduce those risks, but prion diseases present a unique diagnostic challenge. There is no significant host immune response to the infecting agent—an understandable situation, given that the prion hypothesis predicts that it is simply a misfolded host protein. Any new conformational epitopes that may be created in the misfolded infectious prions are apparently not sufficient to induce an effective host immune response. Thus, the host immune response offers no help in diagnosing the disease or in fighting it. Diagnosis is further confounded by the relatively limited distribution of prions and pathology. Prion concentrations are highest in the brain and spinal cord, with generally only low concentrations found in tissues outside the central nervous system, including blood, urine, and feces. Although peripheral distribution of prions varies in different prion diseases, pathology is most evident in the central nervous system, which limits the practicality of tissue biopsies as a routine diagnostic procedure. The diagnosis of prion disease in a patient (or animal) is usually presumptive and confirmed only upon postmortem examination using histopathology, immunohistopathology, or Western immunoblotting techniques.

Prions themselves offer a better, but still difficult, diagnostic target. The prion protein is the only known macromolecule that is specifically associated with the infectious particle. Prion protein can be detected with highly specific antibodies but diagnostic tests must distinguish it from its cellular form. The two forms of prion protein can be differentiated by various chemical and physical properties; these techniques complicate the diagnostic procedure. Other molecular targets have been suggested, but none is as specific to these diseases as the prion protein. Prions appear not to contain a specific nucleic acid, so PCR and similar techniques to detect these molecules are not applicable.

There is an immediate need for good prion diagnostic tests to serve four basic purposes: (1) screening food animals at slaughter, (2) screening live animals, (3) screening biological products or ingredients, and (4) detecting infection in humans at an early stage. Rapid postmortem tests that use specific antibodies to detect the misfolded prion protein are currently being used in some countries to screen food animals at the slaughterhouse. That application obviously reduces the possibility for transmission to humans and also helps to monitor the incidence of disease. A simple antemortem test for animal prion diseases would extend our current capability and permit routine disease monitoring in cattle and sheep, as well as other captive ruminants. Ideally, an antemortem test should detect prion infection prior to onset of clinical disease. Such a test would permit strategic monitoring of wild animal populations as well as domesticated animals, thus providing a scientific basis for epidemic control and eradication. Highly sensitive tests are needed to screen biological products or ingredients, since the amount of the product available for testing is relatively small compared to the product batch size. Probably the

most difficult challenge ahead is the preclinical diagnosis of human prion disease. To be of practical use, the test must be minimally invasive (samples from blood, urine, throat swab, etc.), highly sensitive, and very specific. Ideally, the test should be capable of detecting infection far in advance of clinical disease to maximize the effectiveness of any therapeutic intervention that may eventually be developed.

The importance of developing improved prion diagnostic tests is reflected in the contents of this book, which focuses on the current status of prion diseases and their diagnosis. Chapters 1–5 review aspects of prion diseases to put the diagnostic issues in proper perspective, and Chapters 6–14 describe a variety of approaches to detecting prions and prion protein. These contributions describe the state of the art as well as promising avenues for new investigations. This field is rapidly evolving and the ultimate answer to the problem of prion diagnosis may not be within these pages. While our primary goal in publishing these reports is to convey current information, we also hope that this book will inspire others to see beyond the ideas presented here—perhaps to view the problem from an entirely new perspective. For it is through revolutionary vision that science advances.

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David C. Bolton, Ph.D.
Department of Molecular Biology
New York State Institute for Basic Research
in Developmental Disabilities
Staten Island, New York, U.S.A.

REFERENCES

1. Wells GAH, Scott AC, Johnson CT, Gunning RF, Hancock RD, Jeffrey M, Dawson M, Bradley R. A novel progressive spongiform encephalopathy in cattle. Vet Rec 1987; 121:419–420.
2. Will RG, Ironside JW, Zeidler M, Cousens SN, Estibeiro K, Alperovitch A, Poser S, Pocchiari M, Hofman A, Smith PG. A new variant of Creutzfeldt-Jakob disease in the UK [see comments]. Lancet 1996; 347: 921–925.
3. Williams ES, Young S. Chronic wasting disease of captive mule deer: a spongiform encephalopathy. J Wildlife Dis 1980; 16(1):89–98.

Preface

The discovery in May 2003 of a cow testing positive for bovine spongiform encephalopathy (BSE), or mad cow disease, in Canada has brought the issue of mad cow to worldwide attention yet again. Mad cow has become a *cause célèbre* in the United Kingdom and Europe since the mid- to late-1980s and early 1990s. Certainly, every country that has begun routine monitoring has found some levels of mad cow. There does not, at the moment, appear to be a way to stop the continued spread of this disease, other than by destroying infected cattle. Human consumption of beef derived from cows with mad cow appears to be a potential cause of Creutzfeldt-Jakob disease (CJD), a very slow-progressing disease that eventually leads to dementia, loss of mental powers and basic bodily functions, and, without exception, death. Mad cow or CJD takes a long time to express itself and there are few validated analytical methods to test for the presence of the disease in animals or humans. The diagnosis can be confirmed only by a postmortem test: Analysis of the brain matter would then indicate the sponginess associated with mad cow. Analytical approaches have evolved recently, almost all involving immunoassays, which can be used to detect the prion protein in a variety of matrices. This work focuses on a variety of analytical techniques, either currently employed or being developed for routine, validated use in the future, designed to detect mad cow and other transmissible spongiform encephalopathies (e.g., chronic wasting disease (CWD) or scrapie) postmortem or, more usefully, while the animal is still alive. The ideal test would be a rapid, preclinical test from a matrix that could be easily collected (e.g., feces, urine, or mucus).

While we are clearly not yet there, the contributors to *Prions and Mad Cow Disease* are at the forefront of the current analytical chemistry of mad

cow disease. After the introductory chapters (Chapters 1 and 2), the Harvard Risk Assessment, which estimates the statistical likelihood of a mad cow pandemic in the United States, is presented in Chapter 3. This is followed by a discussion of the measures used to prevent the spread of BSE (Chapter 4) and an overview of CWD in the United States (Chapter 5). Chapters 6 through 13 discuss some of the major analytical techniques being used for the analysis of BSE samples from a variety of matrices, including bioassay, cyclic amplification of prions, immunoassay, differential extraction and DELFIA, in vivo diagnosis, multispectral techniques, and capillary electrophoresis. The final chapter, Chapter 14, presents a very comprehensive review of pharmaceutical prion removal.

This book is devoted to a coverage of the analytical aspects of prions, rather than to the biology and medical aspects. It grew from the symposium "It's a Mad, Mad, Mad, Mad Cow: Detection and Quantification of Prions" for the Pittsburgh Conference (PittCon) in New Orleans, Louisiana, March 2001, organized by Brian Nunnally. We have tried to acquire contributions from all of the originally invited lecturers, as well as from others who have published in the areas of prion analysis.

Quite naturally, we thank all the contributors and acknowledge that any errors or misunderstandings that remain are the responsibility of the editors and not the contributors. We especially thank Russell Dekker, who showed a serious interest in this project from the first discussion. We hope our readers enjoy this book, and hope that it stimulates their own analytical thinking about improved methods for the analysis of prions of all types.

Brian K. Nunnally
Ira S. Krull

Contents

Contributors

Andrew Alpert PolyLC, Inc., Columbia, Maryland, U.S.A.

Emmanuel A. Asante MRC Prion Unit, Department of Neurodegenerative Disease, Institute of Neurology, University College London, National Hospital for Neurology and Neurosurgery, London, England

Geoff Barnard Centre for Veterinary Science, Department of Clinical Veterinary Medicine, University of Cambridge, Cambridge, England

David C. Bolton, Ph.D. Department of Molecular Biology, New York State Institute for Basic Research in Developmental Disabilities, Staten Island, New York, U.S.A.

Richard I. Carp, V.M.D., Ph.D. Department of Virology, New York State Institute for Basic Research in Developmental Disabilities, Staten Island, New York, U.S.A.

Joshua T. Cohen, Ph.D. Harvard Center for Risk Analysis, Harvard School of Public Health, Boston, Massachusetts, U.S.A.

John Collinge, M.D., F.R.C.P., FRC.Path., F.Med.Sci. MRC Prion Unit, Department of Neurodegenerative Disease, Institute of Neurology, University College London, National Hospital for Neurology and Neurosurgery, London, England

Emmanuel E. Comoy, D.V.M., Ph.D. Medical Research Department, Atomic Energy Commission, Fontenay-aux-Roses, France

Jean-Philipe Deslys Medical Research Department, Atomic Energy Commission, Fontenay-aux-Roses, France

Linda A. Detwiler, D.V.M. Animal and Plant Health Inspection Service, U.S. Department of Agriculture, Robbinsville, New Jersey, U.S.A.

Keith Duggar Harvard Center for Risk Analysis, Harvard School of Public Health, Boston, Massachusetts, U.S.A.

Ruth Gabizon, Ph.D. Department of Neurology, The Agnes Ginges Center for Human Neurogenetics, Hadassah University Hospital, Jerusalem, Israel

Jacques Grassi Medical Research Department, Atomic Energy Commission, Fontenay-aux-Roses, France

George M. Gray, Ph.D. Harvard Center for Risk Analysis, Harvard School of Public Health, Boston, Massachusetts, U.S.A.

Perry C. Gray Los Alamos National Laboratory, Los Alamos, New Mexico, U.S.A.

Hatim Gubara Center for Computational Epidemiology, School of Veterinary Medicine, Tuskegee University, Tuskegee, Alabama, U.S.A.

Tsegaye HabteMariam Center for Computational Epidemiology, School of Veterinary Medicine, Tuskegee University, Tuskegee, Alabama, U.S.A.

Andrew F. Hill MRC Prion Unit, Department of Neurodegenerative Disease, Institute of Neurology, University College London, National Hospital for Neurology and Neurosurgery, London, England

Sam D. Holland, D.V.M. South Dakota Animal Industry Board, Pierre, South Dakota, U.S.A.

Richard J. Kascsak, Ph.D. New York State Institute for Basic Research in Developmental Disabilities, Staten Island, New York, U.S.A.

Jae-Il Kim New York State Institute for Basic Research in Developmental Disabilities, Staten Island, New York, U.S.A.

Silvia Kreindel Harvard Center for Risk Analysis, Harvard School of Public Health, Boston, Massachusetts, U.S.A.

Douglas C. Lee, Ph.D. Department of Preclinical Research and Pathogen Safety, Biological Products Division, Bayer Corporation, Research Triangle Park, North Carolina, U.S.A.

Jeanette L. C. Miller, Ph.D.* Department of Pathogen Safety and Research, Biological Products Division, Bayer Corporation, Research Triangle Park, North Carolina, U.S.A.

David Oryang Center for Computational Epidemiology, School of Veterinary Medicine, Tuskegee University, Tuskegee, Alabama, U.S.A.

Stephen R. Petteway, Jr., Ph.D. Department of Preclinical Research and Pathogen Safety, Biological Products Division, Bayer Corporation, Research Triangle Park, North Carolina, U.S.A.

Burt Pritchett, D.V.M., M.S. Center for Veterinary Medicine, Food and Drug Administration, U.S. Department of Health and Human Services, Rockville, Maryland, U.S.A.

Richard Rubenstein, Ph.D. Molecular and Biochemical Neurovirology, Department of Virology, New York State Institute for Basic Research in Developmental Disabilities, Staten Island, New York, U.S.A.

Riona Sayers, B.Sc., M.An.Sc., P.M.P. Operations, Enfer Scientific, Newbridge, Ireland

Mary Jo Schmerr Virus and Prion Diseases of Livestock Research Unit, National Animal Disease Center, Agricultural Research Service, U.S. Department of Agriculture, Ames, Iowa, U.S.A.

Gideon M. Shaked, D.V.M., M.S. Department of Neurology, The Agnes Ginges Center for Human Neurogenetics, Hadassah University Hospital, Jerusalem, Israel

* *Current affiliation*: Research and Product Development, Meridian Bioscience Inc., Cincinnati, Ohio, U.S.A.

Claudio Soto Serono International, S.A., Geneva, Switzerland

Christopher J. Stenland, Ph.D. TSE/NAT Research, Biological Products Division, Bayer Corporation, Research Triangle Park, North Carolina, U.S.A.

Man-Sun Sy Case Western Reserve University School of Medicine, Cleveland, Ohio, U.S.A.

Berhanu Tameru Center for Computational Epidemiology, School of Veterinary Medicine, Tuskegee University, Tuskegee, Alabama, U.S.A.

Gary C. Tisone[†] TW Research Associates, Albuquerque, New Mexico, U.S.A.

[†]Deceased.

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Taking Aim at the Transmissible Spongiform Encephalopathies Infectious Agents

Richard I. Carp and Richard J. Kascsak
New York State Institute for Basic Research in Developmental Disabilities, Staten Island, New York, U.S.A.

I. INTRODUCTION

The major focus of this book is to detail the various approaches that are being developed to rapidly and accurately diagnose the transmissible spongiform encephalopathies (TSE), also known as prion diseases. This aim has acquired increased urgency because of the emergence first of bovine spongiform encephalopathy (BSE), and then of the related human disease, referred to as new-variant Creutzfeldt-Jakob disease (nvCJD) (1,2). Evidence obtained from several laboratories, using different approaches, established that nvCJD was caused by the ingestion of BSE-contaminated material; this fact emphasizes the importance of being able to assess the BSE status of cattle prior to entrance into the food chain or processing industry (3,4). The extensive use of both human and bovine material for either therapy or prophylaxis of human diseases points to the need for rapid diagnosis of both BSE and CJD. A partial list of products and procedures used in humans that are based on obtaining material from cows or humans includes factor VIII, various heterologous transplants from humans, such as kidney and bone marrow, hyperimmune human IgG preparations, therapeutic enzymes, gonadotropin, surfactants, collagen, etc.

The aim of this introductory chapter is to set the stage for the presentations on diagnosis of TSEs that will form the focal point of this

book. In this chapter, there will be a brief history of the research on TSEs, followed by an examination of the abnormal protein, termed the prion protein (PrP^{Sc}), with specific focus on evaluating its potential as a diagnostic marker. This will be followed by a discussion of a potential misconception concerning PrP^{Sc} preparations that often surreptitiously enters the thinking of those who observe this field of study, and even infects those who participate in such studies. Finally, we will briefly set the stage for the diagnostic aims and approaches to be described elsewhere in this book.

II. HISTORY OF RESEARCH

A. Early Studies

The description of the behavioral and physical changes that literate farmers of the 1700s in Great Britain ascribed to some of their sick sheep matches the modern description of clinically affected scrapie-positive sheep (5). Thus, it is safe to say that scrapie has existed for at least 250 years and, in fact, almost surely predates the rise in literacy in Western Europe. The disease remained a mysterious problem for sheep farmers, and their major aim for the succeeding centuries was to avoid and/or eliminate the disease from their flocks. The first break in the mystery was the demonstration by Cuillé and Chelli (6) that sheep injected with material from affected animals became sick with disease. In the subsequent two decades, efforts of D.R. Wilson and others established many of the unusual physical and chemical characteristics that we have come to associate with scrapie and scrapie-type agents (7). This effort, particularly that of D.R. Wilson, was prodigious because not only are the animals large and relatively expensive to maintain, but also the proportion of animals that developed disease, even after injection of high concentrations of brain material, was variable and only rarely reached 100%. An examination of the genetic component of susceptibility was also initiated during this period by J. Stanp, A.G. Dickinson, and others (8,9).

B. The 1950s

The 1950s saw two important conceptual contributions. In the first, B. Sigurdsson put forth the concept of slow infections (10). His studies of scrapie and visna in the sheep population of Iceland prompted him to suggest that these are examples of a class of infectious diseases in which there is a long preclinical phase followed invariably by severe clinical manifestations of disease and then death. It is interesting that these two

diseases, scrapie and visna, are the archetypes of two devastating groups of diseases that are now a major concern to health professionals: the transmissible spongiform encephalopathies and AIDS, respectively. The other key observation of the 1950s was the suggestion that the pathological similarity between scrapie and an obscure disease that was devastating the Fore tribe in New Guinea, called kuru, might mean that an etiological relationship exists. W. Hadlow argued that kuru should be viewed as a slow infection similar to scrapie and that attempts to isolate an infectious agent should involve an extended period of observation (11). To fully appreciate the significance of Hadlow's contribution, it is worthwhile quoting the final sentence of his 1959 *Lancet* paper: "Thus, it might be profitable, in view of veterinary experience with scrapie, to examine the possibility of the experimental induction of kuru in a laboratory primate, for one might surmise that the pathogenetic mechanisms involved in scrapie—however unusual they may be—are unlikely to be unique in the province of animal pathology." This spiked the interest of the medical profession in the scrapie field and led to suggestions that the pathology of CJD might warrant its inclusion with scrapie and kuru (12). With this as background, D.C. Gajdusek, C.J. Gibbs Jr. and their colleagues injected primates with brain material from kuru and CJD and maintained these animals well beyond the time routinely used for assaying agents that caused acute infections (13,14). Positive findings in these studies launched the human and animal diseases along similar tracks with aims of defining the nature of the agents and understanding their interactions with, and effects on, their hosts. These findings also emphasized the necessity for diagnostic approaches to prevent additional transmissions.

C. The 1960s and 1970s

1. Assay in Animals

A major advance in the effort to take aim at the animal portion of these studies, specifically with the scrapie agent, was made in 1961: R.L. Chandler showed that infected sheep material could cause disease in the mouse (15). This provided for the use of an inexpensive, rapidly reproducing animal model that was amenable to precise assays of infectivity and to thorough assessment of the role of genetics of agent and host in disease pathogenesis. This led to a series of important advances over the next two decades on the role of genetics of host and agent in the progression of disease, in analysis of the pathogenesis of the disease in various scrapie strain-host (primarily mouse and hamster) interactions, and in attempts to purify and characterize the infectious agent.

2. Strains of Agent

In an early observation of the natural disease, a difference in clinical manifestations was observed in scrapie-positive goats; the salient clinical features of these two forms could be summed up by the words "scratchy" and "drowsy" (16). Over a number of years, A. Dickinson and his colleagues in Edinburgh, using well-characterized inbred mouse strains, carefully and clearly established the existence of a number of mouse-adapted scrapie strains that had distinctive characteristics with regard to incubation period, lesion profile of vacuoles in the brain, the frequency of amyloid plaques in the brain, the development of obesity, and a number of other parameters that were a function of the interaction of agent with host (17–20). These studies also demonstrated that strains could differ in the rate at which they were inactivated by heat (21). The presence of strains, previously questioned by some, is now accepted by all and is a phenomenon that must be taken into account when taking aim at the TSE infectious agents.

3. Pathogenesis

During this time period, important advances in understanding the pathogenesis of scrapie and related diseases were made by detailed analysis of the interaction between infectious agents and hosts. Key findings were made by C.M. Eklund and colleagues (22), R.H. Kimberlin and colleagues (23–25), H. Fraser, A.G. Dickinson (25,26), and their associates. The relationship between route of infection and the pathogenesis of the disease process was explored (22–26). In other studies, it was established that following peripheral [non-central nervous system (CNS)] injection, the infectious agent accumulates first in lymphoid organs (22–24,27,28). In fact, either surgical removal or genetic absence of the spleen led to a lengthening of incubation period compared to mice with an intact spleen (29). Further studies showed that following intraperitoneal injection, increased titer was found first in the spleen and later in the thoracic cord at the point of entrance of the splanchnic nerve into the spinal cord (23,24). Infectivity then spread anteriorly and posteriorly along the spinal cord, eventually reaching the posterior portion of the brain and finally the anterior brain areas. The types of cells of the lymphoreticular system that are involved were addressed by showing that cells with a low rate of replication were important (26,27). These early studies established the framework for more recent studies that have established roles for various cell types in replication and transport of agent within the periphery and the CNS. These mechanisms will be discussed later in this chapter and explored in detail in other chapters of this book.

4. "Clinical Target Areas" and "Species Barrier"

The concepts of "clinical target areas" and "species barrier" were also established during this period (25,30). The former can be viewed as the concept that the induction of damage in specific brain areas can more readily lead to clinical disease than effects in other areas and, as a corollary, that replication of agent in the latter areas might be irrelevant to the progression of disease. The additional concept of species barrier is based on the finding that there is an impediment to the replication of infectious agent during passage from one species to another (31). The barrier is made manifest by the long incubation period on first passage from species A to species B, with a shortening of incubation on second passage of the specific agent in species B. Also, the barrier phenomenon is often exemplified by the fact that a dose that causes disease in 100% of species A may affect only a limited proportion of animals on initial passage in species B, and on a different time scale.

D. The 1980s and 1990s

1. Fibrillary Structures (SAF)

The early 1980s yielded two developments that focused our attempts at understanding these mysterious agents. In the first, fibrillary structures were described in partially purified preparations of scrapie and CJD by P.A. Merz and her colleagues (32,33). These structures, termed scrapie associated fibrils (SAF), were in all scrapie and CJD samples with high levels of infectivity, increased in number in parallel with increases in the number of infectious units, and were viewed as a marker of the disease or even the physical manifestation of the infectious agent (32–34). Treatments that mimicked the histopathological changes seen in scrapie-type diseases failed to cause the development of SAF. In subsequent years, SAF have been established as a marker of TSE disease. In a sense, the identification of SAF represented the first "test tube" or in vitro assay for TSE diseases. Unknowingly, this finding would also merge with the next major discovery in this field.

2. Protease-Resistance Protein (PrP^{Sc}, PrP^{res})

The next finding involved a protein that copurified with infectivity and that resisted protease digestion, a protein first identified in the brains of scrapie-infected animals (35,36). Extensive examination and scrutiny of this protein, termed PrP, has established its importance and diagnostic value for this group of diseases (37,38). Studies conducted by many laboratories firmly

establish PrP^{Sc} (protease-resistant PrP) as a key marker in what has developed into a range of immunodiagnostic assays. It is now known that SAF are composed of the polymerized form of PrP^{Sc}, one of a number of PrP^{Sc} forms (see later discussion). The scrapie form of this protein has been purported to be the sole macromolecule of the infectious entity. This protein forms the basis of the prion hypothesis, which will be discussed in detail in Chapter 2, by Dr. D. Bolton.

3. Physical-Chemical Studies of PrP

Studies on this "new, disease-specific" protein marker moved rapidly with initial sequencing leading to the demonstration that the genetic information for this protein was contained in a single open reading frame of a gene in the host genome (39). Subsequent studies established that the protein was expressed in normal brain as well as in scrapie brain, but that the physical-chemical properties of the normal and scrapie isoforms of the protein differed (Table 1). The normal protein has been termed PrP^{C} or PrP^{sen}, whereas the scrapie form is referred to as PrP^{Sc} or PrP^{res}. PrP^{C} is not sedimentable and is sensitive to protease digestion. In contrast, PrP^{Sc} is sedimentable and is relatively resistant to proteinases. The characteristic shift in the molecular size of the diglycosylated, monoglycosylated, and unglycosylated forms of PrP^{Sc} following protease digestion forms the basis for Western blot diagnosis (35,37,38,40–42) and for typing of different forms of human TSEs. The specific identification of PrP^{Sc} in a wide range of

Table 1 Comparison of PrP^{C} and PrP^{Sc}

Characteristic tested	PrP^{C}	PrP^{Sc}
Normal cells	+	–
Scrapie-infected cells	+	+
Protease sensitivity (lysates)	+	–
Protease sensitivity (intact cells)	+	–
PIPLC sensitivity (intact cells)	+	–
Biosynthesis ($t_{1/2}$)	<1 h	15 h
Degradation ($t_{1/2}$)	3–6 h	>24 h
Aggregation	–	+
Cell surface biotinylation	+	–
Cytoplasmic immunofluorescent labeling, different locations	+	+
Protease resistance	No	Yes
Conformational differences	Low amount of β sheet High amount of α helical	Increased β sheet Decreased α helical

immunoassays forms the basis for the immunodiagnosis of human and animal TSEs (43–45). As will be discussed in several chapters in this book, the ability of PrP^{Sc} to serve as a diagnostic marker relies on both the specificity and sensitivity of various parameters within such assays.

The levels of expression of PrP messenger RNA in normal and scrapie-infected animals are the same. The amino acid sequences of PrP^{C} and PrP^{Sc} are identical, and there are no differences in biochemical types of post-translation modifications (39,46,47). The differences in the two isomers appear to be based upon conformational differences in which PrP^{C} contains primarily α-helix regions and very little β-sheet structure, whereas the PrP^{Sc} molecule has extensive β-sheet structure and reduced α-helix areas (Table 1). Topographical, structural, and biophysical differences between these isoforms will be discussed in detail elsewhere in this book. Circular dichroism (CD) and Fourier transform infrared spectroscopy (FTIR) have confirmed the α-helical and random-coil-rich structure of PrP^{C} and its conversion into the β-sheet-enriched and highly aggregated conformation of PrP^{Sc} (48,49).

4. The Role of PrP

The role of PrP in the development of TSEs was firmly established by demonstrating that the barrier that reduces the susceptibility of a species to infectious preparations from another species could be eliminated by introducing a PrP^{C} transgene from the infecting species genome (50). The length of the incubation period was reduced relative to PrP transgene expression (51). The importance of PrP was further established in PrP null (knockout) mice. Animals that do not express PrP are no longer susceptible to TSE disease, i.e., fail to support agent replication (52), and are no longer tolerant with regard to their immune response to the homologous PrP immunogen (45). It has also been established that transgenic animals overexpressing PrP^{C} (8- to 10-fold above normal expression levels) have remarkably shorter incubation times following infection (50,53,54), the increased availability of PrP^{C} precursor apparently leading to a more rapid conversion to, and accumulation of, the pathogenic PrP^{Sc} isoform (53,54).

5. PrP and the Familial Forms of Disease

Additional studies established that alterations or mutations in the PrP protein are associated with the induction of neurological disease in humans: In diseases such as Gerstmann-Straussler-Scheinker syndrome (GSS), fatal familial insomnia (FFI), and other familial forms of CJD, mutations in the coding sequence for PrP^{C} lead to a malformed protein that accumulates in diseased brain tissue (55). The abnormal PrP found in brains of these patients had the general characteristics of PrP^{Sc}. Disease is transmissible to

animals or individuals that do not possess the PrP mutation. The concept that PrP^{Sc}, in and of itself, could cause the disease was supported by the production of transgenic mice, in which multiple copies of the mutant protein found in GSS were expressed; these mice developed neurodegeneration and clinical disease reminiscent of TSE in mice (54). The mutation in GSS is a change from proline to leucine, at position 101 in terms of the mouse genome. The finding that these mice contained infectious TSE agent has not been repeated. Furthermore, neither histopathological changes, nor clinical signs, nor infectious agent was found in transgenic mice containing a single copy of the mutated PrP protein in which the gene was incorporated in the mouse genome at the appropriate chromosomal location (homologous recombination) (56). Interestingly, the mice with the P101L GSS homologous recombination often show a different barrier height to infection with TSE agent from other species (species barrier) compared to mice with a nonmutated PrP genome (57). These studies provide insight into the distinction between the pathogenic role for PrP^{Sc} and the ability to transmit disease (see later discussion).

III. PrP^{Sc} AS A TARGET FOR DIAGNOSIS

A. Markers: PrP^{Sc} and Surrogates

The unique properties of PrP^{Sc} appeared to provide a facile target for diagnostic attack. Its characteristics allow it to remain after its normal counterpart and other normal proteins have been eliminated. Such characteristics have been well established, and multiple monoclonal and polyclonal antibodies have been raised against PrP (40,41,43,45). The aims of much of the work reported in this book have been to develop approaches that: (1) can detect PrP^{Sc} and readily distinguish it from the normal PrP^{C} isoform, (2) are highly sensitive, (3) can be performed using tissue or body fluids that are easily accessible antemortem, and (4) can be performed quickly and economically. The emphasis on PrP^{Sc} as a TSE diagnostic marker is certainly appropriate at the present time in that its absolute specificity to these diseases has been firmly established (40,41,45). Other changes, however, that occur during TSE disease have been lumped into a category referred to as surrogate markers and these hold promise as diagnostic tools (58–60). An example of one such marker is the dramatic decrease in expression of the erythroid-differentiation-related factor in spleen and bone marrow of TSE-positive animals (58). Another surrogate marker is the up-regulation of laminin receptor in TSE-infected material. Microarray technology may provide the sensitivity to examine a large number of such markers that may be up- or down-regulated during the TSE disease process

(59). While any one or two of such markers may well not be specific for these diseases, the signature pattern of hundreds of these markers may specifically define the group of TSE diseases.

B. Diagnostic Problems with PrP^{Sc}—Spectrum of Conformations

Certainly, PrP^{Sc} has proven to be an excellent diagnostic target, but many challenges remain. Ideally, such a test would rely on uniform characteristics and a consistent, clear-cut difference from any normal protein. This condition is exemplified in the cartoon shown in Fig. 1a, with the solid green representing constant characteristics, and the sharp-edged circle representing a clear-cut difference between PrP^{Sc} and PrP^{C}. In Fig. 1b, the cartoon illustrates the fact that the characteristics of PrP^{Sc} are not uniform. In fact, Safar et al. (61) have stated that PrP^{Sc} exists as a "spectrum of conformations" with inter- and intrastrain variations. The question of how many conformations PrP^{Sc} can adopt was raised by Safar et al. (61), but the answer is not yet known. It has been assumed that each agent strain–host strain combination may have a unique conformation; however, even within a specific combination, there appears to be a spectrum of PrP^{Sc} conformations, as noted above by Safar et al. (61) and by others (62,63). Thus, one could argue that in aiming for a diagnostic breakthrough, the PrP^{Sc} protein must be considered a "moving target." Even under defined conditions of a single host

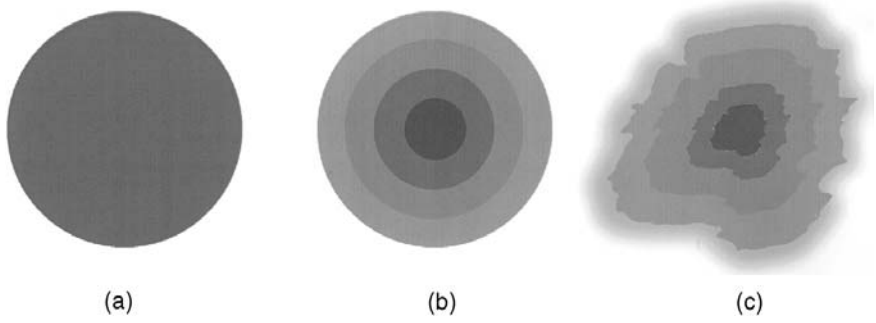

Figure 1 A cartoon representation of a population of PrP^{Sc} molecules with (a) uniform characteristics, (b) a spectrum of physical-chemical characteristics, and (c) a spectrum of physical-chemical characteristics that, at one extreme, approaches the characteristics associated with PrP^{C}. Almost certainly, the (b) and (c) cartoons represent the situation in nature, which adds to the complexity involved in targeting PrP^{Sc} for diagnosis.

PrP genotype and single TSE strain, PrP^{Sc} molecules may display a range of characteristics. Furthermore, there are strains of TSE agents that differ dramatically in the sensitivity of their PrP^{Sc} to protease (19,37,40,41,64), rate of PrP^{Sc} conversion and accumulation, and ability of PrP^{Sc} to form amyloid deposits. These observations call into question the use of a rigid, unchanging set of parameters within a defined diagnostic protocol.

C. Issues Involving the Ratio of PrP^{Sc} to Infectivity

Another difficulty is that there can be marked differences in the ratio of PrP^{Sc} to infectious agent, as shown in the cartoons in Figs. 2a and 2b. Using PrP^{Sc} as a marker of infection, it is clear that a test that is sufficiently sensitive to detect PrP^{Sc} in the Fig. 2a scenario might fail to hit the mark in Fig. 2b. A number of years ago, initial studies by Merz et al. (64) established the variable quantitative relationship between PrP^{Sc}, SAF, and infectivity. For example, the ratio of infectivity to micrograms of purified PrP^{Sc} can differ by as much as 100-fold depending on the method of purification (65). There are also examples of TSE-infected material in which the protease resistance of PrP^{Sc} is much less than that found in most infected animals or tissue culture cells (66). In fact, the PK sensitivity of what is termed PrP^{Sc} can often approach that of PrP^{C}, which suggests the picture presented in Fig. 1c in which the border between the target and normal material is fuzzy or nonexistent. Physiological and environmental influences, such as pH,

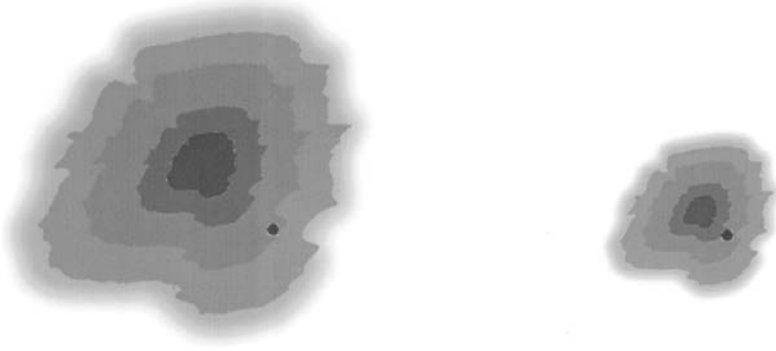

Figure 2 A cartoon in which the ratio of PrP^{Sc} molecules to infectious units ranges from (a) high to (b) low. As a marker of the infectious process, the diagnostic target (PrP^{Sc}) is easier to hit in (a) than it is in (b).

ionic strength, protease exposure, etc., appear to also influence the "fuzzy" distinctions among isoforms (61–66).

The properties of infectivity and PrP^{Sc} toxicity can be separated under certain conditions. Toxic forms of PrP^{Sc} need not be infectious. Toxicity has been associated with oxidative processes and/or alterations in metabolism (67). Under conditions of agent persistence and adaptation, infectivity can be demonstrated in the absence of detectable PrP^{Sc} (68,69). In primary isolation of BSE agent in mice, it was shown that all injected mice showed clinical changes and neuronal death, but more than half of these mice had no detectable PrP^{Sc} (68). In subsequent passages, increasing proportions of clinically positive mice were also positive for PrP^{Sc}. Such studies caution against relying solely on detection of PrP^{Sc} as a means of declaring biologics "safe." On the opposite end of the spectrum, two recent studies appear to identify PrP^{Sc}-like molecules that may not be associated with infectivity. A PrP isolated from urine, termed $UPrP^{Sc}$ (70), possesses characteristics of PrP^{Sc} but is not infectious. The cyclic amplification assay (71) also generates PrP^{Sc}-like molecules in vitro akin to nucleic acid amplification using PCR, but without evidence that infectivity has increased.

D. Cell Types Involved in Transport and/or Production of PrP^{Sc}

The complex interrelationship between agent replication and increased concentrations of PrP^{Sc} is mirrored in the confusion over the role of various cell types vis-à-vis the transport and/or replication of the infectious agent. Several cell types, such as B cells and dendritic cells and, under certain conditions, macrophages, may serve as agent transport vehicles (72–74). Other studies have identified the agent replication roles of follicular dendritic cells and neurons but have not ruled out a potential replication role for macrophages and dendritic cells (75–77). Other cell types may bind agent or PrP^{Sc} and not allow dissemination or propagation (78,79). It appears that agent replication involves a complex interaction of a number of cell types and PrP^{Sc} forms, which interact in both replication and transport modalities. Any one of these steps that involve PrP^{Sc} formation may indeed offer diagnostic promise (see other chapters in this book).

IV. FURTHER MISCONCEPTIONS AFTER THE TARGET IS HIT

The work on PrP^{Sc} has led some, both interested observers and active researchers, to make erroneous assumptions concerning the relationship between findings on the physical-chemical and immunological character-

istics of PrP^{Sc} preparations and the infectious unit. The key point is that although the ratio of PrP^{Sc} to infectivity varies, the generally accepted ratio of PrP^{Sc} molecules to infectious units is 100,000 to 1 (61,80). This means that those analyses that measure the characteristics of the mass of PrP^{Sc} molecules are not necessarily identifying characteristics of the infectious unit. This issue assumes importance in a variety of studies, which include in vitro chemical conversion assays, physical and chemical assessment of preparations of PrP^{Sc}, analysis of cellular distribution of PrP^{Sc} in infected tissue culture cells, as well as the distribution in the organs of infected animals. In each of these examples, the data provide evidence for the characteristics or distribution of the bulk of PrP^{Sc} molecules, but *may not* shed light on the characteristics of the particle or particles that form the infectious entity (see previous section). Ironically, although the high ratio of PrP^{Sc} molecules to infectivity may compromise analysis of the characteristics of the macromolecules associated with the infectious agent, it may well provide an advantage in developing protocols aimed at diagnosis.

As noted above, the situation is further complicated by the findings that each strain of scrapie is composed of a spectrum of PrP^{Sc} conformations as demonstrated by limited protease digestion, by Gdn-HCl denaturation, and by influences of pH, ionic strength, and perhaps other environmental factors (61–66). This situation is represented in Fig. 3a. Again, with the shades of green as a target, if the aim is the "red" infectious

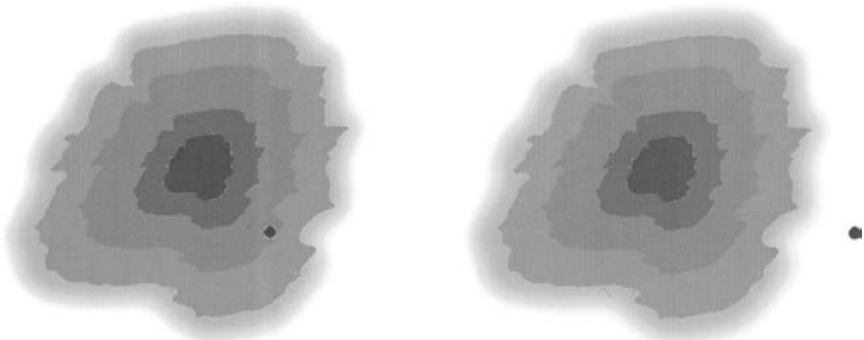

Figure 3 A cartoon in which the physical-chemical characteristics of the infectious units (a) are within the spectrum of the bulk of PrP^{Sc} molecules and (b) are different from the majority of PrP^{Sc} molecules. Assessing the characteristics of PrP^{Sc} may (a) or may not (b) hit the mark with regard to the physical-chemical characteristics of the infectious unit.

unit, analysis of green areas may not tell us very much about the characteristics of the red dot. Thus, even the analysis of the bulk of PrP^{Sc} molecules presents a range of characteristics, a spectrum that may be in a state of flux. How is one to determine the characteristics of the infectious entity in this heterogeneous set? In fact, it is possible that the cartoon shown in Fig. 3b represents the situation. The infectious unit may be composed of PrP^{Sc}, but associated molecules or other influences may have conferred upon it characteristics that remove it from the target area, represented by the bulk of PrP^{Sc} molecules; hitting the "green target" of PrP^{Sc} molecules may miss the mark with regard to the "red" infectious unit. Hitting the red or green target may be sufficient for diagnosis, but therapeutic or prevention strategies must hit the true "red" infectious unit. In summary, we think it is important to keep in mind that one cannot extrapolate results obtained in analyses of PrP^{Sc} characteristics and distribution to define the nature of the infectious unit, nor can these analyses track with certainty the progression of infectivity throughout the body. The only way you can be sure that the infectious unit is being analyzed is when you assay for infectivity.

V. THE FOCUS OF THIS BOOK

As noted previously, most of this book is devoted to approaches being pursued with the aim of developing a quick, sensitive, economical, and precise assessment of the presence of PrP^{Sc}. If possible, the test should be based upon tissue or body fluid that can be obtained antemortem, prior to the time when this material would enter the food chain or the pathway to either therapeutic or transplantation uses. The goal is to develop a method that is as sensitive as bioassay of infectivity in a homologous host or a host with the homologous PrP sequence. The problem with bioassay is the long incubation period, even in transgenic animals expressing multiple PrP^{C} copies. It is possible that with regard to the diagnostic aim, the high ratio of PrP^{Sc} molecules to infection units noted previously could prove to be advantageous. Of course, the problems noted previously with regard to the variation in the characteristics of PrP^{Sc} from different sources and the shifting properties of a population of PrP^{Sc} molecules must be kept in mind. The ability of PrP^{Sc} to attain a specific conformational state may be influenced by several factors, including environmental influences, association with other molecules or compounds, and conformational variation among strains. These influences may well impact upon the properties of PrP^{Sc} as a diagnostic target.

A variety of approaches to assay PrP^{Sc} will be described in this book including HPLC, capillary electrophoresis, Western blot, a spectral

method, ELISA, capture ELISA, and time-resolved fluorescence immunoassay (DELFIA).

There are several other presentations on a number of pertinent issues such as bioassay of prions, a recently described method to amplify the number of PrP^{Sc}-like molecules, and an assay for non-infectious $UPrP^{Sc}$ in urine. There will also be a discussion of the prion hypothesis and an overview of TSE infection of elk and mule deer in the United States.

We believe that improving our capacity to accurately and quickly assess the TSE status of animals and humans is of vital importance to our public health and agricultural prosperity.

REFERENCES

1. Wells GAH, Scott AC, Johnson CT, Gunning RF, Hancock RD, Jeffrey M, Dawson M, Bradley R. A novel progressive spongiform encephalopathy in cattle. Vet Rec 1987; 121:419–420.
2. Will RG, Ironside JW, Zeidler M, Cousens SN, Estibeiro K, Alperovitch A, Poser S, Pocchiari M, Hoffman A, Smith PG. A new variant of Creutzfeldt-Jakob disease in the UK. Lancet 1996; 347:921–925.
3. Bruce ME, Will RG, Ironside JW, McConnell I, Drummond D, Suttie A, McCardle L, Chree A, Hope J, Birkett C, Cousens S, Fraser H, Bostock CJ. Transmissions to mice indicate that "new variant" CJD is caused by the BSE agent. Nature 1997; 389:498–501.
4. Hill AF, Desbruslais M, Joiner S, Sidle KCL, Gowland I, Collinge J. The same prion strain causes vCJD and BSE. Nature 1997; 389:448–450.
5. Brown P, Bradley R. 1755 and all that: a historical primer of transmissible spongiform encephalopathy. Br Med J 1998; 317:1688–1692.
6. Cuillé J, Chelle P-L. La maladie dite "tremblante" du mouton; est-elle inoculable? C R Acad Sci 1936; 203:1552–1554.
7. Wilson DR, Anderson RD, Smith W. Studies in scrapie. J Comp Pathol 1950; 60:267–282.
8. Dickinson AG, Fraser H. An assessment of the genetics of scrapie in sheep and mice. In: Prusiner SB, Hadlow WJ, eds. Slow Transmissible Diseases of the Nervous System. New York: Academic Press, 1979:Vol.1, 367–385.
9. Dickinson AG. Scrapie in sheep and goats. In: Kimberlin RH, ed. Slow Virus Diseases of Animals and Man. Amsterdam: North-Holland, 1977:209–241.
10. Sigurdsson SB. Rida, a chronic encephalitis of sheep with general remarks on infections which develop slowly and some of their special characteristics. Br Vet J 1954; 110:341–354.
11. Hadlow WJ. Scrapie and kuru. Lancet 1959; 2:289–290.
12. Beck E, Daniel PM. Kuru and Creutzfeldt-Jakob disease: neuropathological lesions and their significance. In: Prusiner SB, Hadlow WJ, eds. Slow

Transmissible Diseases of the Nervous System. New York: Academic Press, 1979:Vol. 1, 253–270.
13. Gajdusek DC, Gibbs CJ Jr, Asher DM, David E. Transmission of experimental kuru to the spider monkey (Ateles geoffreyi). Science 1968; 162:693–694.
14. Gajdusek DC, Gibbs CJ Jr. Transmission of two subacute spongiform encephalopathies of man (kuru and Creutzfeldt-Jakob disease) to New World monkeys. Nature 1971; 230:588–591.
15. Chandler RL. Encephalopathy in mice produced with scrapie brain material. Lancet 1961; i:1378–1379.
16. Pattison IH, Millson GC. Scrapie produced experimentally in goats with special reference to the clinical syndrome. J Comp Pathol 1961; 71:101–108.
17. Dickinson AG, Outram GW. Genetic aspects of unconventional virus infections: the basis of the virino hypothesis. In: Bock G, Marsh J, eds. Novel Infectious Agents and the Central Nervous System. Chichester: Wiley, 1986:63–83.
18. Fraser H. Neuropathology of scrapie: the precision of the lesions and their diversity. In: Prusiner SB, Hadlow WJ, eds. Slow Transmissible Diseases of the Nervous System. New York: Academic Press, 1979:Vol. 1, 387–406.
19. Bruce ME, Dickinson AG. Genetic control of amyloid plaque production and incubation period in scrapie-infected mice. J Neuropathol Exp Neurol 1985; 44:285–294.
20. Carp RI, Rubenstein R. Diversity and significance of scrapie strains. Semin Virol 1991; 2:285–294.
21. Dickinson AG, Taylor DM. Resistance of scrapie agent to decontamination. N Engl J Med 1978; 299:1413–1414.
22. Eklund CM, Kennedy RC, Hadlow WJ. Pathogenesis of scrapie virus infection in the mouse. J Infect Dis 1967; 117:15–22.
23. Kimberlin RH, Walker CA. Pathogenesis of mouse scrapie: dynamics of agent replication in spleen, spinal cord and brain after infection by different routes. J Comp Pathol 1979; 89:551–562.
24. Kimberlin RH, Walker CA. Pathogenesis of mouse scrapie: evidence for neural spread of infection to the CNS. J Gen Virol 1980; 51:183–187.
25. Kimberlin RH, Walker CA. Pathogenesis of scrapie (strain 263K) in hamsters infected intracerebrally, intraperitoneally or intraocularly. J Gen Virol 1986; 67:255–263.
26. Fraser H, Davies D, McConnell I, Farquhar CF. Are radiation-resistant, post-mitotic, long-lived (RRPMLL) cells involved in scrapie replication? In: Court LA, Cathala F, eds. II Symposium International sur les Virus Non Conventionnels du Système Nerveux Central. Commissariat à l'Énergie Atomique, Département de Protection Sanitaire, Service de Documentation, Fontenay-aux-Roses, Cedex, 1986:563–574.
27. Fraser H, Farquhar CF. Ionising radiation has no influence on scrapie incubation period in mice. Vet Microbiol 1987; 13:211–223.
28. Fraser H, Dickinson AG. Pathogenesis of scrapie in the mouse: the role of the spleen. Nature 1970; 226:462–463.

29. Dickinson AG, Fraser H. Scrapie: effect of Dh gene on incubation period of extraneurally injected agent. Heredity 1972; 29:91–93.
30. Kimberlin RH, Walker CA. Invasion of the CNS by scrapie agent and its spread to different parts of the brain. In: Court LA, Cathala F, eds. Virus Non-conventionnels et Affections du Système Nerveux Central. Paris: Masson, 1983:15–33.
31. Pattison IH. Experiments with scrapie with special reference to the nature of the agent and the pathology of the disease. In: Gajdusek DC, Gibbs CJ, Alpers MP, eds. Slow, Latent and Temperate Virus Infections. NINDB Monogr. Washington DC: US Gov Print Off, 1965; 2:249–257.
32. Merz PA, Somerville RA, Wisniewski HM, Iqbal K. Abnormal fibrils from scrapie-infected brain. Acta Neuropathol (Berl) 1981; 54:63–74.
33. Merz PA, Somerville RA, Wisniewski HM, Manuelidis L, Manuelidis EE. Scrapie associated fibrils in Creutzfeldt-Jakob disease. Nature 1983; 306:474–476.
34. Merz PA, Rohwer RG, Kascsak RJ, Wisniewski HM, Somerville RA, Gibbs CJ Jr, Gajdusek DC. Infection-specific particle from the unconventional slow virus diseases. Science 1984; 225:437–440.
35. Bolton DC, McKinley MP, Prusiner SB. Identification of a protein that purifies with the scrapie prion. Science 1982; 218:1309–1311.
36. Prusiner SB. Novel proteinaceous infectious particles cause scrapie. Science 1982; 216:136–144.
37. Prusiner SB. Molecular biology of prion diseases. Science 1991; 252:1515–1522.
38. Collinge J. Prion diseases of humans and animals: their causes and molecular basis. Annu Rev Neurosci 2001; 24:519–550.
39. Oesch B, Westaway D, Walchli M, McKinley MP, Kent SB, Aebersold R, Barry RA, Tempst P, Teplow DB, Hood LE, Prusiner SB, Weissman C. A cellular gene encodes scrapie PrP 27–30 protein. Cell 1985; 40:735–746.
40. Kascsak RJ, Rubenstein R, Merz PA, Tonna-DeMasi M, Fersko R, Carp RI, Wisniewski HM, Diringer H. Mouse polyclonal and monoclonal antibody to scrapie-associated fibril proteins. J Virol 1987; 61:3688–3693.
41. Kascsak RJ, Tonna-DeMasi M, Fersko R, Rubenstein R, Carp RI, Powers JM. The role of antibodies to PrP in the diagnosis of transmissible spongiform encephalopathies. In: Brown F, ed. Dev. Biol. Stand. Transmissible Spongiform Encephalopathies—Impact on Animal and Human Health. Basel: Karger, 1993:Vol. 80, 141–151.
42. Bendheim PE, Potempska A, Kascsak RJ, Bolton DC. Purification and partial characterization of the normal cellular homologue of the scrapie agent protein. J Infect Dis 1988; 158:1198–1208.
43. Bendheim P, Barry RA, DeArmond SJ, Stiles DP, Prusiner SB. Antibodies to a scrapie prion protein. Nature (London) 1984; 310:418–421.
44. Aucouturier P, Kascsak RJ, Frangione B, Wisniewski T. Biochemical and conformational variability of human prion strains in sporadic Creutzfeldt-Jakob disease. Neurosci Lett 1999; 274:33–36.

45. Zanusso G, Liu D, Ferrari S, Hegyi I, Yin X, Aguzzi A, Hornemann S, Liemann S, Glockshuber R, Manson JC, Brown P, Petersen RB, Gambetti P, Sy M-S. Prion protein expression in different species: analysis with a panel of new mAbs. Proc Natl Acad Sci USA 1998; 95:8812–8816.
46. Hope J, Morton LJD, Farquhar CF, Multhaup G, Beyreuther K, Kimberlin RH. The major polypeptide of scrapie-associated fibrils (SAF) has the same size, charge distribution and N-terminal protein sequence as predicted for the normal brain protein (PrP). EMBO J 1986; 5:2591–2597.
47. Kretzschmar HA, Prusiner SB, Stowring LE, DeArmond SJ. Scrapie prion proteins are synthesized in neurons. Am J Pathol 1986; 122:1–5.
48. Pan K-M, Baldwin M, Nguyen J, Gasset M, Serban A, Groth D, Mehlhorn I, Huang Z, Fletterick RJ, Cohen FE, Prusiner SB. Conversion of α-helices into β-sheets features in the formation of the scrapie prion proteins. Proc Natl Acad Sci USA 1993; 90:10962–10966.
49. Riek R, Hornemann S, Wider G, Billeter M, Glockshuber R, Wüthrich K. NMR structure of the mouse prion protein domain PrP(121–231). Nature 1996; 382:180–182.
50. Scott M, Foster D, Mirenda C, Serban D, Coufal F, Wälchli M, Torchia M, Groth D, Carlson G, DeArmond SJ, Westaway D, Prusiner SB. Transgenic mice expressing hamster prion protein produce species-specific scrapie infectivity and amyloid plaques. Cell 1989; 59:847–857.
51. Telling GC, Scott M, Mastrianni J, Gabizon R, Torchia M, Cohen EG, DeArmond SF, Prusiner SB. Prion propagation in mice expressing human and chimeric PrP transgene implicating the interaction of cellular PrP with another protein. Cell 1995; 83:79–90.
52. Büeler H, Aguzzi A, Sailer A, Greiner R-A, Autenried P, Auget M, Weissmann C. Mice devoid of PrP are resistant to scrapie. Cell 1993; 73:1339–1347.
53. Telling GC. Prion protein genes and prion diseases: studies in transgenic mice. Neuropathol Appl Neurobiol 2000; 26:209–220.
54. Hsiao KK, Scott M, Foster D, Groth DF, DeArmond SJ, Prusiner SB. Spontaneous neurodegeneration in transgenic mice with mutant prion protein. Science 1990; 250:1587–1590.
55. Gambetti P, Petersen RB, Parchi P, Chen SG, Capellari S, Goldfarb L, Gabizon R, Montagna P, Lugaresi E, Peccardo P, Ghetti B. Inherited prion diseases. In: Prusiner SB, ed. Prion Biology and Diseases. New York: Cold Spring Harbor Laboratory Press, 1999:509–583.
56. Manson JC, Jamieson E, Baybutt H, Tuzi NL, Barron R, McConnell I, Somerville R, Ironside J, Will R, Sy M-S, Melton DW, Hope J, Bostock C. A single amino acid alteration (101L) introduced into murine PrP dramatically alters incubation time of transmissible spongiform encephalopathy. EMBO J 1999; 18(23):6855–6864.
57. Barron RM, Thomson V, Jamieson E, Melton DW, Ironside J, Will R, Manson JC. Changing a single amino acid in the N-terminus of murine PrP alters TSE incubation time across three species barriers. EMBO J 2001; 20(18): 5070–5078.

58. Miele G, Manson J, Clinton M. A novel erythroid-specific marker of transmissible spongiform encephalopathies. Nature Med 2001; 7:361–364.
59. Schena M, Shalon D, Davis RW, Brown PO. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science 1995; 270: 467–470.
60. Dandoy-Dron F, Benboudjema L, Guillo F, Jaegly A, Jasmin C, Dormont D, Tovey MG, Dron M. Enhanced levels of scrapie responsive gene mRNA in BSE-infected mouse brain. Mol Brain Res 2000; 76:173–179.
61. Safar J, Wille H, Itri V, Groth D, Serban H, Torchia M, Cohen FE, Prusiner SB. Eight prion strains have PrP(Sc) molecules with different conformations. Nat Med 1998; 4:1157–1165.
62. Collinge J. Human prion diseases and bovine spongiform encephalopathy (BSE). Hum Mol Genet 1997; 6:1699–705.
63. Jackson GS, Hosszu LLP, Power A, Hill AF, Kenney J, Saibil H, Craven CJ, Waltho JP, Clarke AR, Collinge J. Reversible conversion of monomeric human prion protein between native and fibrilogenic conformations. Science 1999; 283:1935–1937.
64. Merz PA, Rubenstein R, Carp RI, Kascsak RJ. Evidence to implicate SAF as a form of the infectious agent. In: Court LA, Dormont D, Brown P, Kingsbury DT, eds. Unconventional Virus Diseases of the Central Nervous System. II Symposium International sur les Virus Non Conventionnels du Système Nerveux Central. Commissariat à l'Énergie Atomique, Département de Protection Sanitaire, Service de Documentation, Fontenay-aux-Roses, Cedex, 1988: 563–574.
65. Rubenstein R, Kascsak R, Merz P, Carp R, Papini M, Scalici C. Scrapie associated fibrils (SAF): analysis of their components and infectivity. Abstr. American Society for Virology, Annual Meeting, University of North Carolina, Chapel Hill, NC, May 31–June 4, 1987.
66. Lehmann S, Harris DA. Two mutant prion proteins expressed in cultured cells acquire biochemical properties reminiscent of the scrapie isoform. Proc Natl Acad Sci USA 1996; 93:5610–5614.
67. Chiesa R, Harris DA. Prion diseases: what is the neurotoxic molecule? Neurobiol Dis 2001; 8:743–763.
68. Lasmézas CI, Deslys J-P, Robain O, Jaegly A, Beringue V, Peyrin J-M, Fournier J-G, Hauw J-J, Rossier J, Dormont D. Transmission of the BSE agent to mice in the absence of detectable abnormal prion protein. Science 1997; 275:402–405.
69. Race R, Raines A, Raymond GJ, Caughey B, Chesebro B. Long-term subclinical carrier state precedes scrapie replication and adaptation in a resistant species: analogies to bovine spongiform encephalopathy and variant Creutzfeldt-Jakob disease in humans. J Virol 2001; 75(21):10106–10112.
70. Shaked GM, Shaked Y, Kariv-Inbal Z, Halimi M, Avraham I, Gabizon R. A protease-resistant prion protein isoform is present in urine of animals and humans affected with prion diseases. J Biol Chem 2001; 276:31479–31482.

71. Saborio G, Permanne B, Soto C. Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding. Nature 2001; 411:810–813.
72. Aucouturier P, Carp RI, Carnaud C, Wisniewski T. Short Analytical Review: Prion disease and the immune system. Clin Immunol 2000; 96(2):79–85.
73. Shlomchik M, Radeboid K, Duclos N, Manuelidis L. Neuroinvasion by a Creutzfeldt-Jakob disease agent in the absence of B cells and follicular dendritic cells. Proc Natl Acad Sci USA 2001; 98:9289–9294.
74. Aucouturier P, Geissmann F, Damotte D, Saborio G, Meeker HC, Kascsak R, Kascsak R, Carp RI, Wisniewski T. Infected splenic dendritic cells are sufficient for prion transmission to the CNS in mouse scrapie. J Clin Invest 2001; 108(5):703–708.
75. Brown KL, Stewart K, Ritchie DL, Mabbott NA, Williams A, Fraser H, Morrison WI, Bruce ME. Scrapie replication in lymphoid tissues depends on prion protein-expressing follicular dendritic cells. Nat Med 1999; 5:1308–1312.
76. Mabbott NA, Mackay F, Minns F, Bruce ME. Temporary inactivation of follicular dendritic cells delays neuroinvasion of scrapie. Nat Med 2000; 6: 719–720.
77. Jeffrey M, McGovern G, Goodsir CM, Brown KL, Bruce ME. Sites of prion protein accumulation in scrapie-infected mouse spleen revealed by immune-electron microscopy. J Pathol 2000; 191:323–332.
78. Carp RI, Callahan SM. Effect of mouse peritoneal macrophages on scrapie infectivity during extended in vitro incubation. Intervirology 1982; 17:201–207.
79. Holada K, Vostal JG. Different levels of prion protein (PrP^C) expression on hamster, mouse and human blood cells. Br J Haematol 2000; 110(2):472–480.
80. Prusiner SB, McKinley MP, Bowman KA, Bolton DC, Bendheim PE, Groth DF, Glenner GG. Scrapie prions aggregate to form amyloid-like birefringent rods. Cell 1983; 35:349–358.

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Prions, the Protein Hypothesis, and Scientific Revolutions

David C. Bolton
New York State Institute for Basic Research in Developmental Disabilities, Staten Island, New York, U.S.A.

I. INTRODUCTION

The protein hypothesis of the structure of transmissible spongiform encephalopathy agents (or prions[1]) radically altered the scientific view of infectious pathogens. Thomas Kuhn, in his classic analysis of science history and philosophy *The Structure of Scientific Revolutions* (1), has described this type of change as a *paradigm shift* and the entire process as a *scientific revolution*. In this chapter, I examine the transition from a conventional microbiological paradigm (the virus hypothesis) to a new paradigm (the protein hypothesis) within Kuhn's framework and show that the transition to the prion paradigm follows the pattern of previous scientific revolutions. Anomalous observations accumulated and precipitated a crisis in the field, from which radical new hypotheses appeared. A period of extraordinary science ensued wherein five major hypotheses for the structure of these unusual agents (the virus, membrane, virino, protein, and genetic hypotheses) competed to explain the key inconsistencies. Over time, the membrane and genetic hypotheses lost ground and the virus, virino, and prion hypotheses came to predominate. Now, after more than three decades, the revolution appears to be drawing to a close and it seems likely that the prion hypothesis will be the new paradigm that succeeds in displacing the previously accepted virus hypothesis. This transition, however, does not depend on formal proof of the prion hypothesis

or on disproof of the virus or virino hypotheses; rather, the paradigm shift has occured because a majority of scientists believe that the prion hypothesis provides the best model for explaining key observations. In viewing the virus to prion paradigm shift from Kuhn's perspective, we gain an important insight into the nature of scientific discovery.

Physicist Richard Feynman wrote, "The question of doubt and uncertainty is what is necessary to begin; for if you already know the answer there is no need to gather any evidence about it" (2). Questions spawned in the void of the unknown feed the insatiable appetite of the human mind for knowledge. This is especially true of the scientific mind. The scientific enterprise is driven by the desire of individual scientists to understand their world: how things work and what causes the various elements of nature to behave physically, chemically, and biologically the way they do. Scientists do this in a specific way, using the scientific method, to avoid as much as possible interpreting natural events in the context of superstition, religious edict, or emotion. Our quest for knowledge is guided by the understanding that all knowledge is necessarily uncertain to some degree. Factual knowledge is unambiguously valid only within the narrow confines of the specific system and conditions of study. We must also recognize, however, that we interpret our observations through a set of rules based on existing biases. When the observations agree with our expectations based on the rules of interpretation, we believe we know something about the way the world behaves. Ironically, the best experimental results are often those that don't meet the expectations of the investigator. Unexpected results may raise questions that challenge the premise on which the discipline is based. If the anomalies persist they can lead to a process that Kuhn described as a scientific revolution (1). Research on TSE agents during the last four decades provides a contemporary example of one such scientific revolution.

Kuhn formulated his theory of scientific revolutions after observing that science progresses through periods of orderly accumulation of knowledge punctuated by discontinuous transitions characterized by major upheavals in both theory and methodology. He separates the process of scientific discovery into three phases, which I will summarize as *normal science*, *crisis*, and *revolution*.

A. Normal Science

Kuhn defined normal science as "research firmly based upon one or more past scientific achievements, achievements that some particular scientific community acknowledges for a time as supplying the foundation for its further practice." The foundation of normal science is the "paradigm"[2] that is shared by that particular scientific community. The shared paradigm

defines the fundamental characteristics of a particular field, i.e., what is true and what is false, but the paradigm also defines which questions are worth asking, which methods are appropriate, the standards for scientific training, and the criteria for evaluating ideas. The accepted paradigm is the central dogma; it establishes the standard view of the world. Kuhn argues that scientists spend most of their time doing normal science, contributing important but incremental advances in scientific knowledge. He states "normal science, ... is a highly cumulative enterprise, eminently successful in its aim, the steady extension of scope and precision of scientific knowledge" (1). Normal science is largely a puzzle-solving activity, whose questions are defined by the accepted paradigm. Much of the success of normal science comes from defending the paradigm on which it is based and thus, normal science "often suppresses fundamental novelties because they are necessarily subversive of its basic commitments" (1).

Each new scientific discipline coalesces around a "founding" paradigm. Some disciplines arise anew from a preparadigm period that is without an accepted basis for conducting investigations in a scientific manner. Kuhn states that the preparadigm period is characterized by competition between disparate theories and the absence of an accepted common set of techniques and methodologies. In other cases, a new discipline will branch off from an established field following a crisis and revolution that generates a new paradigm. In both the newly created and evolving disciplines, investigators entering the developing field often come from different scientific backgrounds, bringing with them different techniques, methods of interpreting data, and different opinions as to which questions should be addressed. Acceptance of a founding paradigm brings coherence to the discipline and orderliness to the research effort. What is lost, however, is the freedom to challenge the fundamental basis for the paradigm, as that has become accepted knowledge. The chaos of uncertain knowledge is exchanged for the order imposed by the framework of the adopted paradigm.

While normal science is an enterprise primarily focused on confirming and extending the founding paradigm, it also provides an extremely effective platform for producing new discoveries (Kuhn's "novelties of fact") and inventions (Kuhn's "novelties of theory"). It is able to do so because it "leads to a detail of information and to a precision of the observation-theory match that could be achieved in no other way" (1). Advances in normal science require increasingly sophisticated equipment, often built for a specific purpose, to accurately determine measurements whose expected value, framed by the accepted paradigm, is known with precision. When an observation consistently fails to match that precise expectation, it creates the opportunity for doubt in those prepared to perceive the importance of the anomaly.

The route to recognizing novelties and acting upon them is not direct, though. Ironically, the communal confidence placed in the accepted factual knowledge creates a major impediment to recognizing an anomalous observation (1). Kuhn states,

> In science, ... novelty emerges only with difficulty, manifested by resistance, against a background provided by expectation. Initially, only the anticipated and usual are experienced even under circumstances where anomaly is later to be observed (1).

A further irony is that the leading scientists in the field may be the least likely to recognize anomaly or to accept a new paradigm because they are entrenched in the accepted paradigm. This is due at least in part to their substantial role in creating and governing the major institutions of the contemporary normal science establishment.

B. Crisis

When normal science encounters anomalies that cannot be explained within the accepted paradigm, things stop making sense and a crisis emerges. Exactly when a discipline moves into the crisis phase may be difficult to determine, but the transition is characterized by an accumulation of anomalous experimental observations. New observations may fail to match existing theoretical predictions, existing observations may not be explained by the developing theory, or both. As these anomalies mount, new versions of the current theory are created to explain the special circumstances that cannot be explained otherwise. Often special cases of the accepted theory will proliferate until the theory becomes clumsy and unwieldy. This crisis creates the opportunity for a novel theory to emerge from the surrounding chaos.

In the crisis phase, the sharp focus of the accepted paradigm is blurred. The accepted paradigm is modified ad hoc and the strict rules of normal science are relaxed. In this way the crisis phase resembles the period before the founding paradigm was accepted. The period of crisis ends in one of three ways according to Kuhn. First, the problems can be resolved within the current paradigm by normal science and the discipline continues essentially as it did before the crisis. Second, the problems can be shelved after a failure to resolve them despite radical approaches. In this case, the discipline essentially concludes that the current methods and apparatus are not capable of resolving the problem and leaves it for future generations to solve. Third, a new paradigm arises and displaces the old one. This last possibility moves the discipline into the revolutionary phase.

C. Revolution

Kuhn defines scientific revolutions as "those non-cumulative developmental episodes in which an older paradigm is replaced in whole or in part by an incompatible new one"[3] (1). Revolutions involve a change in paradigm (a "paradigm shift") and are discontinuous by definition. Revolution is distinct from the cumulative normal science period and produces extraordinary science. Scientific revolutions need not be major in scope (i.e., on the order of Newtonian mechanics or Einstein's general relativity) but only need to be revolutionary to those whose paradigms are directly affected. In the prion example, most biologists might not be disturbed by the protein theory of TSE agent structure since their fundamental paradigms are not much affected. Proteins are still translated from mRNA, modified by the accepted posttranslational processes, and so on. Anfinsen's concept that each protein has only a single stable conformation (3) is challenged, but that concept is fundamental only to a relatively specialized group of biologists and its interpretation is undergoing revision. On the other hand, virologists could be expected to be very resistant to this theory because it directly challenges their established concepts of host and pathogen, as well as the concepts of infection and replication. Thus, what is seen as revolutionary for one group of scientists is not a significant issue for another group. This helps to explain why outsiders to a discipline in crisis can accept a new paradigm more readily than those who are already invested in the accepted paradigm.

Kuhn's work makes it clear that a new view of the world is essential to initiate a scientific revolution. Initially, only one or a few individuals see that view. Kuhn shows that those individuals usually have two circumstances in common. He states that "their attention has been intensely concentrated upon the crisis-provoking problems" and that "they are men so young or so new to the crisis-ridden field that practice has committed them less deeply than most of their contemporaries to the world view and rules determined by the old paradigm." Conversely, those who have a long history in the old paradigm are much less likely to rapidly switch to the new one.[4] Often, those most entrenched are never able to see the world in this new way.

The revolutionary phase is brief, historically lasting one generation or less. The old paradigm is replaced with the new one and a new phase of normal science begins. The old paradigm may persist to the extent that its advocates remain active in the field, but it dies out as they retire or expire.

D. Paradigm Shift

A fundamental element of Kuhn's thesis is that paradigm selection is not simply a matter of the old paradigm being disproved by new observations.

Instead the old and new paradigms (and any other competing paradigms) are compared with each other for their ability to explain the natural observations. A new theory is required to displace the old one in part because in its absence the defenders of the older theory will continue to construct ad hoc modifications to explain any anomalous observations. In the absence of a better theory for comparison, the modified hypothesis remains acceptable, if imperfect. The battle between paradigms can be viewed as a pseudo-Darwinian struggle for survival between the existing paradigms. The best paradigm survives this struggle to become the foundation for a new period of normal science. It does not have to be the best of all possible paradigms; in fact it does not even have to be correct, but simply better than the others to which it is compared at the time. Indeed, most paradigms are eventually found lacking in some way and are replaced by better ones (e.g., Aristotle's theories were replaced by Galileo's, which were replaced by Newton's, which were replaced by Einstein's). Thus, the process of scientific revolution is never final but continues in a cycle of normal science, anomaly, crisis, revolution, and normal science.

Scientists on opposing sides of the issue begin to collect evidence in support of the old and new paradigms using essentially the same techniques, reagents, and methods but they employ distinct approaches and interpret the data differently. In addition, the opposing factions in this paradigm struggle do not necessarily agree on the rules of competition. Each side cites certain facts that they believe must be explained, but the sets of facts are often not the same for both sides. Different data sets are proposed by each side to support their theories or the same data are interpreted in different ways by each side. Those behaviors reflect the fact that the participants see the world differently: "Both are looking at the world, and what they look at has not changed. But in some areas they see different things, and they see them in different relations one to the other" (1). Kuhn goes on to state that before these two groups "can hope to communicate fully, one group or the other must experience the conversion that we have been calling a paradigm shift" (1). The new view of the world is reminiscent of the computer-generated color pictures [Magic Eye pictures (4)] wherein a picture is hidden within a complex pattern. Viewers first see those pictures as an arbitrary mixture of lines, swirls, and dots in two dimensions but one's perception can instantly switch to reveal a coherent three-dimensional picture of an object. The picture is the same before and after the shift in perception occurs but the transformation creates a dramatic new insight for the viewer. Some observers attain this new insight quickly while others are never able to see the alternate perception. The perceptual switch is immediate and discontinuous; usually it is not possible to see part of the two-dimensional pattern and part of the three-dimensional picture. Similarly, scientific paradigm shifts are discontinuous and rapid. Once the

new view of the world is seen it is difficult, if not impossible, to go back to seeing the world from the old perspective.

II. NORMAL TSE SCIENCE

A. Accepted Paradigm

The prion revolution begins under the accepted paradigms of microbiology, virology, and genetics in the middle of the twentieth century. The founding paradigm of microbiology goes back to the discovery of microbes by Aton van Leeuwenhoek in the middle of the seventeenth century and proceeds through Louis Pasteur's revolution in the mid-nineteenth century, which overthrew the paradigm of spontaneous generation. The field of virology sprung from microbiology in the late nineteenth century with the discovery of filterable viruses independently by Ivanowski and Beijerinck. A substantial research effort over a period of decades was required, however, before their initial discoveries were refined to the current virological paradigm that distinguishes between the true viruses and filterable cellular organisms (mycoplasmas, rickettsiae, and chlamydiae). In parallel with the development of microbiology and virology, the field of genetics received its founding paradigm in 1865 with Mendel's observations on heredity. The chemical basis of heredity was not resolved until much later, however, and even as late as 1940 few scientists seriously considered nucleic acids as the chemical basis for genes (5,6). The accepted view was that *proteins* were the chemical basis for inheritance. Nucleic acids, owing to their relatively simple chemical composition, were thought to be incapable of encoding the massive amounts of information required to encode a complex organism. Tikvah Alper illustrates this point,

> In 1940, the proteinaceous nature of genes was "common knowledge." At the Cold Spring Harbor Symposium that year, our own Alexander Hollaender might well have questioned that belief and maintained that the essential constituent of the genome must be nucleic acid. With Emmons, he showed that the UV wavelength most effective for producing toxic and genetic effects in a fungus was that which was selectively absorbed by nucleic acid. But at that time it was thought that, with only four bases, nucleic acids could not possibly have the specificity to account for the innumerable genes of living organisms. (7)

The "protein-as-genetic-substrate" paradigm was overthrown in a scientific revolution sparked by the observations of Avery, MacLeod, and McCarty, who demonstrated that DNA was the transforming principle in *Streptococcus pneumoniae* (8,9). They demonstrated that the transforming principle was DNA and not protein by showing that it was destroyed by

incubation with highly purified deoxyribonucleases but was not affected by exposure to proteases. The technique they used is relevant to this discussion; others would later use protease inactivation of the scrapie agent in support of the protein hypothesis (10), but their conclusions were not received with similar enthusiasm.

Within a decade of the work of Avery, MacLeod, and McCarty the structure of DNA was known and the implications for transfer of genetic information were clear. Microbiologists and virologists in particular played important roles in illuminating the structure of genes and the mechanisms for their expression and replication. From that knowledge sprang the new paradigm of molecular biology: DNA makes RNA makes protein. That paradigm, which is also known as the central dogma of molecular biology, gave biologists absolute confidence that nucleic acids, not proteins, were the sole substrate for storage of genetic information.[5] It is often forgotten that the opposite view was the common wisdom only 20 years earlier.

Within a decade of the genetic revolution anomalies in the scrapie field would suggest that an agent could replicate without benefit of a nucleic acid genome. Had those anomalies been observed from the perspective of the genetic paradigm of 1940, the suggestion that an infectious agent was composed only of protein would not have been controversial. One could speculate that the protein hypothesis might even have provided the preferred structure for any infectious agent at that time. From the perspective of the new DNA-based genetics, however, the idea that a replicating agent did not contain nucleic acid was essentially inconceivable. The new genetic paradigm held by most microbiologists, virologists, and geneticists could be summarized as follows:

1. All living cells and replicating subcellular agents require and transmit genetic information from generation to generation.
2. Genetic information is contained in genes, which are made of DNA or RNA.
3. Scrapie agents replicate.
4. Therefore, scrapie agents must contain either DNA or RNA.

The virus hypothesis for the structure of the scrapie agent is a product of that accepted paradigm. Viruses can be defined as obligate intracellular parasites that contain a DNA or RNA genome that encodes at least one viral coat protein. Viruses depend on the host cell for energy, biosynthetic intermediates and the biosynthetic machinery for protein synthesis. Viruses may or may not encode enzymes that aid in their replication. Viruses may integrate their genome into that of the infected host but they can also exist as independent particles capable of infecting a new host cell and replicating within it. Defective virus particles may require the coinfection by a complete,

or helper, virus. All viruses have a regular unitary structure that defines individual viral particles. While not all viral particles are infectious, the unitary structure demands that the minimal infectious unit is one viral particle. Under special circumstances, some viral nucleic acids (viral genomes) are infectious alone. Under those special circumstances, the minimal infectious unit may be considered one intact viral genome but the product of replication is a complete virus particle.

B. Scientific Progress

The virus hypothesis adequately explained all that was known about the scrapie agent until about 1965 and normal research under the virus paradigm produced important advances in this field. Significant among those were the transmission of sheep scrapie to goats (11,12) and later to laboratory rodents (13,14). Other significant studies defined many of the parameters of scrapie pathogenesis and identified strains of the scrapie agent (15–18). Those studies were all conducted within the standard microbiological/virological paradigm and produced significant incremental advances in the field. In turn, the studies also refined the available methodology and increased the level of precision expected of subsequent observations. In particular, transmission of scrapie to mice increased the number of experiments that could be performed and improved the precision of the observations through the use of endpoint titrations to quantify the amount of scrapie agent.

C. Anomalies Arise

While research under the normal paradigm produced cumulative advances in knowledge, significant anomalies were also being identified during the period from about 1946 through 1967 (Table 1). As I will discuss below, most of

Table 1 Scrapie Agent Anomalies Under the Standard Virological Paradigm

Observation	Ref.
Resistance to formalin inactivation	30
Resistance to heat inactivation	136
Lack of an immune response in infected host	137
Unusually long asymptomatic incubation periods	138–141
Failure to visualize a viral particle	100–104
Very small ionizing radiation target size	20
UV inactivation spectrum different from nucleic acid	21,22,32

those anomalies, when considered individually, were not sufficiently unusual to demand a novel scrapie agent structure, but their cumulative effect created uncertainty in the minds of scientists in the field. Two observations, however, stood out as particularly troubling because they directly challenged the primacy of nucleic acids as the chemical substrate for the scrapie agent genome. Those two observations, the size of the scrapie agent estimated from ionizing radiation inactivation studies and its ultraviolet (UV) inactivation spectrum, precipitated a crisis that permanently changed the field.

III. CRISIS

> This shows that there is no reason to fear that the existence of a protein agent would cause the whole theoretical structure of molecular biology to come tumbling down.
>
> —J.S. Griffith (19)

Alper and colleagues demonstrated that the scrapie agent was more resistant to both UV and ionizing radiation than expected for known viruses (20–22). The size of the agent they estimated from the ionizing radiation data (7 nm or 150 kDa) (20) was too small to be compatible with structures of even the smallest known viruses. The UV inactivation data confirmed the apparent small size and the inactivation spectrum was not consistent with the absorbance profile of nucleic acids. This led them to suggest that nucleic acids were not required for scrapie agent replication (20–22).

The fact that several new theories for the structure of the scrapie agent appeared within a few years of Alper's publications (Table 2) indicates the beginning of the crisis phase. While all of the new hypotheses are of some interest, I will focus on four of these and compare them to the previously accepted virus hypothesis. The four to be discussed are the membrane hypothesis, the virino hypothesis, the protein hypothesis, and the genetic hypothesis. Other hypotheses have been discussed elsewhere (23,24).

Table 2 New Theories of Scrapie Agent Structure Appear During Crisis

Theory	Year proposed	Ref.
Genetic hypothesis	1960	35
Membrane hypothesis	1967	25
Protein hypothesis	1967	19
Replicating polysaccharide hypothesis	1967	25
Unconventional virus hypothesis	1971	141,142
Virino hypothesis	1979	26,28,29

A. The Membrane Hypothesis

In 1967, Gibbons and Hunter reviewed four current hypotheses of scrapie agent structure (virus, protein, polysaccharide, and membrane hypotheses) and proposed the membrane hypothesis as the best of those (25). Their definition of the membrane hypothesis was broad:

> If the determining factor in scrapie pathogenesis is a steric arrangement of the membrane—and we specifically suggest that it is likely to be a rearrangement in the sugar or oligosaccharide residues attached to it—the agent of scrapie would behave, . . . in the very curious ways observed. (25)

Their hypothesis was vague as to the exact structure and did not specify a mechanism of agent replication, but the proposed structure was clearly not a virus. The authors believed that the hypothesis addressed the significant anomalous observations, in particular resistance to UV and ionizing radiation, relative resistance to proteolysis, resistance to heat and formalin, sensitivity to periodate, and lack of a host immune response. In addition, the proliferation of abnormal membranes explained the neuronal vacuolation and spongiform pathology seen in scrapie. The hypothesis also attempted to explain the apparent inability to separate scrapie infectivity from host cell components.

It is ironic that Tikvah Alper, whose work precipitated the crisis that led to the protein hypothesis paradigm shift, favored the membrane hypothesis and not the protein hypothesis. She supported a modification of the membrane hypothesis because she believed that the UV inactivation data, which failed to show an inactivation peak around 280 nm that is characteristic of most proteins, was more compatible with destruction of membrane components than pure protein (7). As data pointing to the importance of the prion protein accumulated, she recognized that the original membrane hypothesis had to be updated. She stated, "Clearly the membrane hypothesis, in its original form, cannot accommodate the results of a decade of experimental work on PrP; it must be modified accordingly" (7). Rather than reject the membrane hypothesis, she attempted to adjust it to explain the newer observations. She did this by adapting a theory of membrane biogenesis by Palade in which he states, "Traffic control enables each membrane to recognize 'like' proteins and accept them as 'self' in its existing matrix. Unlike proteins are not recognized as 'self' and are not accepted" (quoted from Ref. 7). Alper proposed that mutant PrP (and presumably PrP^{Sc}) fails to be correctly incorporated into the cell membrane and accumulates on the interior surface of the plasma membrane. When the cells die, vesicles are released that contain abnormal PrP inside but lack PrP^{C} on the surface. This membrane fragment infects new cells when it is incorporated into the plasma membrane. The infecting membrane, which lacks PrP^{C} on its sur-

face, is incapable of incorporating PrP^C synthesized by the new cell because there is no "like" protein in the membrane. Thus, membrane free of PrP^C is "replicated."

Analysis of the membrane hypothesis reveals several problems. First, the original membrane hypothesis did not provide a clear structural definition against which to compare experimental observations. Gibbons and Hunter did not identify which specific membrane components were essential and did not indicate how those components would replicate the change in the membrane, except to suggest sugars or oligosaccharides were somehow rearranged. Alper modified the hypothesis by proposing a replication mechanism that identified one specific membrane component (PrP) but the net effect made PrP the biologically active principle without clarifying the role of other membrane components, which presumably give the agent its nonprotein characteristics. The membrane hypothesis is no better than the virus, virino, or protein hypotheses in explaining the anomalous properties of the scrapie agent. It currently has few adherents and is not likely to endure.

B. The Virino Hypothesis

Dickinson and Outram first proposed the virino hypothesis (26) on the presumption that others had demonstrated the requirement for a DNA molecule in the scrapie agent (27). They defined virinos, by analogy with neutrinos, as "small, immunologically neutral particles with high penetration properties but needing special criteria to detect their presence" (26). As with the original membrane hypothesis, their initial definition of the virino lacks any structural criteria. In addition, subsequent studies by several investigators failed to verify that a DNA molecule is required so that the hypothesis was, strictly speaking, unnecessary. Despite those shortcomings, the theory was soon modified to define virinos as small noncoding or regulatory nucleic acid genomes protected by a host protein coat[6] (28,29). The virino hypothesis presents an intermediate position between the protein hypothesis and the virus hypothesis, making it an attractive alternative for many scientists. Having the virino coat protein encoded by the host allows the virino genome to be very small. Its only required functions are to be replicated and to produce the strain-specific characteristics seen in the infected host. The lack of an immune response to virino infection can be explained by the host's tolerance to the virino coat protein, which is encoded by a host gene.

Although the original virino hypothesis was structurally vague, its current form provides a clear structural model against which experimental data can be compared. A virino can be distinguished from a virus by the small size and noncoding nature of its genome and by the fact that its coat protein is host derived. Similarly, a virino is differentiated from a prion (protein agent) by the presence of a small noncoding nucleic acid in the former but not in the

latter. As is discussed below, this hypothesis remains viable but appears to have much less support than the protein hypothesis.

C. The Protein Hypothesis

Pattison recognized the unusual characteristics of the scrapie agent early on. In 1965 he suggested, "If the transmissible agent of scrapie is a living virus, it is a virus of a kind as yet unrecognized" (30). He later compared the scrapie agent with the properties of encephalitogenic factor of allergic encephalomyelitis and found them to be similar. Since encephalitogenic factor was thought to be a basic protein, he suggested, "The scrapie transmissible agent may be, or may be associated with, a small basic protein" (31). Pattison provided some speculations on how the protein might cause disease by analogy to histone proteins but no mechanism of replication was advanced at that time. Pattison published his paper at about the same time as Alper and colleagues (20–22,32) but while Alper's data supported the conclusion that the scrapie agent had an unusual structure (i.e., different from a conventional virus), they did not propose any specific structural model.

The peculiar properties of the scrapie agent attracted other scientists to the field. Among them was J.S. Griffith, a mathematician and neuroscientist who apparently had only a passing interest in scrapie. In his only publication in this area, he discussed the self-replication of proteins and argued "that there are at least three distinct kinds of way in which it could occur" (19). It is again historically ironic that Griffith's only publication in this field established a theoretical benchmark that even today marks the paradigm shift in TSE research. Griffith's three "ways" or specific protein hypotheses are described in the following paragraphs.

Griffith's first way stated that if the product of a normally suppressed gene could induce its own expression, then introducing the protein by inoculation would induce synthesis in a manner indistinguishable from replication. He proposed that disease would result as a consequence of the gene being expressed in the particular cell or tissue in which it was not normally expressed. The hypothesis provided an elegant and logical mechanism to explain a process that gave the appearance of protein self-replication. It was the theory favored by many (I for one) during the period immediately following the discovery that PrP^{Sc} was encoded by a normal host gene (33). That it subsequently was shown to be incorrect in this case* does not detract from its beauty.

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*PrP^{C} is expressed in many tissues, including the brain, in normal animals and its expression is not significantly altered by infection with prions (33,143).

The second of Griffith's protein hypotheses is both simpler than the first and at the same time more complex. Griffith suggested that if a protein is produced as a normal cellular constituent, an abnormal form of the same protein could bind to it and convert the normal protein to the abnormal form. Being a mathematician he put this in the form of an equation:

> Given protein sub-units α which can undergo the following reactions
>
> $$\begin{aligned} \alpha_2 + \alpha &\rightarrow \alpha_3 \\ \alpha_3 + \alpha &\rightarrow \alpha_4 \\ \alpha_4 &\rightarrow 2\alpha_2 \end{aligned} \qquad (1)$$
>
> it is clear that the net result is that the monomer α gets converted into the dimer α_2. If we suppose also that the reaction
>
> $$\alpha + \alpha \rightarrow \alpha_2 \qquad (2)$$
>
> cannot take place directly, it follows that α can only dimerize to α_2 under the catalytic influence of molecules of α_2 which are already present.
>
> Next we show that this reaction scheme could be realized through the assignment of physically reasonable properties to the protein sub-units. *Let the reactive sub-unit α be a different conformation of the stable structure, α', say, of the protein.* (19) [Emphasis mine.]

Griffith went on to show how this process is possible thermodynamically. His "second way" described the conversion of a host protein from its normal conformation to a diseased conformation by the diseased protein. This model provided the foundation for all subsequent modern versions of the protein hypothesis, although that fact is often not recognized (34). Griffith published just this one paper in this field (19) but it forever changed the way the world was viewed. The fact that Griffith was an outsider to this field validates an important element of Kuhn's theory of scientific revolutions.

Griffith's third way is of less interest than the other two, in part because it was unlikely to be correct even at the time it was proposed. It stated that an antibody could exist that had as its epitope (binding site) a portion of the same antibody molecule. Introducing the antibody by injection would stimulate the immune system to produce more of the antibody, giving the appearance of replication. Accumulation of the antibodies presumably would cause disease. Griffith recognized this as being unlikely: "It seems probable, however, that scrapie does not use the third way because there is good experimental evidence that the disease is not antigenic" (19).

D. Genetic Hypothesis

At least one other hypothesis from this period requires mention. H.B. Parry observed that scrapie occurred within flocks in a familial pattern and pro-

posed that "scrapie is primarily a hereditarily determined disease" and that the "results are consistent with the hypothesis that scrapie may be due to a single pair of alleles behaving in a simple Mendelian autosomal recessive manner" (35). He did not deny the transmissibility of scrapie, however, and suggested that "in the homozygous recessive individual a substance, the transmissible scrapie agent, is formed which is closely associated with the development of the degenerative changes in several organ systems" (35). Parry's genetic hypothesis, though not well received at the time, correctly predicted inherited forms of TSEs, but while he viewed scrapie as an autosomal recessive disease, the genetic TSEs are autosomal dominant. Parry's view of natural sheep scrapie as a genetic disease that was secondarily transmissible contrasted with that of others who saw it as a contagious disease with a wide range of susceptibility that was determined by host genotype. Both views are accommodated by the protein hypothesis, wherein natural prion diseases span the spectrum from heritable to infectious.

IV. REVOLUTION

> I have shown the existence of at least three classes of replication mechanisms and that, therefore, the occurrence of a protein agent would not necessarily be embarrassing although it would be most interesting.
>
> — J.S. Griffith (19)

A. New Directions

Significant technological advances and a remarkable diversity in research approaches mark the period following the publication of the new hypotheses. This period of scientific revolution begins with little factual knowledge of the physical and chemical nature of the agent. Its anomalous biological properties had raised important questions but the analytical tools to obtain the answers were not yet available. The desire to obtain those answers motivated scientists to develop innovative new research tools.

Many new techniques were brought to this field in the period from the crisis phase to the present. Some of the techniques were adapted from other disciplines for use in this field. Some examples are purification methods, techniques for creating chimeric genes and transgenic mice, brain grafting, and methods for determining protein structure. Scientists in the TSE/prion disease field also developed new techniques that may be useful in other fields. Some examples are lesion profiling to characterize scrapie agent strains, the incubation time period assay (36), histoblotting (37), and return refocusing gel electrophoresis (38). Still other techniques were developed specifically based on the protein hypothesis and may be of only limited use outside the

TSE field. These include the proteinase K digestion assay for PrP^{Sc} (10,39), the in vitro PrP^{C} conversion assay (40), and protein cyclic amplification of protein misfolding (41).

Specific new reagents were also developed during the crisis period, including polyclonal and monoclonal antibodies to PrP, and existing reagents found prominent new uses (proteinase K, phosphatidylinositol phospholipase C, endoglycosidase-H, endoglycosidase-F, Sarkosyl, and others). Importantly, one class of reagent that has not yet been developed is any specific probe for the TSE agent nucleic acid genome.[7]

Scientists used the new tools in new ways. Many studies did not just attempt to refine or extend the accepted paradigm but had as their primary goal the validation or invalidation of a specific hypothesis. Thus, the practice of normal science pointed out anomalies, which produced a crisis that led to creation of new methodologies to investigate them. The outcome of the ongoing scientific revolution depends on many factors, including the observations made using these new techniques and their interpretation. Some of that knowledge is analyzed in the next sections as it relates to the three primary structural hypotheses.

B. Direct Evidence

During the period of revolution rival camps gathered data that either supported their chosen hypothesis or contradicted an opposing hypothesis. This is not to say that normal science was neglected during this period. In fact, most science in the field was of that type, and thus, did not directly address the essential issue of the revolution, i.e., the nature of the TSE agents. The fact that much of the published science either did not address this issue or was unable to contribute to its resolution makes it irrelevant to this discussion. Instead, the following sections focus on studies that directly address three aspects of the nature of the TSE agents: size, chemical composition, and ultrastructural morphology.

1. Size Matters

The scrapie agent exhibits a wide range of apparent sizes (reviewed in Ref. 42) that can be explained by the aggregation of small individual infectious particles. Agent size is important because different hypothetical structures can be excluded when the measured size of the infectious particle falls below a specific minimum. For example, filterability essentially eliminated cellular organisms from consideration early on because the agent was too small to fit in that class. During the revolutionary phase, ionizing and UV radiation studies (discussed below) indicated that the scrapie agent was significantly

smaller than the smallest known viruses. Those results supported elimination of a virus structure and suggested that the scrapie agent must have a simpler structure. The protein hypothesis arose in part because of the requirement for a very small particle size. Both the virino and prion hypotheses allow for a wider range of particle sizes than are allowed by a virus structure but neither hypothesis restricts the infectious particle to that extremely small size. Thus, prions and virinos are not as easily excluded by size as are viruses. In addition, they cannot be differentiated from each other by size because the size of the regulatory nucleic acid molecule in the hypothetical virino could be as small as a few nucleotides.

How do estimates for the size of the scrapie agent compare with the smallest known viruses? The porcine circovirus (PCV) establishes a lower size limit for known viruses with a diameter of about 17 nm and a total particle mass of about two million Da[8] (43). It has a circular single-stranded genome of 1759 bases (approximate mass of 580 kDa) that encodes at least two proteins (44). One of the proteins appears to be involved in DNA replication (ORF V1) and the second is probably the capsid protein (ORF C1). The capsid protein is predicted to be 231 amino acids long with a mass of 27,600 Da.

It is clear that PCV has a very simple structure but could viruses exist that are even smaller and simpler? The theoretical minimum size for a virus can be very roughly estimated based on the size of the putative capsid protein. For example, if we assume that a capsid protein might be as small as 10 kDa then the nucleic acid genome encoding that protein would have to be approximately 100 kDa.[9] Assuming very efficient packing and a very thin protein coat, the spherical particle would have a diameter of about 10 nm and a total mass of approximately 440 kDa.[10] Reducing the capsid protein size to 5 kDa only reduces the particle size slightly to about 9 nm with a total mass of 290 kDa. These estimates put a lower limit on theoretical virus size that is about 10 times smaller than the smallest known viruses.[11]

A number of different methods have been used to determine the minimum size of the scrapie agent. Unfortunately, the accuracy of most of these measurements is disputed because of various technical or theoretical issues. For example, size estimates from filtration studies are brought into question by the possibility of filter leakage or variation in the pore size. Particle shape, aggregation, and affinity for the column matrix affect size determinations made by size exclusion chromatography while particle shape, buoyant density, and aggregation affect estimates obtained by sedimentation. Reliable physical measurement of particle size could theoretically be obtained from direct visualization in the electron microscope but there has been no reliable visual evidence on which to base a morphological definition of the scrapie agent particle (see below). Two methods are discussed below

that provide more reliable size estimates by avoiding many of the deficiencies described above.

Ionizing Radiation Studies. Ionizing radiation has been used to estimate sizes of biological complexes in a way that is relatively independent of assumptions about the chemical composition of the complex. The basic concept is that ionizing radiation deposits energy randomly along its path and that depositing a sufficient amount of energy within the volume of a biological complex (the target volume) will inactivate it.[12] The dose that inactivates all but e^{-1} (36.8%) of the relevant activity is called the D_0 (or sometimes the D_{37}). The "target theory" of inactivation has three restrictions that are relevant here: the samples should be irradiated (1) dry, (2) near room temperature, and (3) in the presence of oxygen. Samples are dried to prevent indirect inactivation by radiolysis products (primarily of water). Some investigators have attempted to overcome the radiolysis problem by irradiating frozen samples at very low temperatures. This introduces other complications, however, because temperature has a differential effect on the inactivation curves of different macromolecules. The differences disappear around room temperature, which is why that temperature is selected to determine the size of a complex independent of its composition (45). The presence of oxygen is required to "fix" the radiation damage in the dried molecule. Irradiation in the absence of oxygen requires a higher dose because other reactions, such as intramolecular donation of a nearby hydrogen atom, can restore function. If the sample is irradiated in solution instead of in the dry state, the presence of oxygen has differential effects depending on the chemical composition of the complex (45). Oxygen tends to protect proteins and nucleic acids irradiated in solution but it sensitizes lipid membranes.

Alper has estimated the size of the scrapie agent to be between 110 kDa and 210 kDa based on six separate experiments under the conditions required by target theory (45). Others have estimated the size to be as small as 55 kDa (46) under conditions that fall outside those specified by target theory (samples in solution irradiated at $-135°C$). In either case, the size estimated by ionizing radiation is far smaller than any known virus and at most about two-thirds the size estimated above for the smallest theoretical virus particle. This interpretation would exclude a virus structure of TSE agents.

Rohwer, who analyzed published ionizing radiation inactivation data from several sources, challenged Alper's interpretation of the ionizing radiation data. He interpreted the data he analzyed separately in the context of RNA and DNA viruses to yield two different larger estimates of scrapie agent size (47). Alper subsequently rebutted this approach (48) and Rohwer issued a counterrebuttal (49). Their exchange illustrates a classic example of

Kuhn's concepts of crisis and conflict in revolutionary science wherein a scientific dispute cannot be resolved because the opposing parties view the world differently.

UV Radiation Studies. Inactivation by UV irradiation provides a different view of the structure of the scrapie agent. While inactivation by ionizing radiation can be considered random, UV irradiation inactivates molecules that absorb light within a relatively narrow band of wavelengths depending on their chemical composition. Nucleic acids absorb strongly around 260 nm and below 230 nm with a minimum absorbance at around 240 nm while most proteins absorb strongly at 280 nm and below 240 nm. Thus, when molecular sizes are determined from UV inactivation data, the estimates depend on certain assumptions about the chemical composition of the target.

Inactivation by UV irradiation has mostly been used to determine the sizes of nucleic acids by irradiating them at 254 nm for two reasons: nucleic acids are fairly uniformly sensitive to that wavelength and inexpensive germicidal lamps at that wavelength are readily available. Several groups have determined values of D_0 for the scrapie agent irradiated at 254 nm, with a consensus at around 20,000 J/m^2 (21,22,32,45,50). When the inactivation is interpreted as resulting from destruction of a nucleic acid, the estimated size of the "active" molecule would be very small. Bellinger-Kawahara et al. estimated that the maximum size would be four nucleotides if single stranded and 30–45 base pairs if double stranded (50). When the inactivation is assumed to be due to protein, the D_0 value for the scrapie agent compares favorably to known proteins in the size range estimated for the scrapie agent using ionizing radiation (45). Neither interpretation is compatible with a virus structure for the scrapie agent.

2. Chemical and Molecular Composition

Hypotheses based on the structure of an agent can best be validated by structural and compositional analyses of the biologically active particles. Knowledge of both the chemical composition of the particle (percentage of protein, nucleic acid, carbohydrate, etc.) and the identity of the molecular components (specific proteins, nucleic acids, carbohydrates, etc.) is crucial for evaluating proposed hypotheses. While this concept is obvious on its face, applying it in the real world has been difficult. This is true at least in part because the scientists from different camps hold incompatible views of the problem. For example, separation of the agent from host components was impossible from the perspective of Gibbons and Hunter but that objective was essential for Prusiner, who argued that purification of TSE agents was a necessary prerequisite for their characterization. Even those

who agreed that purification and biochemical analyses of TSE agents were important goals often could not agree on how one assessed the degree of purification in enriched samples. The specific issues regarding purification of the scrapie agent will not be discussed here but have been reviewed elsewhere (51).

Proteins. Essentially nothing was known about the chemical composition of the scrapie agent through the 1970s. By 1980, published reports indicated that the scrapie agent was inactivated by proteases (52–54) or by treatments that modified or denatured proteins (42,55). Those studies demonstrated that the scrapie agent required a protein but did little to discriminate it structurally from almost all other infectious entities.[13] The knowledge that a protein was involved undoubtedly motivated scientists to improve both the methods for purifying the scrapie agent and the techniques for identifying agent-specific proteins.

Important advances in purifying the scrapie agent (54,56,57) accelerated the search for a scrapie-specific protein and ultimately led to the discovery of the protease-resistant form of prion protein, PrP-27–30 (39,57). Subsequent studies demonstrated that PrP-27–30 is the partial degradation product of a larger protein, PrP^{Sc},[14] which is derived from a normal cellular protein, PrP^{C}. Some contrasting properties of PrP^{C} and PrP^{Sc} are shown in Table 3.

Discovery of PrP^{Sc} and PrP^{C} initiated a period of remarkable progress in this field by providing a necessary technological breakthrough: a physical marker for TSE agents/prions. From Kuhn's perspective it is important to recognize that PrP^{Sc} and PrP^{C} were discovered at the point where two critical factors coincided: (1) observed anomalies motivated scientists to look

Table 3 Some Properties of PrP^{C} and PrP^{Sc}

Property	PrP^{C}	PrP^{Sc}
Present in normal brain	+	–
Present in diseased brain	+	+
Present in prion preparations	–	+
Present in SAF/amyloid	–	+
Purified proteins cause disease	–	+
Cellular localization	Cell surface, Golgi	Intracytoplasmic vesicles
Released from cell by PIPLC	+	–
Resistant to proteolysis	–	+
Soluble in sarkosyl	+	–
Primary protein conformation	α-helical	β-sheet

for specific proteins, and (2) purification methods had advanced to the point of being informative.

The number and power of the observations supporting PrP^{Sc} as an essential component of TSE agents (i.e., prions) have grown over the last two decades (Table 4). These observations are well accepted by the proponents of the protein hypothesis. Proponents of the virino hypothesis also accept most of those arguments because their hypothesis accommodates PrP^{Sc} as the virino coat protein and thus its proponents find no need to dispute them.

One can safely assume that the proponents of the virus hypothesis reject most of the observations listed in Table 4. The virus hypothesis is incompatible with most of those statements and they must be disputed or accounted for in another way for the hypothesis to remain viable. Since specific arguments for and against the virus or protein hypotheses have already been published previously (58–61) I will not revisit them here. Rather, my purpose is to show that the logical force of the arguments is largely irrelevant because those who support the virus hypothesis find the facts irrelevant. Instead, they construct individual ad hoc arguments to counter each supporting fact proposed by the advocates of the protein hypothesis.

One of the common ad hoc arguments is essentially that of denial, i.e., that the true scrapie virus has not been identified and that the association of PrP with infectivity is coincidental. Another argument, i.e., that PrP^{Sc} is

Table 4 Arguments That PrP^{Sc} Is a Constituent of the Scrapie Agent

PrP^{Sc} purifies with infectivity by several different methods.
PrP^{Sc} is the only macromolecule consistently found in purified agent preparations.
Physical and chemical properties of PrP^{Sc} and scrapie agent are similar.
Concentration of PrP^{Sc} correlates with scrapie agent infectivity.
Nondenatured PrP^{Sc} has not been separated from scrapie agent infectivity.
Accumulation of PrP^{Sc} or abnormalities in PrP^{C} metabolism are associated with all TSEs.
Mutations in the PrP gene are linked to familial spongiform encephalopathies.
PrP^{C} expression is required for agent replication, pathology, and clinical disease.
Incubation times are inversely proportional to PrP^{C} expression.
Changes in PrP sequence alter TSE agent properties, extending even to production of artificial prions not previously found in nature.
Species-specific variations in PrP sequence are major determinants of the barrier to interspecies transmission.
PrP^{Sc} binds to PrP^{C} in vitro and converts it to a PrP^{res} form.
Differences in PrP^{Sc} conformation correlate with well-defined agent strains.
Prions and PrP^{Sc} show similar kinetics of degradation by proteases in vitro.
Antibodies to PrP prevent or clear infection in cultured cells.

merely a product of the pathology but is not a component of the infectious particle (62,63), frequently accompanies the first argument. Virus proponents explain that virus particles are not detected in purified preparations because the preparations are "contaminated with large amounts of prion protein" (63). The argument concludes that nothing is known of the structure and composition of the agent (usually assumed to be a virus) and further research is required to identify its constituent macromolecules. The requirement for expression of PrP^{C}, implied by the complete resistance of $PrP^{0/0}$ mice to scrapie infection, is accounted for by proposing that PrP^{C} is the receptor for the scrapie virus (62,63). The fact that the putative receptor (PrP^{C}) is altered during infection to produce PrP^{Sc}, which subsequently purifies with the virus, is left unexplained except as a coincidence. Also unexplained is the observation that the altered virus receptor (PrP^{Sc}) is readily identified in purified preparations but the virus or its constituent macromolecules cannot be seen. Such arguments may be sufficient to retain those who already believe the virus hypothesis but are unlikely to persuade uninitiated scientists to adopt it.

Nucleic Acids. The search for a specific nucleic acid associated with the scrapie agent contrasts remarkably with the search for a specific protein. While the identification of a single protein, PrP, led to extensive research and substantial advances within the field, the many attempts to identify a specific nucleic acid have yet to yield a single strong candidate (27,38,64–99). Interest in the search for scrapie-agent-specific nucleic acids appeared to increase as the protein hypothesis gained momentum. This was undoubtedly a reflection of the various participants' desire to either prove one hypothesis or disprove the opposing hypothesis. The search for TSE-agent-specific nucleic acids seems to have declined in recent years. This may be due either to the perceived validity of specific negative results (38,82,85,86,91,94,97), which might have converted some scientists to a belief in the protein hypothesis, or to the belief that the current techniques have been exhausted without solving the problem.

The failure to identify a specific nucleic acid emphasizes the point made earlier regarding the asymmetry of certain experimental findings. First, I propose that there is an asymmetry of perceived achievement, i.e., how the field and one's peers view each result. If a specific nucleic acid exists, its discovery would be of paramount importance for both practical and theoretical reasons. Thus, the value of identifying the scrapie nucleic acid should provide substantial motivation for interested scientists. Failure to find the nucleic acid, or perhaps succeeding in not finding the nucleic acid, does not have equivalent value. This leads to the second asymmetry, i.e., that of result. Identification of a specific nucleic acid is a definitive result that can

be extended to other experiments. In contrast, even the best-designed experiment is problematic if the nucleic acid is not found because one cannot determine whether the experiment failed because no specific nucleic acid is present or because the methods applied were inadequate. These possible outcomes compete in the minds of scientists, providing incentive and disincentive at the same time.[15] The balance currently appears to favor the disincentives but future economic or scientific events could alter this balance.

The current status of the science is that several studies rule out large nucleic acids but no study has excluded a very small nucleic acid. The most informative results from direct searches limit the putative nucleic acid from less than 80 nucleotides to 240 nucleotides in length (38,82,85). That size range essentially excludes the viruses as a viable model but does not exclude either the virino or prion hypotheses.

3. Ultrastructure

The inability to identify a TSE-agent-specific structure was an important anomalous observation that contributed to the eventual crisis in this field. Early studies revealed either no identifiable structure (100–104), abnormal membranes (101,102,105,106), electron-dense particles (106,107), amyloid fibrils (108), or tubulofilamentous particles (109–111). None of those candidate structures was consistently observed and the question of TSE agent ultrastructure remained unsettled.

The discovery of scrapie-associated fibrils (SAF) (112–114) provided the first ultrastructural observation that achieved some degree of consensus. SAF were verified by several laboratories and were similar, but not identical, to prion rods that were described shortly thereafter (115,116). A consensus on the issue of SAF/prion rod identity was not achieved, however, because the theoretical perspective of each relevant group significantly shaped its interpretation of those observations. Some in the virus group viewed SAF as the filamentous scrapie virus or later as part of the tubulofilamentous nemavirus structure (90,99,117) while others viewed SAF as a product of pathology but not part of the virus. The virino group believed SAF could be the virino particle, concealing a small nucleic acid inside (29,118), or it could be a pathological by-product. The prion group saw SAF and prion rods as either related forms of the same phenomenon, i.e., aggregates of PrP^{Sc} (29) or unrelated structures (119,120), but in both cases the structures were considered unnecessary for prion biological activity. This is because neither prion rods nor SAF are present after sonication or in liposome preparations even though high prion titers and PrP^{Sc} remained (121,122).

Of course, other more conventional structures for TSE agents have been proposed. Reports identifying specific virus particles or nucleoprotein com-

plexes (74,80,84,93,96,117,123–128) have not been substantiated by others, however, and their relevance to TSE agent structure remains in doubt.

One may ask if the size limits imposed by the ionizing radiation experiments severely constrain the prevailing theories of TSE agent structure. While a virus structure is not compatible with those data (as noted above), virino and prion structures remain formal possibilities. Several hypothetical virino structures are compared with a single hypothetical prion structure in Table 5. These structures were devised to meet the size restrictions imposed by the ionizing radiation data assuming a target size of 150 kDa without considering some of the more complex issues of macromolecular assembly, packing, or symmetry (129). I have simply assumed that such a structure can be assembled from multiple copies of a single protein molecule and a nucleic acid, where indicated. Average values for the dimensions and densities of proteins and nucleic acids were used.

Table 5 Hypothetical Physical Parameters of Different Virino and Prion Models

Physical model[a]	Target[b]	Coat[c] (nm)	PrP's[d]	V_i[e] (cm^3)	V_t[f] (cm^3)	NTs[g]	Dimensions[h] (nm)	Mass[i] (kDa)	Density (g/cm^3)
Spherical virino	P + N	1	4	7.4×10^{-20}	2.0×10^{-19}	201	7.2 (d)	160	1.34
Spherical virino	P + N	2	6	1.7×10^{-20}	2.0×10^{-19}	47	7.2 (d)	150	1.27
Spherical virino	N	2	17	1.7×10^{-19}	6.6×10^{-19}	455	11 (d)	540	1.36
Spherical virino	N	5	79	1.7×10^{-19}	2.5×10^{-18}	455	17 (d)	2,000	1.33
Tubular virino[j]	N	2	54	1.3×10^{-18}	2.9×10^{-18}	455	13 (w) × 23 (l)	2,100	1.20
Tubular virino[j]	N	5	155	1.3×10^{-18}	5.9×10^{-18}	455	18 (w) × 23 (l)	4,400	1.24
Spherical prion	P	NA	6–7	NA	1.9×10^{-19}	0	7.2 (d)	150	1.31

[a] Physical models based on the assumption that the target size is limited to about 150 kDa.
[b] Target molecule that is inactivated by ionizing radiation: P = protein; N = nucleic acid.
[c] Thickness of the protein coat in nm.
[d] Number of PrP^{Sc} molecules in the protein coat.
[e] Volume enclosed by the protein coat.
[f] Total volume.
[g] Number of nucleotides in the genome.
[h] Particle dimensions where (d) is particle diameter, (w) is the width, and (l) is the length.
[i] Total particle mass.
[j] The tubular virino model is based on packing dimensions of TMV and thus includes a void volume in the interior that is assumed to be filled by water.

The first two models assume that the target is the entire virino particle, i.e., that deposition of energy anywhere within the volume of the particle causes inactivation. The largest genome that could be accommodated is about 200 nucleotides, assuming a protein coat thickness of only 1 nm. The second model assumes a more reasonable coat thickness and thus accommodates only a 47-nucleotide genome. The next four models use more liberal interpretation of target theory, which assumes that only the nucleic acid is affected by ionizing radiation.[16] Each of those models allows for a genome of 455 nucleotides and requires a substantially larger virino particle. In those cases, the permissible amount of PrP^{Sc} is not restricted because it does not figure into the target size. Note, however, that the size of the genome is now more than twice that determined from direct analysis of nucleic acids in TSE agent preparations (38,82,85). The prion model assumes that inactivation is due solely to reaction with the protein component of PrP^{Sc}, ignoring any contribution by the GPI anchor and oligosaccharide chains.

The minimum theoretical sizes of virino and prion particles shown in Table 5 are distinct from those of the smallest known viruses but they are smaller by only about an order of magnitude. This presents another case of the asymmetry of experimental results. In one case, identification of a particle similar to that of a known virus family would probably be viewed as conclusive evidence for a virus structure. On the other hand, visual identification of particles that are dissimilar to viruses would probably be judged as inconclusive. Neither the virino nor prion model requires a defined unitary structure nor it is possible to predict a priori an ultrastructural morphology. Reports of virus particles have not been confirmed to date and the particles that have been seen are consistent with either virino or prion structures. Further technological developments may be required before a conclusive definition of a TSE agent particle can be obtained.

C. Indirect Evidence

The following paragraphs address arguments that are often given in support of one or another hypothesis but that really only indirectly address the critical issues. On first glance these arguments usually appear to have considerable merit but a careful analysis reveals that they cannot conclusively distinguish between two or more hypotheses.

1. Diverse Agent Phenotypes (Agent Strains)

Three biological criteria are used to define agent strains: incubation times, distribution of vacuoles throughout the brain, and distribution of amyloid. There should be little doubt that different TSE isolates exhibit distinct

phenotypic or strain differences and thus the existence of more than one TSE agent phenotype in a single genetically identical host can be accepted as fact.

This fact is interpreted in different ways to support different TSE agent structures, however. The proponents of the virus and virino hypotheses argue that the expression of different phenotypes requires an "informational" molecule that can only be nucleic acid. Prion proponents argue that different conformations of PrP^{Sc} can explain the known strains and specific examples of this have been reported (130,131). Critics of the protein hypothesis cite the existence of at least 20 different strains and argue that different conformations of PrP^{Sc} cannot account for all of these. The prion proponents counter with the following two arguments. First, most of the strains cited have not been characterized in a single inbred host, so the actual number of unique strains remains in question. Second, PrP^{Sc} is actually a population of molecules having at least 400 different glycosylation variants. Therefore, combinations of protein conformation and glycosylation could (at least numerically) account for all of the strains known at this time.

In the case of the virus and virino supporters, the DNA-based paradigm of information transfer appears to provide an accepted foundation for their position. The virus hypothesis appears untenable on other grounds, however, and the virino hypothesis provides no mechanistic basis for the different phenotypes; i.e., the hypothesis does not explain how a nucleotide of ≤ 240 bases produces the observed phenotypes. The protein hypothesis proposes that the different phenotypes result from differences in the interactions between PrP^{C} and different conformers of PrP^{Sc}. Each of these arguments has strengths and weaknesses but none can directly address the question of the chemical basis of the informational molecule. Thus, the chemical basis for variation in TSE agent phenotype remains in dispute and cannot be determined solely on the basis of the existence of strains.

2. Mutations in Agent Phenotype

Murine scrapie agents have been classified into three stability groups (132, 133). Class I agents are stable over multiple passages in either of two mouse PrP genotypes ($sinc^{s7}$ or Prn-p^{a} and $sinc^{p7}$ or Prn-p^{b}). Strain ME7 is the prototype for Class I agents. The phenotypic stability of the Class I agents was noted by Bruce and Dickinson: "This apparent immutability of the ME7 agent is in itself remarkable if, as now seems more likely, the informational molecule of scrapie is a DNA" (132).

Class II agents are stable when propagated in the host strain of origin but are unstable in the other strain. The prototype of the Class II agents is strain 22A. During multiple passages in the C57BL host (not the host of

origin) strain 22A gradually changes into strain 22F. According to Bruce and Dickinson, "C57BL passage of 22A has always resulted in the same agent, 22F, which is unlike any of our other agents" (132).

Class III agents are unstable in either strain and change phenotype in a single discontinuous event. Strain 87A is prototypic of the Class III agents. Strain 87A (and other Class III agents) always change to a stable strain, 7D, that appears to be identical to the most common mouse scrapie strain, ME7 (132).

Virus and virino proponents argue that these changes in phenotype represent mutations in a nucleic acid genome. This argument suffers from the same weaknesses described above for strains, i.e., chemical composition cannot be inferred from this biological behavior. Additionally, the observed changes in phenotype, especially those of the Class II agents, appear more like host-induced modifications than mutation followed by selection. For example, it is difficult to explain why strain 87A always changes into the same strain, 7D/ME7, apparently regardless of the host genotype. Also unexplained is the fact that chemical and physical mutagens, classic tools in molecular genetics that cause mutations through defined interactions with nucleic acids, have not been reported to induce mutations in TSE agents. As above, these arguments fail to address the chemical basis for the phenotypic changes and cannot discriminate between these hypotheses.

3. Host Genotype Effects

The PrP genotype of the host is the major determinant of susceptibility and incubation time within those species that have been carefully studied. PrP genotype is also a major factor in determining the efficiency of interspecies transmission and expression of PrP transgenes can make the host susceptible to TSE agents to which it was previously resistant. Modifying PrP transgenes has created novel TSE agents that were not previously found in nature (134,135). As important as these observations are, they do not exclude the requirement for a small nucleic acid and thus cannot discriminate between the prion and virino hypotheses.

4. Unusual Agent Properties

It may be surprising that the atypical physical, chemical, and biological properties that have become accepted markers of TSE agents and diseases are not really informative regarding agent structure. Disease characteristics such as long incubation periods and lack of inflammatory and immune responses set these diseases apart from many others but can be accommodated within each of the major hypotheses. The virus, virino, or prion models can also explain characteristics of the agents such as resistance to heat, resist-

ance to various chemical and enzymatic treatments, and inefficient replication in cell culture. Even the observation that human TSEs can have a genetic as well as a transmissible etiology can be explained within these three hypotheses, though perhaps not equally well.

V. CONCLUSIONS

If the virus hypothesis is not formally disproved, it has performed unsatisfactorily in several critical areas. Perhaps the contradictory data are wrong or have been misinterpreted, but that seems unlikely on the whole. The question of whether TSE agents are viruses could be resolved with current technology. Application of the techniques of molecular biology in conjunction with the published virus genome sequences should allow detection of nucleic acids related to any known virus. Virus sequences should be readily detectable in hamster brains infected with the 263K scrapie agent or in purified preparations. The fact that there is no enthusiasm for such a project suggests that few believe that a positive result would be obtained. Similarly, established electron microscopic techniques should detect virus particles similar to any known virus in 263K scrapie agent samples prepared using current purification methods. Again, there seems to be little enthusiasm for such studies. Proponents of the virino or protein hypothesis might refrain from pursuing such studies for fear that the results might not be interpretable under their hypothesis but the proponents of the virus hypothesis should expect an unambiguous result. Of course, the argument can be made that TSE agents are true viruses that are unrelated to any known virus but the available evidence appears to weigh against that possibility.

The virino hypothesis remains a viable alternative and is perhaps more attractive in some ways than the protein hypothesis. There is little doubt, however, that it receives far less attention than the protein hypothesis and appears to have fewer adherents. This may be explained in several ways. First, the attention given to the protein hypothesis may derive from its early classification as "heretical" (and therefore inherently newsworthy) or from the substantial effort made to popularize the term "prion".[17] Second, the lack of a proposed mechanism by which the small virino nucleic acid creates phenotypic variation among TSE agents makes that aspect of the virino hypothesis more ephemeral than the protein hypothesis. Different protein conformations may seem an unlikely basis for the observed agent strain differences but at least they provide a mechanism that relies on plausible interactions between known components of the disease process. Third, the protein hypothesis may be seen to adequately explain the observed anomalies and thus the nucleic acid that sets the virino hypothesis apart may

appear irrelevant. Finally, the lack of direct evidence for the virino nucleic acid or the data limiting its size may have discouraged proponents of the theory even though those data do not disprove it.

The TSE/prion revolution appears to be nearly over but this does not mean that the protein hypothesis has been proved. It does indicate that the protein hypothesis is widely accepted as explaining the most important anomalies that were not satisfactorily explained by the previous hypothesis. In addition, the protein hypothesis correctly predicted aspects of disease that were not anticipated by the virus hypothesis: genetic disease, species barriers that correlate with PrP sequence differences, and spontaneous disease. Griffith anticipated this when he wrote about the hypothetical protein agent:

> It may be passed between animals but be actually a different protein in different species. Finally, in either case there is the possibility of spontaneous appearance of the disease in previously healthy animals. (19)

The virino hypothesis remains viable in theory and its longevity may depend more on the persistence of its proponents than on the factual evidence that supports it. It is possible that the TSE field will postpone resolution of the prion-versus-virino issue. This could happen, for example, by adopting a composite "pririno" model, in which the presence of a nucleic acid is allowed but not essential or by allowing both virino and prion models to survive in parallel until the correct model (if either is correct) is discerned.

Prusiner (24) has expressed the idea that the protein hypothesis might have been more readily accepted if the role of PrP in genetic disease had been discovered before its role as an infectious agent. While this suggestion may be correct, I believe that it discounts the fact that the scientific crisis and revolution occurred within the virology community and not within the genetic community. Thus, while the geneticists probably would have readily accepted the relevant genetic aspects of the protein hypothesis (as they apparently have now), the conflict with the virologists would have surfaced once the similarities with the infectious diseases were identified. This is true because there is an inherent conflict between the protein paradigm and the virus paradigm and this conflict does not exist between the genetic and protein paradigms. The collision between virus and protein paradigms of TSE agent structure was therefore unavoidable.

Finally, the prion or protein revolution in scrapie research occurred approximately contemporarily with the publication of Kuhn's theory of the revolutionary nature of scientific discovery. While it is clear that at least some, and perhaps many, of the participants in the TSE/prion revolution were aware of Kuhn's work, it seems not to have affected the overall course of the process. It appears that in this case insight into the mechanism of scientific revolutions did not affect the behavior of those involved in the

process. It may be that those two events, Kuhn's hypothesis and the TSE/prion revolution, were too closely juxtaposed for Kuhn's ideas to be grasped and applied in a timely manner.[18] Alternatively, the processes he describes may reflect fundamental elements of human behavior that are not easily modified, even when the participants have certain knowledge of their effects.

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NOTES

1. Nomenclature in this field is both controversial and confusing. In this chapter, I will use the term "prion" to refer to the class of hypothetical particles that contains an essential protein but does not contain or require an exogenous (nonhost) nucleic acid. The prion model is distinguished from the "virino" model in that the latter particle is proposed to contain an essential nonhost nucleic acid that does not code for a protein. I will use the term "agent" when referring to the infectious particle without bias toward any particular structure. I will also use the acronym "TSE" in referring to the diseases when a specific structural hypothesis is not relevant.
2. Kuhn defines "paradigm" in a somewhat circular fashion. A paradigm is the belief system, apparatus, methods, etc., that are shared by the members of a scientific community and, conversely, the scientific community are those who share a common paradigm.
3. Kuhn often uses the term "old" to identify the accepted paradigm during the crisis phase as well as after it. I will continue that usage but the reader should recognize that during the crisis phase it might also be referred to as the "accepted" or "current" paradigm.
4. This is a two-edged sword. While a steadfast belief in the accepted paradigm may impede conversion to a correct new paradigm, it also keeps the discipline from randomly switching paradigms without ample justification. In this sense, intractable established scientists create a barrier to transition that prevents unnecessary paradigm shifts.
5. The fact that the new genetic paradigm soon required modification to accommodate the retroviruses (RNA makes DNA makes RNA makes

protein) did little to diminish confidence in the new nucleic-acid-based genetic paradigm.

6. Broadening the definition of the virino hypothesis to include a gene encoding a virino coat protein essentially turns it into a virus.

7. Motivation to develop such reagents must come from those that follow the virus or virino hypotheses since the target genome does not exist for the adherents of the prion hypothesis.

8. I have estimated that a circovirus with a diameter of 17 nm and a density of 1.37 g/cm^3 (43) has a volume of 2.6×10^{-18} cm^3 and a mass of 3.5×10^{-18} g/particle. This gives a particle mass of 2.1×10^6 Da.

9. We could assume that the genome could be about one third that size if the mRNA is produced by three rounds of transcription from a circular genome. This seems extraordinarily unlikely and unnecessary, as will be seen below.

10. This estimate was calculated using an average amino acid molecular mass of 110 Da and 91 amino acids in the putative capsid protein. The gene would require 276 nucleotides to encode 91 amino acids and one stop codon. Assuming a minimum amount of 5′ and 3′ untranslated region and an average mass of 330 Da/nucleotide, the genome size is rounded to 300 bases with a mass of approximately 100 kDa. A 100-kDa nucleic acid would occupy a volume of about 1.1×10^{-19} cm^3 (assuming a density of 1.5 g/cm^3) and have a radius of about 3 nm. A 2-nm protein shell made from 34 copies of a 10-kDa protein could encapsulate this nucleic acid genome to give a particle with a diameter of about 10 nm, assuming a protein density of about 1.3 g/cm^3. The virus particle would have a total mass of approximately 440 kDa, of which 77% is protein and 23% is nucleic acid, and a density of about 1.4 g/cm^3.

11. For comparison, a protein agent with a density of 1.3 gm/cm^3 and a mass of 150 kDa would have a volume of $\sim 2 \times 10^{-19}$ cm^3 and a diameter of about 7.2 nm (see Table 5).

12. A detailed discussion of the theoretical and experimental constraints on the use of ionizing radiation to determine the size of macromolecular complexes is beyond the scope of this chapter but has been presented elsewhere (45).

13. This illustrates an asymmetry to certain scientific discoveries. The discovery that a protein was required eliminated viroids (naked nucleic acid agents) as a hypothetical structure for TSE agents but left viable almost all other hypothetical structures. If the results had indicated that

the agent did not require a protein, however, all protein-requiring agents would have been eliminated, leaving only a few possible structures. Thus these two results are mutually exclusive and clearly unequal in their ultimate impact.

14. The prion protein nomenclature has a tortured history. I originally coined the term "PrP" as an acronym for protease-resistant protein and the term "PrP-27–30" denoted a protease-resistant protein with an apparent mass of 27–30 kDa (39). Prusiner soon adapted the term "PrP" to mean prion protein and this modification has endured. PrP-27–30 was also called p26 and SAF protein by some investigators and is now often referred to as PrP^{res} by others. The larger precursor has been called PrP^{Sc}, Sp33–37, and Gp34 (and probably other names as well). The host protein has been called PrP^{C}, Cp33–37, and PrP^{sen}. The PrP^{C}/PrP^{Sc} nomenclature has been widely used but it is not without complications that may confuse the uninitiated. First, the name PrP as an acronym for prion protein seems inappropriate for the cellular form, which is not a "prion" protein in that state. Second is the difficulty of referring to other prion disease proteins (e.g., Creutzfeldt-Jakob disease, Gerstmann-Sträussler-Scheinker disease, etc.) using the term PrP^{Sc}, which refers to the sheep disease scrapie. It is not clear that the terms PrP^{sen} and PrP^{res} provide any more clarity because they differentiate PrP isoforms based on a property that is relative (rather than absolute) and may not be universal to all abnormal PrP's.

15. A scientist contemplating the quest for the TSE agent nucleic acid would have to balance two substantial benefits, the potential accolades expected from a successful search and being in on the ground floor of an important area of research, against the possibility that a negative result will bring no reward at all. The decision will thus be heavily influenced by data that suggest that a negative result is likely.

16. Alper's discussion of target theory indicates that inactivation correlates with size independent of the composition of the particle but it is possible that the effects of energy deposited within the protein coat might have only a minimal biological effect. Thus, the true target size would be due primarily to the nucleic acid genome.

17. Terminology alone may play some role here. While the terms "prion" and "virino" are probably equivalent in cache and ease of use, the term "prion disease" has clear advantages over its counterpart "transmissible spongiform encephalopathy," which is commonly used by the virino proponents.

18. The edition of Kuhn's book that I have used was published in 1996 but the first edition was published in 1962 and revised in 1970. Also, the book evolved from ideas conceived almost 15 years prior to its initial publication.

REFERENCES

1. Kuhn TS. The Structure of Scientific Revolutions. 3rd ed. Chicago and London: University of Chicago Press, 1996:1–212.
2. Feynman RP. The role of scientific culture in modern society. In: Robbins J, ed. The Pleasure of Finding Things Out. Cambridge, MA: Helix Books, 1999: 97–115.
3. Anfinsen CB. Principles that govern the folding of protein chains. Science 1973; 181(96):223–230.
4. Magic Eye: A New Way of Looking at the World. Kansas City, MO: Andrews & McMeel, a Universal Press Syndicate Company, 1993.
5. Darnell JE, Lodish HF, Baltimore D. Molecular Cell Biology. 2nd ed. New York: Scientific American Books, 1990.
6. Stent GS. Molecular Genetics. San Francisco: W.H. Freeman & Company, 1971.
7. Alper T. The scrapie enigma: insights from radiation experiments. Radiat Res 1993; 135:283–292.
8. Avery OT, MacLeod CM, McCarty M. Studies on the chemical nature of the substance inducing transformation of pneumococcal types: induction of transformation by a desoxyribonucleic acid fraction isolated from pneumococcus type III. J Exp Med 1944; 79:137–158.
9. McCarty M, Avery OT. I. Studies on the chemical nature of the substance inducing transformation of pneumococcal types. II. Effect of deoxyribonuclease on the biological activity of the transforming substance. J Exp Med 1946; 83:89–96.
10. McKinley MP, Bolton DC, Prusiner SB. A protease-resistant protein is a structural component of the scrapie prion. Cell 1983; 35:57–62.
11. Stamp JT, Brotherston JG, Zlotnik I, MacKay GMJ, Smith W. Further studies on scrapie. J Comp Pathol 1959; 69:268–280.
12. Zlotnik I. The histopathy of the brain of goats affected with scrapie. J Comp Pathol 1961; 71:440–450.
13. Chandler RL. Encephalopathy in mice produced by inoculation with scrapie brain material. Lancet 1961; 1:1378–1379.
14. Zlotnik I, Rennie JC. The pathology of the brain of mice inoculated with tissues from scrapie sheep. J Comp Pathol 1962; 72:360–365.
15. Dickinson AG. Scrapie in sheep and goats. In: Kimberlin RH, ed. Slow Virus Diseases of Animals and Man. Amsterdam: North-Holland, 1976:211–241.

16. Fraser H. The pathology of a natural and experimental scrapie. Front Biol 1976; 44:267–305.
17. Dickinson AG, Outram GW. Genetic aspects of unconventional virus infections: the basis of the virino hypothesis. Ciba Found Symp 1988; 135:63–83.
18. Bruce ME, Fraser H. Scrapie strain variation and its implications. Curr Top Microbiol Immunol 1991; 172:125–138.
19. Griffith JS. Self-replication and scrapie. Nature 1967; 215:1043–1044.
20. Alper T, Haig DA, Clarke MC. The exceptionally small size of the scrapie agent. Biochem Biophys Res Commun 1966; 22:278–284.
21. Alper T, Cramp WA, Haig DA, Clarke MC. Does the agent of scrapie replicate without nucleic acid? Nature 1967; 214:764–766.
22. Latarjet R, Muel B, Haig DA, Clarke MC, Alper T. Inactivation of the scrapie agent by near monochromatic ultraviolet light. Nature 1970; 227:1341–1343.
23. Adams DH. Does the infective agent of scrapie replicate without nucleic acid? An assessment. Med Hypoth 1991; 35:253–264.
24. Prusiner SB. Prions. Proc Natl Acad Sci USA 1998; 95(23):13363–13383.
25. Gibbons RA, Hunter GD. Nature of the scrapie agent. Nature 1967; 215: 1041–1043.
26. Dickinson AG, Outram GW. The scrapie replication-site hypothesis and its implications for pathogenesis. In: Prusiner SB, Hadlow WJ, eds. Slow Transmissible Diseases of the Nervous System. Vol. 2. New York: Academic Press, 1979:13–31.
27. Marsh RF, Malone TG, Semancik JS, Lancaster WD, Hanson RP. Evidence for an essential DNA component in the Scrapie agent. Nature 1978; 275:146–147.
28. Kimberlin RH. Scrapie agent: prions or virinos? [news]. Nature 1982; 297:107–108.
29. Carp RI, Kascsak RJ, Wisniewski HM, Merz PA, Rubenstein R, Bendheim PE, Bolton DC. The nature of the unconventional slow infection agents remains a puzzle. Alzheimer Dis Assoc Disord 1989; 3:79–99.
30. Pattison IH. Resistance of the scrapie agent to formalin. J Comp Pathol 1965; 75:159–164.
31. Pattison IH, Jones KM. The possible nature of the transmissible agent of scrapie. Vet Rec 1967; 80:2–9.
32. Haig DA, Clarke MC, Blum E, Alper T. Further studies on the inactivation of the scrapie agent by ultraviolet light. J Gen Virol 1969; 5:455–457.
33. Oesch B, Westaway D, Wälchli M, McKinley MP, Kent SB, Aebersold R, Barry RA, Tempst P, Teplow DB, Hood LE, Prusiner SB, Weissmann C. A cellular gene encodes scrapie PrP 27–30 protein. Cell 1985; 40:735–746.
34. Bendheim PE, Bolton DC. Scientific correspondence. Nature 1991; 352:291–292.
35. Parry HB. Scrapie: a transmissible hereditary disease of sheep. Nature 1960; 185:441–443.
36. Prusiner SB, Cochran SP, Groth DF, Downey DE, Bowman KA, Martinez HM. Measurement of the scrapie agent using an incubation time interval assay. Ann Neurol 1982; 11:353–358.

37. Taraboulos A, Jendroska K, Serban D, Yang SL, DeArmond SJ, Prusiner SB. Regional mapping of prion proteins in brain. Proc Natl Acad Sci USA 1992; 89:7620–7624.
38. Kellings K, Meyer N, Mirenda C, Prusiner SB, Riesner D. Analysis of nucleic acids in purified scrapie prion preparations. Arch Virol 1993; 7(suppl): 215–225.
39. Bolton DC, McKinley MP, Prusiner SB. Identification of a protein that purifies with the scrapie prion. Science 1982; 218:1309–1311.
40. Kocisko DA, Come JH, Priola SA, Chesebro B, Raymond GJ, Lansbury PT, Caughey B. Cell-free formation of protease-resistant prion protein [see comments]. Nature 1994; 370:471–474.
41. Saborio GP, Permanne B, Soto C. Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding. Nature 2001; 411(6839): 810–813.
42. Prusiner SB. Novel proteinaceous infectious particles cause scrapie. Science 1982; 216:136–144.
43. Tischer I, Gelderblom H, Vettermann W, Koch MA. A very small porcine virus with circular single-stranded DNA. Nature 1982; 295(5844):64–66.
44. Meehan BM, Creelan JL, McNulty MS, Todd D. Sequence of porcine circovirus DNA: affinities with plant circoviruses. J Gen Virol 1997; 78(pt 1): 221–227.
45. Alper T. Radio- and photobiological techniques in the investigation of prions. In: Prusiner SB, McKinley MP, eds. Prions: Novel Infectious Pathogens Causing Scrapie and Creutzfeldt-Jakob Disease. San Diego, CA: Academic Press, 1987:113–148.
46. Bellinger-Kawahara CG, Kempner E, Groth D, Gabizon R, Prusiner SB. Scrapie prion liposomes and rods exhibit target sizes of 55,000 Da. Virology 1988; 164:537–541.
47. Rohwer RG. Scrapie infectious agent is virus-like in size and susceptibility to inactivation. Nature 1984; 308:658–662.
48. Alper T. Scrapie agent unlike viruses in size and susceptibility to inactivation by ionizing or ultraviolet radiation [letter]. Nature 1985; 317:750.
49. Rohwer RG. Estimation of scrapie nucleic acid MW from standard curves for virus sensitivity to ionizing radiation [letter]. Nature 1986; 320:381.
50. Bellinger-Kawahara C, Cleaver JE, Diener TO, Prusiner SB. Purified scrapie prions resist inactivation by UV irradiation. J Virol 1987; 61:159–166.
51. Bolton DC, Bendheim PE. Purification of scrapie agents: how far have we come? Curr Top Microbiol Immunol 1991; 172:39–55.
52. Cho HJ. Requirement of a protein component for scrapie infectivity. Intervirology 1980; 14:213–216.
53. Cho HJ. Inactivation of the scrapie agent by pronase. Can J Comp Med 1983; 47:494–496.
54. Prusiner SB, McKinley MP, Groth DF, Bowman KA, Mock NI, Cochran SP, Masiarz FR. Scrapie agent contains a hydrophobic protein. Proc Natl Acad Sci USA 1981; 78:6675–6679.
55. McKinley MP, Masiarz FR, Prusiner SB. Reversible chemical modification of the scrapie agent. Science 1981; 214:1259–1261.

56. Prusiner SB, Groth DF, Cochran SP, Masiarz FR, McKinley MP, Martinez HM. Molecular properties, partial purification, and assay by incubation period measurements of the hamster scrapie agent. Biochemistry 1980; 19:4883–4891.
57. Prusiner SB, Bolton DC, Groth DF, Bowman KA, Cochran SP, McKinley MP. Further purification and characterization of scrapie prions. Biochemistry 1982; 21:6942–6950.
58. Bendheim PE, Bolton DC. The transmissible agent causing scrapie must contain more than protein: against the proposition. Rev Med Virol 1991; 1:139–144.
59. Somerville RA. The transmissible agent causing scrapie must contain more than protein: for the proposition. Rev Med Virol 1991; 1:131–139.
60. R Somerville. Do prions exist? PBS/NOVA, 2001. http://www.pbs.org/wgbh/nova/madcow/prions.html.
61. DC Bolton. Do prions exist? PBS/NOVA, 2001. http://www.pbs.org/wgbh/nova/madcow/prions.html.
62. Manuelidis L, Sklaviadis T, Manuelidis EE. Evidence suggesting that PrP is not the infectious agent in Creutzfeldt-Jakob disease. EMBO J 1987; 6:341–347.
63. Manuelidis L. Dementias, neurodegeneration, and viral mechanisms of disease from the perspective of human transmissible encephalopathies. Ann NY Acad Sci 1994; 724:259–281.
64. Marsh RF, Semancik JS, Medappa KC, Hanson RP, Rueckert RR. Scrapie and transmissible mink encephalopathy: search for infectious nucleic acid. J Virol 1974; 13:993–996.
65. Hunter GD, Collis SC, Millson GC, Kimberlin RH. Search for scrapie-specific RNA and attempts to detect an infectious DNA or RNA. J Gen Virol 1976; 32:157–162.
66. Manuelidis L, Manuelidis EE. Search for specific DNAs in Creutzfeldt-Jakob infectious brain fractions using "nick translation. Virology 1981; 109:435–443.
67. Diener TO, McKinley MP, Prusiner SB. Viroids and prions. Proc Natl Acad Sci USA 1982; 79:5220–5224.
68. Manning EJ, Millson GC. Infectivity of liposomally encapsulated nucleic acids isolated from EMC virus and scrapie-infected mouse brain. Intervirology 1983; 20:164–168.
69. Dees C, McMillan BC, Wade WF, German TL, Marsh RF. Characterization of nucleic acids in membrane vesicles from scrapie-infected hamster brain. J Virol 1985; 55:126–132.
70. German TL, McMillan BC, Castle BE, Dees C, Wade WF, Marsh RF. Comparison of RNA from healthy and scrapie-infected hamster brain. J Gen Virol 1985; 66:839–844.
71. Borras T, Gibbs CJ Jr. Molecular hybridization studies with scrapie brain nucleic acids I. Search for specific DNA sequences. Arch Virol 1986; 88:67–78.
72. Duguid JR, Rohwer RG, Seed B. Isolation of cDNAs of scrapie-modulated RNAs by subtractive hybridization of a cDNA library. Proc Natl Acad Sci USA 1988; 85:5738–5742.
73. Manuelidis L, Murdoch G, Manuelidis EE. Potential involvement of retroviral elements in human dementias. Ciba Found Symp 1988; 135:117–134.

74. Narang HK, Asher DM, Gajdusek DC. Evidence that DNA is present in abnormal tubulofilamentous structures found in scrapie. Proc Natl Acad Sci USA 1988; 85:3575–3579.
75. Oesch B, Groth DF, Prusiner SB, Weissmann C. Search for a scrapie-specific nucleic acid: a progress report. Ciba Found Symp 1988; 135:209–223.
76. Aiken JM, Marsh RF. The search for scrapie agent nucleic acid. Microbiol Rev 1990; 54:242–246.
77. Aiken JM, Williamson JL, Borchardt LM, Marsh RF. Presence of mitochondrial D-loop DNA in scrapie-infected brain preparations enriched for the prion protein. J Virol 1990; 64:3265–3268.
78. Duguid JR, Dinauer MC. Library subtraction of in vitro cDNA libraries to identify differentially expressed genes in scrapie infection. Nucleic Acids Res 1990; 18:2789–2792.
79. Murdoch GH, Sklaviadis T, Manuelidis EE, Manuelidis L. Potential retroviral RNAs in Creutzfeldt-Jakob disease. J Virol 1990; 64:1477–1486.
80. Narang HK. Detection of single-stranded DNA in scrapie-infected brain by electron microscopy. J Mol Biol 1990; 216:469–473.
81. Sklaviadis T, Akowitz A, Manuelidis EE, Manuelidis L. Nuclease treatment results in high specific purification of Creutzfeldt-Jakob disease infectivity with a density characteristic of nucleic acid-protein complexes. Arch Virol 1990; 112:215–228.
82. Meyer N, Rosenbaum V, Schmidt B, Gilles K, Mirenda C, Groth D, Prusiner SB, Riesner D. Search for a putative scrapie genome in purified prion fractions reveals a paucity of nucleic acids. J Gen Virol 1991; 72:37–49.
83. Narang HK, Millar NS, Asher DM, Gajdusek DC. Increased multimeric mitochondrial DNA in the brain of scrapie-infected hamsters. Intervirology 1991; 32:316–324.
84. Narang HK. Evidence of ssDNA in tubulofilamentous particles: their relationship to scrapie-associated fibrils. Intervirology 1991; 32:185–192.
85. Kellings K, Meyer N, Mirenda C, Prusiner SB, Riesner D. Further analysis of nucleic acids in purified scrapie prion preparations by improved return refocusing gel electrophoresis. J Gen Virol 1992; 73:1025–1029.
86. Sasaki K, Tanaka K, Ito M, Isomura H, Horiuchi M, Ishiguro N, Shinagawa M. Biological activity of subfractions from scrapie-associated fibrils. Virus Res 1992; 23:241–251.
87. Sklaviadis T, Dreyer R, Manuelidis L. Analysis of Creutzfeldt-Jakob disease infectious fractions by gel permeation chromatography and sedimentation field flow fractionation. Virus Res 1992; 26:241–254.
88. Akowitz A, Manuelidis EE, Manuelidis L. Protected endogenous retroviral sequences copurify with infectivity in experimental Creutzfeldt-Jakob disease. Arch Virol 1993; 130:301–316.
89. Narang HK. Molecular cloning of single-stranded DNA purified from scrapie-infected hamster brain. Res Virol 1993; 144:375–387.
90. Narang HK. Evidence that scrapie-associated tubulofilamentous particles contain a single-stranded DNA. Intervirology 1993; 36:1–10.
91. Riesner D, Kellings K, Wiese U, Wulfert M, Mirenda C, Prusiner SB. Prions

and nucleic acids: search for residual nucleic acids and screening for mutations in the PrP-gene. Dev Biol Stand 1993; 80:173–181.
92. Sklaviadis T, Akowitz A, Manuelidis EE, Manuelidis L. Nucleic acid binding proteins in highly purified Creutzfeldt-Jakob disease preparations. Proc Natl Acad Sci USA 1993; 90:5713–5717.
93. Akowitz A, Sklaviadis T, Manuelidis L. Endogenous viral complexes with long RNA cosediment with the agent of Creutzfeldt-Jakob disease. Nucleic Acids Res 1994; 22:1101–1107.
94. Kellings K, Prusiner SB, Riesner D. Nucleic acids in prion preparations: unspecific background or essential component? Philos Trans R Soc Lond B Biol Sci 1994; 343:425–430.
95. Narang HK. Evidence that homologous ssDNA is present in scrapie, Creutzfeldt-Jakob disease, and bovine spongiform encephalopathy. Ann NY Acad Sci 1994; 724:314–326.
96. Manuelidis L, Sklaviadis T, Akowitz A, Fritch W. Viral particles are required for infection in neurodegenerative Creutzfeldt-Jakob disease. Proc Natl Acad Sci USA 1995; 92:5124–5128.
97. Bountiff L, Levantis P, Oxford J. Electrophoretic analysis of nucleic acids isolated from scrapie-infected hamster brain. J Gen Virol 1996; 77:2371–2378.
98. Dron M, Manuelidis L. Visualization of viral candidate cDNAs in infectious brain fractions from Creutzfeldt-Jakob disease by representational difference analysis. J Neurovirol 1996; 2:240–248.
99. Narang HK. Evidence that single-stranded DNA wrapped around the tubulofilamentous particles termed "nemaviruses" is the genome of the scrapie agent. Res Virol 1998; 149(6):375–382.
100. Mould DL, Smith W, Dawson AM. Centrifugation studies on the infectivities of cellular fractions derived from mouse brain infected with scrapie (Suffolk strain). J Gen Microbiol 1965; 40:71–79.
101. Kimberlin RH, Millson GC, Hunter GD. An experimental examination of the scrapie agent in cell membrane mixtures. 3. Studies of the operational size. J Comp Pathol 1971; 81:383–391.
102. Semancik JS, Marsh RF, Geelen JL, Hanson RP. Properties of the scrapie agent-endomembrane complex from hamster brain. J Virol 1976; 18:693–700.
103. Prusiner SB, Hadlow WJ, Garfin DE, Cochran SP, Baringer JR, Race RE, Eklund CM. Partial purification and evidence for multiple molecular forms of the scrapie agent. Biochemistry 1978; 17:4993–4999.
104. Manuelidis EE. Creutzfeldt-Jakob disease. J Neuropathol Exp Neurol 1985; 44:1–17.
105. Lampert PW, Gajdusek DC, Gibbs CJ Jr, Pathology of dendrites in subacute spongiform virus encephalopathies. Adv Neurol 1975; 12:465–470.
106. Chou SM, Payne WN, Gibbs CJ Jr, Gajdusek DC. Transmission and scanning electron microscopy of spongiform change in Creutzfeldt-Jakob disease. Brain 1980; 103:885–904.
107. Baringer JR, Prusiner SB. Experimental scrapie in mice: ultrastructural observations. Ann Neurol 1978; 4:205–211.

108. Wisniewski HM, Bruce ME, Fraser H. Infectious etiology of neuritic (senile) plaques in mice. Science 1975; 190:1108–1110.
109. Narang HK, Shenton B, Giorgi PP, Field EJ. Scrapie agent and neurones. Nature 1972; 240:106–107.
110. Narang HK. Ruthenium red and lanthanum nitrate a possible tracer and negative stain for scrapie "particles"? Acta Neuropathol (Berl) 1974; 29(1):37–43.
111. Kim JH, Manuelidis EE. Ultrastructural findings in experimental Creutzfeldt-Jakob disease in guinea pigs. J Neuropathol Exp Neurol 1983; 42:29–43.
112. Merz PA, Somerville RA, Wisniewski HM, Iqbal K. Abnormal fibrils from scrapie-infected brain. Acta Neuropathol (Berl) 1981; 54:63–74.
113. Merz PA, Somerville RA, Wisniewski HM, Manuelidis L, Manuelidis EE. Scrapie-associated fibrils in Creutzfeldt-Jakob disease. Nature 1983; 306:474–476.
114. Merz PA, Rohwer RG, Kascsak RJ, Wisniewski HM, Somerville RA, Gibbs CJ Jr, Gajdusek DC. Infection-specific particle from the unconventional slow virus diseases. Science 1984; 225:437–440.
115. Prusiner SB, McKinley MP, Bowman KA, Bolton DC, Bendheim PE, Groth DF, Glenner GG. Scrapie prions aggregate to form amyloid-like birefringent rods. Cell 1983; 35:349–358.
116. Barry RA, McKinley MP, Bendheim PE, Lewis GK, DeArmond SJ, Prusiner SB. Antibodies to the scrapie protein decorate prion rods. J Immunol 1985; 135:603–613.
117. Narang H. The nature of the scrapie agent: the virus theory. Proc Soc Exp Biol Med 1996; 212(3):208–224.
118. Hope J. The nature of the scrapie agent: the evolution of the virino. Ann NY Acad Sci 1994; 724:282–289.
119. Gabizon R, Prusiner SB. Prion liposomes. Biochem J 1990; 266:1–14.
120. Prusiner SB. Molecular biology of prion diseases. Science 1991; 252:1515–1522.
121. McKinley MP, Braunfeld MB, Bellinger CG, Prusiner SB. Molecular characteristics of prion rods purified from scrapie-infected hamster brains. J Infect Dis 1986; 154:110–120.
122. Gabizon R, McKinley MP, Prusiner SB. Purified prion proteins and scrapie infectivity copartition into liposomes. Proc Natl Acad Sci USA 1987; 84:4017–4021.
123. Field EJ, Narang HK. An electron-microscopic study of scrapie in the rat: further observations on "inclusion bodies" and virus-like particles. J Neurol Sci 1972; 17:347–364.
124. Narang HK. An electron microscopic study of the scrapie mouse and rat: further observations on virus-like particles with ruthenium red and lanthanum nitrate as a possible trace and negative stain. Neurobiology 1974; 4(6):349–363.
125. Narang HK, Asher DM, Pomeroy KL, Gajdusek DC. Abnormal tubulovesicular particles in brains of hamsters with scrapie. Proc Soc Exp Biol Med 1987; 184:504–509.
126. Narang HK, Asher DM, Gajdusek DC. Tubulofilaments in negatively stained scrapie-infected brains: relationship to scrapie-associated fibrils. Proc Natl Acad Sci USA 1987; 84:7730–7734.

127. Narang HK. Scrapie-associated tubulofilamentous particles in human Creutzfeldt-Jakob disease. Res Virol 1992; 143:387–395.
128. Narang HK. Scrapie-associated tubulofilamentous particles in scrapie hamsters. Intervirology 1992; 34:105–111.
129. Harrison SC. Principles of virus structure. In: Fields BN, Knipe DM, Chanock RM, Hirsch MS, Melnick JL, Monath TP, Roizman B, eds. Virology. New York: Raven Press, Ltd., 1990:37–61.
130. Bessen RA, Marsh RF. Distinct PrP properties suggest the molecular basis of strain variation in transmissible mink encephalopathy. J Virol 1994; 68:7859–7868.
131. Telling GC, Parchi P, DeArmond SJ, Cortelli P, Montagna P, Gabizon R, Mastrianni J, Lugaresi E, Gambetti P, Prusiner SB. Evidence for the conformation of the pathologic isoform of the prion protein enciphering and propagating prion diversity [see comments]. Science 1996; 274:2079–2082.
132. Bruce ME, Dickinson AG. Biological stability of different classes of scrapie agent. In: Prusiner SB, Hadlow WJ, eds. Slow Transmissible Diseases of the Nervous System. Vol. 2. New York: Academic Press, Inc., 1979:71–86.
133. Bruce ME, Dickinson AG. Biological evidence that scrapie agent has an independent genome. J Gen Virol 1987; 68:79–89.
134. Supattapone S, Bosque P, Muramoto T, Wille H, Aagaard C, Peretz D, Nguyen HO, Heinrich C, Torchia M, Safar J, Cohen FE, DeArmond SJ, Prusiner SB, Scott M. Prion protein of 106 residues creates an artifical transmission barrier for prion replication in transgenic mice. Cell 1999; 96(6):869–878.
135. Supattapone S, Bouzamondo E, Ball HL, Wille H, Nguyen HO, Cohen FE, DeArmond SJ, Prusiner SB, Scott M. A protease-resistant 61-residue prion peptide causes neurodegeneration in transgenic mice. Mol Cell Biol 2001; 21(7):2608–2616.
136. Hunter GD, Millson GC. Studies on the heat stability and chromatographic behaviour of the scrapie agent. J Gen Microbiol 1964; 37:251–258.
137. Clarke MC, Haig DA. Attempts to demonstrate neutralising antibodies in the sera of scrapie-affected animals. Vet Rec 1966; 78:647–649.
138. Chandler RL. Experimental scrapie in the mouse. Res Vet Sci 1963; 4:276–285.
139. Dickinson AG, Meikle VM, Fraser H. Identification of a gene which controls the incubation period of some strains of scrapie agent in mice. J Comp Pathol 1968; 78:293–299.
140. Dickinson AG, Fraser H, Outram GW. Scrapie incubation time can exceed natural lifespan. Nature 1975; 256:732–733.
141. Gajdusek DC. Unconventional viruses and the origin and disappearance of kuru. Science 1977; 197:943–960.
142. Field EJ, Joyce G, Keith A. Viral properties of scrapie. Nature [New Biol] 1971; 230:56–57.
143. Bendheim PE, Brown HR, Rudelli RD, Scala LJ, Goller NL, Wen GY, Kascsak RJ, Cashman NR, Bolton DC. Nearly ubiquitous tissue distribution of the scrapie agent precursor protein. Neurology 1992; 42:149–156.

3

A Simulation Model for Evaluating the Potential for Spread of Bovine Spongiform Encephalopathy in Animals or to People

Joshua T. Cohen, Keith Duggar, George M. Gray, and Silvia Kreindel
Harvard School of Public Health, Boston, Massachusetts, U.S.A.

Hatim Gubara, Tsegaye HabteMariam, David Oryang, and Berhanu Tameru
School of Veterinary Medicine, Tuskegee University, Tuskegee, Alabama, U.S.A.

I. INTRODUCTION

In 1998 the U.S. Department of Agriculture asked the Harvard Center for Risk Analysis to evaluate the robustness of U.S. measures to prevent the spread of bovine spongiform encephalopathy (BSE, or "mad cow disease") to animals and humans if it were to arise in this country. BSE is a member of a family of diseases that includes scrapie in sheep and goats, chronic wasting disease in certain North American deer and elk, transmissible mink encephalopathy, and the human ailments Creutzfeldt-Jakob disease, variant Creutzfeldt-Jakob disease, and kuru.

Because nobody has identified BSE-infected cattle in the United States, there are no empirical data that can be used directly to predict how the disease might spread here. We have therefore developed a probabilistic simulation model to help characterize the consequences of introducing BSE into the United States. Our model allows us to predict, for

example, the number of newly infected animals that would result from the introduction of BSE, the time course of the disease following its introduction, and the potential for human exposure to infectious tissues. We can also evaluate key processes and procedures that make the spread of disease more or less likely. Results from the simulation are presented as probability distributions reflecting the probabilistic nature of the model and the processes simulated.

The ability of this model to quantify various aspects of the disease's progression (e.g., the number of animals infected over time, and the amount of transmissible agent in food available for human consumption) distinguishes it from other efforts to characterize BSE risk, such as the European Union's Scientific Steering Committee report on the Geographical Risk of Bovine Spongiform Encephalopathy (1). We have used the simulation model to identify those risk management control options that most influence the spread of disease, and to identify those sources of uncertainty that have the greatest impact on our results. This information can be used to help identify the most promising control measures and to prioritize data collection and research efforts. This chapter describes the BSE model, its predicted consequences for the hypothetical introduction of from one to 500 infected cattle into the United States, and a sensitivity analysis that identifies those assumptions contributing the most to the uncertainty in the model's results. Section II describes our methodology, Section III outlines the scenarios evaluated, and Section IV details our results.

II. MODEL DESCRIPTION AND BASE CASE ASSUMPTIONS

Although the agent responsible for BSE has not been conclusively identified, the leading theory suggests that it is caused by an abnormally configured prion protein that is normally found in the host. In particular, the abnormal form of the protein has the same amino acid sequence as the normal form of the protein, but is folded up differently. The abnormal prion that causes BSE has the following important properties. First, once it is introduced into an animal, it "recruits" additional normal prions, converting them to the abnormal configuration. When a sufficient quantity of these proteins are so converted, they can form lesions that damage tissue and cause disease. Second, the abnormally configured proteins are particularly robust against normal methods of disinfection, including the use of heat to destroy proteins.

The largest outbreak of BSE occurred in the United Kingdom during the late 1980s and early 1990s. Although the origin of the disease is not clear, it appears that the properties just described, together with the

recycling of rendered cattle tissue as cattle feed, were responsible for its rapid spread. In particular, when infected animals were slaughtered, those parts not used for human food were rendered (i.e., ground up and cooked) and ultimately used as protein supplements in cattle feed. Scientists believe that because of the resilience of the abnormally folded prion, a sufficient portion often survived this processing and ultimately infected those cattle consuming this feed.

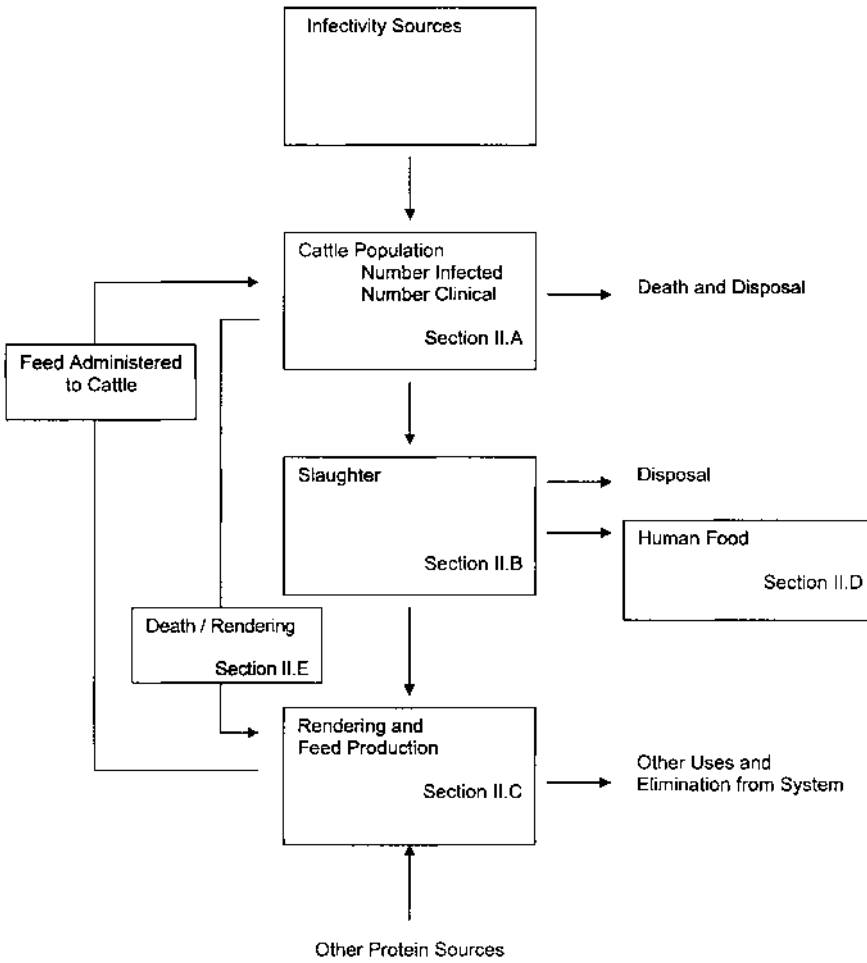

Figure 1 Simulation overview.

Our model reflects the assumption that the recycling of rendered cattle tissue as feed provides a means by which BSE can be spread from one animal to another, although we also allow for the possibility that the disease can also be spread directly from an infected female to her offspring.

The simulation model can be thought of as having five components, as illustrated in Fig. 1. The first component (Section II.A) characterizes the lifecycle of cattle in the United States, including the rates at which they are born, slaughtered, become infected with BSE, or die of natural causes unrelated to BSE. The second component of the model (Section II.B) describes how animals sent to slaughter are processed. Tissue may be disposed of, sent to rendering, or prepared for potential human consumption. The third component of the model (Section II.C) characterizes the disposition of material sent to rendering. That material may be diverted to uses that eliminate the possibility of exposing domestic cattle or the U.S. population (e.g., because it will be disposed of, exported, or used to produce feed for animals other than cattle) or end up in feed that is administered to cattle. The fourth component of the model (Section II.D) quantifies infectivity in material presented for potential human consumption. The final component (Section II.E), which is not represented in Fig. 1, quantifies the proportion of cattle that die prior to being sent to slaughter that are rendered.

A. Cattle Population Dynamics

Figure 2 illustrates the cattle population dynamics component of the simulation model. In particular, this component describes the rate at which cattle are born, the rate at which animals are slaughtered, and the rate at which they die of other causes. Cattle can become infected when they are born as a result of maternal transmission. Alternatively, they can be born uninfected but become infected later by consuming BSE-contaminated feed. Infected animals may proceed to the clinical stage of the disease. Alternatively, they may be slaughtered, or die of other causes. Likewise, animals displaying clinical signs may also be slaughtered or die of other causes, including BSE. This analysis assumes BSE infectivity is introduced into the United States by the import of infected cattle (Section III. A).

The model includes a detailed characterization of the cattle population dynamics because many of the rates influencing disease prevalence and transmission depend on animal age, type (beef or dairy), and gender. For example, the risk of infection via exposure to contaminated feed depends on how much recycled meat and bone meal (MBM) animals consume, and this consumption depends in turn on animal age, type, and gender because these factors influence protein requirements. Infection risk

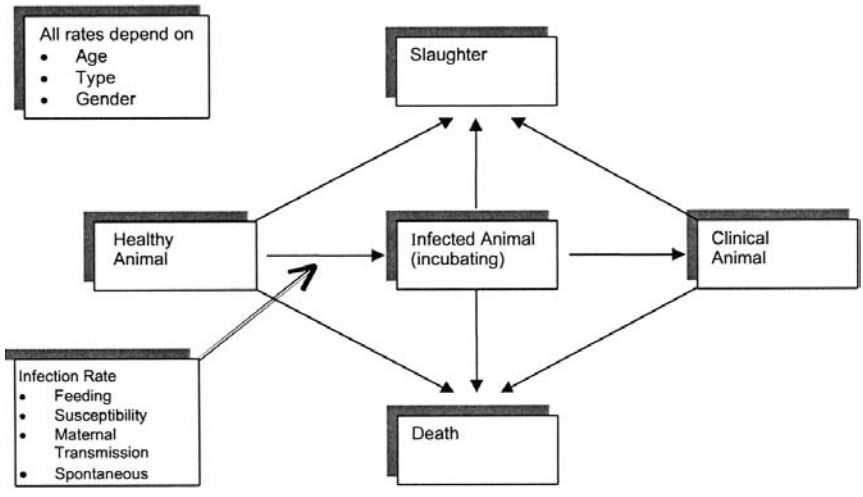

Figure 2 Cattle population dynamics.

also depends on age because young animals appear to be inherently more susceptible to infection than older animals. That is, the same level of exposure is more likely to cause a young animal to become infected than it is to cause an older animal to become infected. Finally, age influences disease transmission because it is related to the probability that an animal will be culled for slaughter, an event that can lead to the recycling of its remains as cattle feed.

The remainder of Section II.A summarizes the following base case assumptions: (1) the number of animals in the U.S. cattle population by age, gender, and type (i.e., dairy or beef, destined for production or breeding), their birth rate, slaughter rate, and rate of death from other causes; (2) cattle consumption of bypass protein and blood meal by age, type, and gender; (3) the dose-response relationship for cattle orally exposed to BSE and the influence of age on this relationship; (4) the rate at which infected cows transmit BSE to their offspring; and (5) the incubation period for BSE (i.e., the time between infection and when clinical signs become apparent) and the time until death following the development of clinical signs.

1. Cattle Population Size, Birth Rates, Slaughter Rates, and Rates of Death Due to Other Causes

The simulation developed for this analysis requires specification of the number of animals by age in months for each gender within each of three

animal classes: dairy, beef, and beef reproductive animals. This last group represents those beef cattle that live beyond the age of 24 months for the purpose of providing beef calves.

Developing this information was complicated by the fact that available data sources do not describe the age distribution in sufficient detail, and in some cases, combine groups that must be characterized separately for the simulation. For example, statistics published by the Food Safety and Inspection Service (FSIS) Animal Disposition Reporting System (ADRS) (2) report the slaughter rate for dairy and beef cows combined, rather than for each group separately. Further complicating the development of this information is the fact that some of the reported statistics do not appear to be consistent with each other. For example, as explained below, the reported number of steers and heifers slaughtered is consistent with birth rates that imply a total cattle population of 140 million, rather than the commonly cited value of approximately 100 million. When forced to diverge from reported statistics for the purpose of maintaining internal consistency, we do so in ways that minimize the impact of distortions on the validity of the simulation results. In the example described in this paragraph, our inflation of the U.S. cattle population should have a minimal impact on simulation results because the rate at which BSE spreads does not in general depend on this statistic.

The possibility of spontaneous development of BSE is the one exception to this generalization because its rate is proportional to the size of the population. By overstating the size of the population, we would therefore overstate the potential impact of spontaneous disease, should it exist.

a. Population Size. Figure 3 plots population size by age for each gender/type combination. Diamonds correspond to the population of production beef animals (i.e., those slaughtered at 24 months to produce food for human consumption), squares correspond to the population of reproductive beef animals, and triangles correspond to the population of dairy animals. Closed symbols correspond to males, and open symbols correspond to females.

We used a spreadsheet to ensure that our population estimates were reasonably consistent with our information regarding birth, death, and slaughter rates (see below), and the assumption that the population size remains stable over time. Our starting point for development of these estimates was the statistics reported by USDA. Our use of these statistics is further documented in Cohen et al. (Appendix 1, Sections 2.9 and 3.9) (3). Because the population we developed was not precisely stable, its size decreased to around 130 million during the simulation. As noted above, this change has a very limited impact on the simulation results.

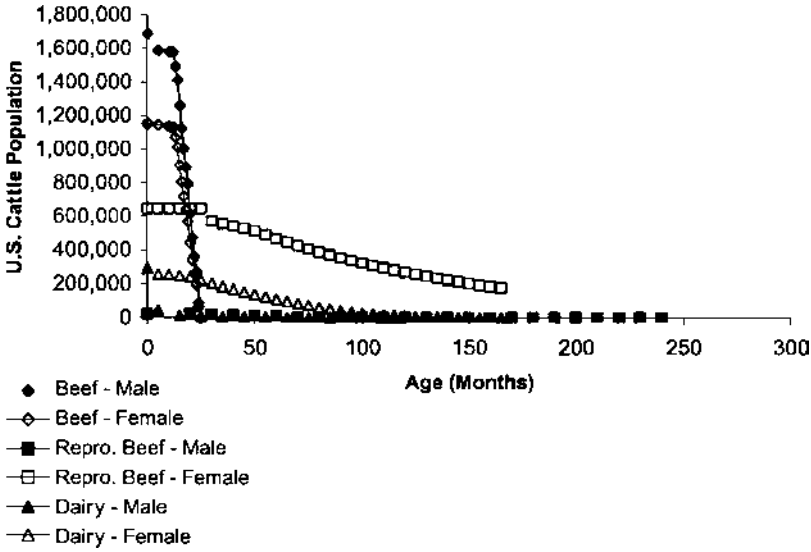

Figure 3 Estimated cattle population size.

b. Slaughter Rate. The rate at which animals are slaughtered depends on their age, type, and gender. We developed estimates for these rates using primarily data from USDA (2) and information from Radostits et al. (4) [see Appendix 1, Sections 2.15 and 3.15 in Cohen et al. (3) for further details]. Figure 4 plots the monthly slaughter rates assumed.

c. Rate of Death for Reasons Other than Slaughter and BSE. The so-called "natural death rate" reflects causes of death due to factors such as disease other than BSE. This rate can influence the transmission of disease for several reasons. First, BSE-infected animals that die due to natural causes prior to reaching clinical status have a smaller quantity of the BSE infective agent than animals that die only after reaching clinical status. Second, the disposition of animals that die due to disease (BSE or otherwise) differs from the disposition of animals that are slaughtered. In particular, animals sent to slaughter are subjected to antemortem inspection prior to slaughter and further processing. That step provides inspectors an opportunity to catch any animals that may be showing signs of BSE, a finding that would lead to the disposal of the animal that would virtually eliminate any chance of exposing other animals. Of course, animals that die prior to being sent to slaughter do not undergo antemortem inspection. A certain fraction of those animals are sent directly to rendering, while others are disposed of in a manner that

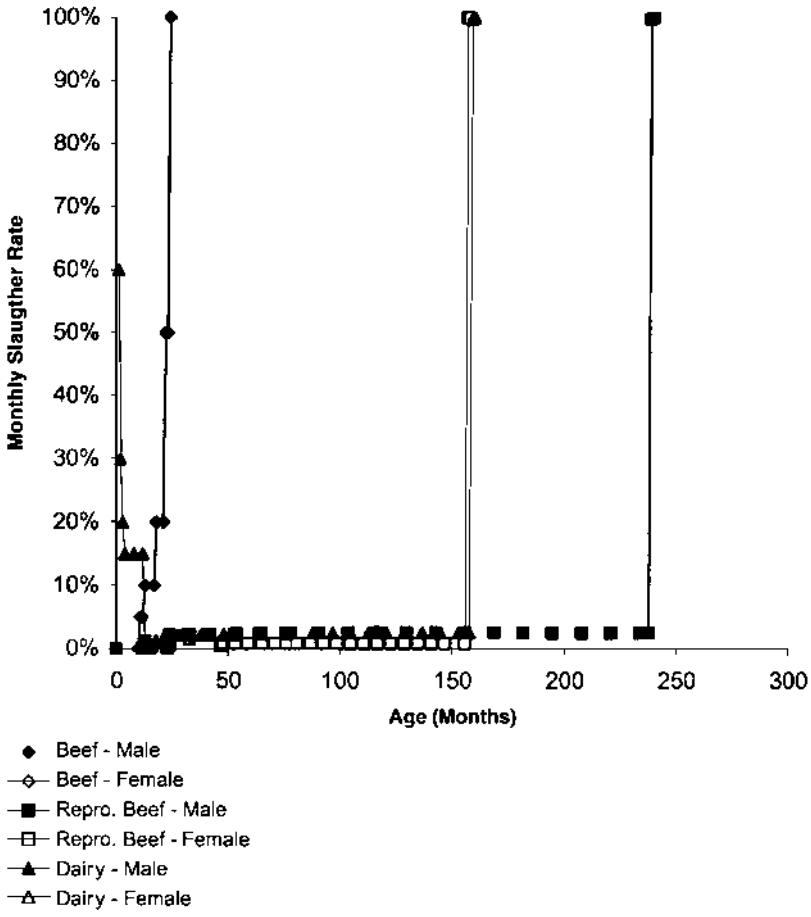

Figure 4 Monthly slaughter rate.

virtually eliminates the possibility of further exposing additional animals (e.g., the carcass may be buried on the farm).

We assume that the rate at which animals die of natural causes other than BSE depends only on age. We developed these rates using information and data from USDA (5,6) and from Radostits et al. (4). Table 1 details the rates we used.

d. Birth Rate Assumptions. The base case assumes that female cattle can calve between the ages of 24 and 180 months. During that time, we assume that they produce one calf once every 12 months on average.

Table 1 Assumed Monthly Death Rate for Causes Other than Slaughter or BSE

Age	Probability of death
0	0
1	0.05
2–33	0.001
34–39	0.0015
40–59	0.003
60–238	0.01
239	1

2. Cattle Consumption of Bypass Protein and Blood Meal

MBM is one source of supplementary protein for livestock feed, although in the United States, other sources are also used, especially vegetable protein derived primarily from soybeans. Cattle also consume blood meal. The amount of blood meal and bypass protein-supplemented feed consumed by an animal depends on the animal's age, type, and gender. For example, dairy cows receive the greatest amount of supplemental bypass protein. Table 2 lists approximate consumption rates (g/day) for bypass protein and blood meal assumed by the base case. Appendix 1 Sections 2.3, 2.13, 3.3, and 3.13 in Cohen et al. (3) provide a more precise description of these rates. Bypass consumption rates were developed using information from Ensminger et al. (7) and the National Research Council (8). Blood meal consumption rates were developed using information from L. Satter (personal communication, USDA-ARS U.S. Dairy Forage Research Center. University of Wisconsin, Madison) and J. Quigley (personal communication, Director of Research and Product Development, APC Company, Inc. Ames, IA).

3. BSE Dose-Response

The dose-response relationship for BSE quantifies the probability that an exposed animal will become infected with BSE as the result of ingesting contaminated materials. The exposure is quantified in terms of the number of susceptibility-adjusted cattle oral ID_{50}s ingested. The susceptibility-adjusted ID_{50} exposure equals the product of an age-specific susceptibility factor and the number of unadjusted ID_{50}s ingested. The base case assumes that the dose-response is linear up to an exposure level of 2.0 adjusted cattle oral ID_{50}s, with an infection probability of zero at an ex-

Table 2 Assumed Daily Consumption of Bypass Protein and Blood Meal

	Consumption (g/day)	
	Bypass protein	Blood meal
Production beef		
Male	0–7 months—0	0–7 months—0
	8–14 months—250 g/day	8–14 months—2.5
	≥15 months—0	≥15 months—0
Female	Same as male	Same as male
Reproductive beef		
Male	0–6 months—0	All ages—0
	7–12 months—100	
	13 months—150	
	≥14 months—0	
Female	Same as male	Same as male
Dairy		
Male	0–6 months—0	0–6 months—0
	7–12 months—100	7–12 months—1
	13 months—150	13–168 months—1.5
	≥14 months—0	≥169 months—0
Female	0–12 months—170	0–12 months—3.3
	13–20 months—0	13–22 months—0
	≥21 months—250	≥23 months—13.3

posure level of zero, and an infection probability of 1.0 at an exposure level of 2.0 adjusted ID_{50}s. For example, an animal that ingests 1.0 susceptibility-adjusted ID_{50}s has a 50% chance of becoming infected. Note that an animal that ingests more than 2.0 adjusted ID_{50}s has a 100% chance of becoming infected.

Our relationship between susceptibility and age (see Fig. 5) is based on the assumption that susceptibility peaks at age 4 months and that it declines exponentially thereafter at a rate of 85% per year, leveling off at approximately 10% of its peak value (9) [see their Eq. (7)]. For animals age 4 months and older, susceptibility [β(a)] is $\beta(a) = 0.1 + 1.8 \times e^{-2a}$, where a is age in years.

4. Maternal Transmission

Although there is no direct evidence of BSE transmission from cow to calf, it is assumed to have occurred when a calf born to a cow incubating BSE contracts the disease in the absence of any other known sources of BSE exposure. There is some evidence that maternal transmission occurs in the

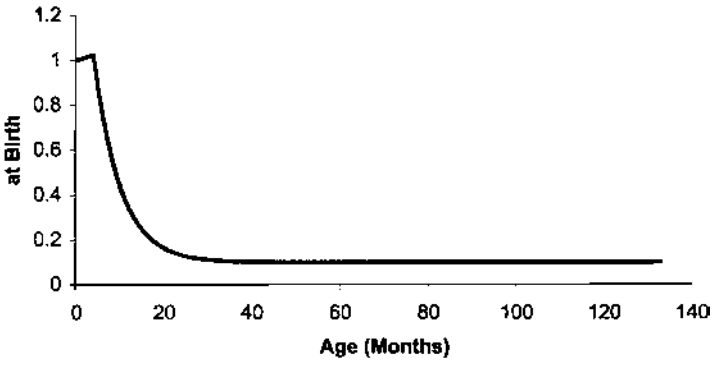

Figure 5 Age-specific relative susceptibility to BSE infection.

case of other TSEs, with the best evidence suggesting that it occurs in the case of scrapie (10–12). Evidence for maternal transmission in the case of BSE comes from several sources (13–17).

The base case assumes that infected cows can transmit BSE to their calves only during the last one-sixth of their incubation period. We assume that a calf born to a cow during this period becomes infected with 10% probability. These assumptions are based on information from the UK Department for Food, Environment, and Rural Affairs (18) and from the Spongiform Encephalopathy Advisory Committee (19).

5. The BSE Incubation Period, and Time Until Death Caused by BSE

The base case assumes that the duration between infection and manifestation of clinical signs follows a distribution inferred by Ferguson et al. (20) from data collected in the United Kingdom. The density is defined by the function $f(t) = \left(\frac{\alpha_2 e^{-t/\alpha_1}}{\alpha_3}\right)^{\frac{\alpha_2^2}{\alpha_3}} e^{\frac{-\alpha_2 e^{-t/\alpha_1}}{\alpha_3}}$, where t is the incubation period in years. The distribution is right-skewed with a 5th percentile of approximately 2.5 years, a median of approximately 4 years, and the 95th percentile of approximately 7 years. Sections 2.17 and 3.17 in Appendix 1 of Cohen et al. (3) provide further detail.

The base case assumes that the time between the manifestation of clinical signs and death is uniformly distributed between 2 and 6 months. Additional information used in the development of this assumption was provided by D. Heim (personal communication, Swiss Federal Veterinary Office, Bern-Liebefeld, Switzerland).

B. The Slaughter Process

The slaughter of an animal with BSE can contaminate tissues destined for potential human consumption with BSE infectivity. In addition, many tissues not used for human consumption go to rendering and may become available to infect other bovines (Section II.C). This section describes the base case assumptions for the slaughter process (Fig. 6). It also describes ways in which infectivity can be diverted from uses that may result in either human or bovine exposure.

1. Level of Infectivity and Distribution of Infectivity Throughout the Carcass

The amount of infectivity that becomes available for potential human consumption or ends up being recycled into cattle feed depends in part on the total amount of infectivity in a slaughtered animal and how that infectivity is distributed through its carcass. Our model assumes these

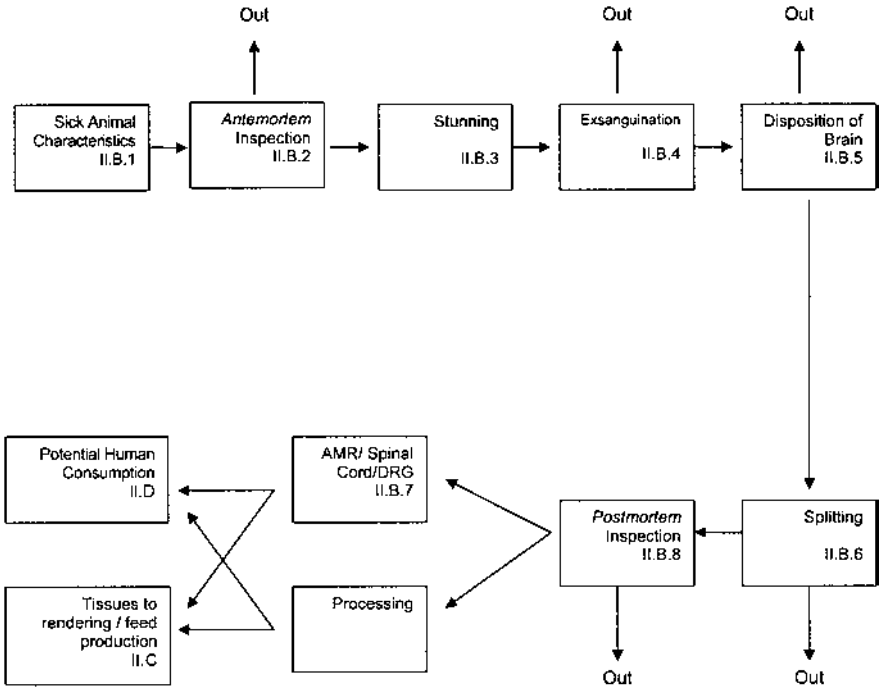

Figure 6 The slaughter process.

factors depend on the amount of time that has passed since the slaughtered animal became infected.

Our description of the distribution of infectivity among the tissues of an infected animal is based on the pathogenesis experiment conducted in the United Kingdom (21,22). This experiment measured the infectivity in each of 44 tissues and fluids after experimental infection of cows with BSE. The experiment found infectivity in the small intestine from 6 to 18 months postinfection, with no detectable infectivity in any other tissues. At the end stage of disease (≥32 months postinfection), infectivity was found in the brain, spinal cord, dorsal root ganglia (DRG), trigeminal ganglia (TGG), and again in the small intestine. We assume that findings of infectivity in bone marrow at one time point were spurious. Table 3 details our specific assumptions.

Note that these assumptions are based on an assumed incubation period of 36 months (as observed in the pathogenesis study). For animals with incubation periods of duration other than 36 months, the time periods postinoculation are scaled accordingly. For example, for an animal with an incubation period of 72 months, there is no infectivity in the brain prior to month 64 (i.e., $32 \times 72/36$).

Table 3 Relative Infectivity of Specific Tissues Specified from an Infected Bovine (Based on SSC, 1999a)[a]

Tissue	Fraction of total infectivity
Brain	No infectivity in cattle <32 months postinoculation (PI) 32 months PI and over: 64.1%
Trigeminal ganglia	No infectivity in cattle <32 months PI 32 months PI and over: 2.6%
Other head (eyes, etc.)	No infectivity in cattle <32 months PI 32 months PI and over: 0.04%
Distal ileum	6–18 months PI: 100% 18–31 months: No infectivity 32 months PI and over 3.3%
Spinal cord	No infectivity in cattle <32 months PI 32 months PI and over: 25.6% infectivity
Dorsal root ganglia	No infectivity in cattle <32 months PI 32 months PI and over: 3.8% infectivity

[a] The postinoculation time values in this table reflect the assumption that the incubation period is 36 months. See text for explanation.

The base case assumes that the total quantity of infectivity in an animal with BSE reaches its maximum level when the animal develops clinically detectable signs (i.e., becomes "clinical") (see Section II.A.5 for a discussion of the incubation period duration). Prior to that time, the total level of infectivity follows the pattern illustrated in Fig. 7. In this example, the animal develops clinical signs 36 months after infection. During the first 5 months of infection, total infectivity in the animal is approximately 0.1% of its maximum value, followed by an increase to approximately 2.5% of its maximum value between 6 and 18 months postinfection. Up until this point, all infectivity is assumed to be in the gut. Starting in month 19, infectivity is assumed to be distributed among several tissues, with the bulk in the brain and the spinal cord, and the remainder divided among the gut, DRG, eyes, and TGG. At this time, total infectivity drops to zero and then grows exponentially until it reaches its maximum level in month 36. For incubation periods other than 36 months, the model scales the horizontal axis (time) in Fig. 7 proportionally. The total amount of infectivity in an animal with clinical BSE is assumed to be 10,000 cattle oral ID_{50}s (23–25). Note that this value has not been adjusted to reflect age-specific susceptibility (see Section II.A.3).

2. Antemortem Inspection

Once the animal is at the slaughter facility, it is inspected for signs of disease. FSIS regulations require that for certain diseases the whole animal is condemned at antemortem (AM) inspection (26). Condemned animals can be rendered or incinerated.

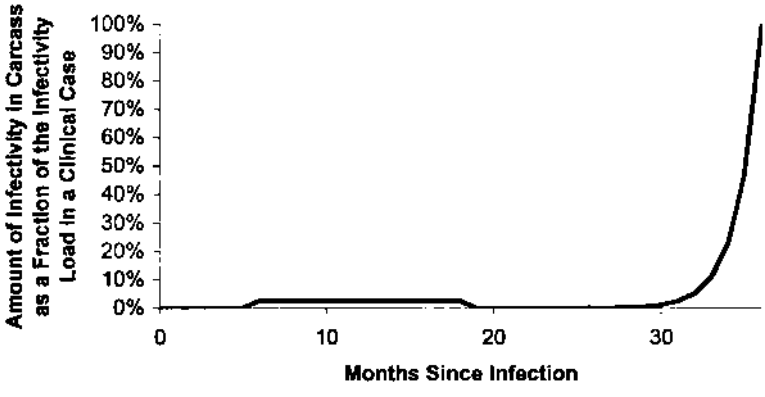

Figure 7 Relationship between time since infection and the fraction of the maximal level of ID_{50}s.

Animals not showing clinical signs during AM inspection are not likely to be condemned for BSE but could be condemned if they show signs of other diseases. The condemnation rates for animals not showing clinical BSE signs depend on age and gender. The rates used in the base case are based on data collected by FSIS for the year 1998 (see Table 4). In particular, the base case assumes that the AM condemnation rate is approximately 1% for animals less than 1 year of age, 0.01% for animals between the ages of 1 year and 31 months, and 0.2% for animals more than 31 months of age.

Animals condemned following AM inspection are usually rendered, although a small proportion are incinerated. The base case assumes that 98% of condemned animals are rendered and that the rest are incinerated. The likelihood that an animal condemned during AM inspection is rendered or incinerated is assumed to be independent of its BSE status. The means of disposal is important because we assume that animals that are incinerated cannot contaminate human food or animal feed.

Animals that do manifest the clinical signs of BSE can be identified by AM inspectors. Because of the implications of a BSE case, from eradication of a herd to drops in beef consumption, it is also possible that farmers might hold back from slaughter animals with BSE signs to prevent the case from being discovered. Although USDA has trained inspectors to make them aware of BSE's signs, it is difficult to estimate how effectively these inspectors would be because the disease has not been detected in this country. Surrogate detection rate estimates cannot be developed using data for other CNS diseases because their true prevalence rates are unknown. Our base case assumes that AM inspectors would detect clinical BSE cases 90% of the time. Because this value is highly uncertain, our sensitivity analysis evaluates the impact of using a wide range of values on the results of our simulation (see Section III.B). The assumptions for AM inspection were developed using information from the USDA Animal Disposition Reporting System (ADRS)

Table 4 Cattle Slaughthered and Condemned (1998)[a]

Cattle age group	Total animals slaughtered	Total animals condemned	Antemortem failure probability	Animals Condemned postmortem	Postmortem failure probability
<12 months	1,483,430	14859	0.0098	13,799	0.0092
12–24 months	32,690,003	2,349	0.0001	22,697	0.0007
≥24 months	7,815,074	21,906	0.0028	11,0172	0.0139

[a] From USDA: Animal Disposition Reporting System.

for the year 1998 (2), Heim and Wilesmith (27), Miller et al. (28), and from D. Heim (personal communication, Swiss Federal Veterinary Office, Bern Liebefeld, Switzerland).

3. Stunning

Stunning humanely renders animals unconscious for slaughter. It is usually performed by mechanical devices, most commonly captive bolts that may or may not penetrate the skull. One type of penetrating captive bolt is referred to as an "air-injected pneumatic stunner" because it injects a jet of air into the brain at the end of the cylinder stroke. Because of the high pressure generated inside the skull by these devices, their use can result in the deposition of central nervous system (CNS) tissue emboli in the blood, heart, lung, and liver. Malfunctions in these devices increase both the probability that emboli will be created and the amount of emboli that will be deposited. However, based on our conversations with USDA personnel (in headquarters and in the field), individuals in the beef packing industry, and others, the base case assumes that air-injected stunning is not currently used in the U.S. cattle industry. Other scenarios evaluating past practices do assume the use of air-injected pneumatic stunning (see Section III.B.2).

There is also some concern that other stunning methods may produce CNS microemboli that could contaminate blood (29). The base case assumes that stunners *not* using air injection can create very small emboli that are found only in blood. The amount of emboli in the blood is not affected by whether the stunner malfunctions.

These assumptions were developed using information from Anil et al. (30), Garland et al. (31), Schmidt et al. (32), N. Bauer (personal communication, Veterinary Scientific Liaison. USDA FSIS. Washington, DC), R. Brewer (personal communication, Associate Deputy Assistant Secretary, Office of Industrial Technologies, USDA FSIS. Washington, DC), C. Shultz (personal communication, USDA FSIS Veterinarian-in-Charge at Taylor Packing, Wyalusing, PA), and C. White (personal communication, Area Supervisor. USDA FSIS. Pickerington, OH). Sections 2.19 and 3.19 in Appendix 1 of Cohen et al. (3) discuss the development of these assumptions in further detail.

4. Exsanguination

After stunning, animals are bled. Bovine blood can be processed for human consumption, processed to make blood meal that can be used in ruminant feed, rendered, or disposed of. The base case assumes that 15% of blood is used to make blood meal that has the potential to be used in cattle feed.

The base case also assumes that blood collected for human consumption is not contaminated with emboli.

Blood collected to produce meal for animal consumption may become contaminated with CNS tissue if some of that tissue drips from the hole created by the stunner. The base case assumes that air-injected pneumatic stunners generate this type of contamination with 30% probability, and that when this contamination does occur, 4% of the infectivity in the brain ends up in the blood being collected. The base case assumes that stunners that do not use air injection never cause this type of contamination. This assumption was developed based on information from N. Bauer (personal communication, Veterinary Scientific Liaison. USDA FSIS. Washington, DC) and C. Shultz (personal communication, USDA FSIS Veterinarian-in-Charge at Taylor Packing, Wyalusing, PA).

5. Disposition of the Brain

After exsanguination, the head is removed from the carcass. USDA has mandated inspection of some parts of the head that are collected for human consumption (e.g., the tongue). Because brain is the tissue with the greatest amount of infectivity in an animal with advanced BSE, the disposition of the head is important. There are no available data on the fraction of brains collected for sale as human food. The base case assumes that 1% of the brains are removed for potential human consumption in the United States and that the rest are rendered.

6. Splitting and Aerosolization

After removal of the head, the carcass is split longitudinally with a saw to facilitate handling and further processing. When the carcass is split, some spinal cord is aerosolized and can contaminate edible meat. Based on data from experiments that measured the amount of spinal-cord-associated protein deposited on the carcass during splitting (33), the base case assumes that approximately 0.001% (2.5 mg) of the spinal cord contaminates edible meat. The base case further assumes that additional carcass treatments, like washing and steaming, do not reduce the amount of contamination.

7. Disposition of the Spinal Cord and Dorsal Root Ganglia

The vertebrae of the animal are arranged in a column that houses and protects the spinal canal. Because the spinal cord and the dorsal root ganglia (DRG), which are nerve ends emerging from the spinal cord, can contain BSE infectivity, their disposition influences the extent to which meat re-

covered for human consumption may become contaminated. The magnitude of this contamination and which selections of meat become contaminated depend on whether a missplit occurs (i.e., the spinal column is not completely split by the saw operator), whether the slaughter plant uses automated technology referred to as advanced meat recovery (AMR) to extract meat from bones, and whether the plant removes the spinal cord from the carcass. The extent to which AMR product becomes contaminated is particularly sensitive to missplits because they can leave behind pieces of spinal cord encapsulated in the vertebral column that are processed by AMR. This section first discusses the frequency of missplits, the proportion of carcasses processed using AMR, and the proportion of carcasses from which the spinal cord is removed. Finally, it discusses how missplitting, AMR, and spinal cord removal influence contamination. Table 5 summarizes the frequency of these events and practices.

a. Missplit Frequency. Missplitting, which refers to the incomplete cutting of the spinal column with a saw, occurs when the cut veers off the vertical and terminates at a point short of the cervical vertebrae (carcasses are split caudal to cranial). The likelihood that missplitting will occur depends on the size and age of the animal (e.g., calves are less likely to be missplit than bulls or cows) and the proficiency of the saw operator. The rate and extent of missplitting influences the potential for spinal cord from an infected animal to contaminate human food, primarily in the AMR process. The base case assumes that among animals below the age of 24 months, missplits occur 5% of the time, whereas for older animals, missplits occur 8% of the time.

b. The Proportion of Cattle Processed Using AMR. Once the carcass is split, the disposition of the spinal cord depends on whether the slaughter facility processes the vertebrae using AMR. AMR machines process bones to recover meat remaining after the hand deboning process is completed. USDA rules allow the AMR product to be labeled as "meat." Approximately 70% of fed cattle and 60% of cows are processed in facilities that use AMR (34). The base case assumes that AMR is used to process no animals below the age of 12 months, 65% of animals between the ages of 12 and 23 months, and 60% of animals 24 months of age or older.

c. Spinal Cord Removal—Plants That Use AMR. An FSIS directive requires that the spinal cord be removed from the vertebral column before the backbones enter the AMR process. The base case assumes that spinal cords are removed with 98% probability in plants using AMR. Spinal cords removed in this manner are rendered. In the event that spinal cord is not removed prior to AMR, it can contaminate the AMR product,

Table 5 Calculation of Probabilities for Combinations of Factors Influencing Contamination of Meat by Spinal Cord and Dorsal Root Ganglia

	Marginal probabilities			
Age	Missplit	AMR	Spinal cord removal	Joint probability
0–11 months	No—95%	No—100%	No—50%	47.5%
			Yes—50%	47.5
		Yes—0%	No—2%	0%
			Yes—98%	0%
	Yes—5%	No—100%	No—50%	2.5
			Yes—50%	2.5%
		Yes—0%	No—2%	0%
			Yes—98%	0%
12–23 months	No—95%	No—35%	No—50%	16.625%
			Yes—50%	16.625%
		Yes—65%	No—2%	1.235%
			Yes—98%	60.515%
	Yes—5%	No—35%	No—50%	0.875%
			Yes—50%	0.875%
		Yes—65%	No—2%	0.065%
			Yes—98%	3.185%
≥24 months	No—92%	No—40%	No—50%	18.4%
			Yes—50%	18.4%
		Yes—60%	No—2%	1.104%
			Yes—98%	54.096%
	Yes—8%	No—40%	No—50%	1.6%
			Yes—50%	1.6%
		Yes—60%	No—2%	0.096%
			Yes—98%	4.704%

although the probability of this occurring is small. In addition, if the carcass is missplit, the spinal cord that remains encapsulated in the spinal canal (usually a small portion of the spinal cord) contaminates AMR product unless it is removed by facility personnel. Whether an AMR processing system is used depends on the size and age of the animal (e.g., AMR is not often used to process calves). The amount of spinal cord left behind that can contaminate edible meat also depends on the age and type of the animal (e.g., for steers and heifers, the lumbar area does not go through AMR because T-bone steaks are highly profitable).

d. Spinal Cord Removal—Plants That Do Not Use AMR. If a facility does not use AMR, FSIS does not require removal of the spinal cord from

the carcass. However, some slaughterhouses choose to remove it and send it to rendering. The base case assumes that spinal cords are removed with 50% probability in plants that do not use AMR. If the spinal cord is not removed, it remains in certain cuts of beef and is hence available for potential human consumption (e.g., T-bone steak). In addition, spinal cord left in the carcass can contaminate the boning table. Finally, a small fraction of the spinal cords removed from steers and heifers is destined for human consumption.

e. Fraction of Spinal Cord and DRG That Contaminate Meat Recovered for Human Consumption. We consider the potential contamination of muscle meat, AMR product, and bone-in cuts of meat by infectivity. Section 3.18.3 in Appendix 1 of Cohen et al. (3) details the proportion of infectivity in DRG and spinal cord that are deposited in each of these types of meat. Table 6 describes their sum contribution to all tissues presented for human consumption, after which we summarize the key factors that went into the determination of these values.

The DRG are firmly attached to the bones of the spinal column and are not removed even if the spinal cord is removed. The disposition of the DRG depends on the cuts of beef recovered for human consumption (which depends on the age of the animal) and on the use of AMR processing systems. For example, some cuts of meat from young animals,

Table 6 Fraction of Spinal Cord Infectivity and DRG Infectivity Deposited in Tissues Presented for Human Consumption

Slaughter house practices and split outcome: AMR used-Spinal cord removed-Spinal cord missplit	Proportion of spinal cord deposited in tissue available for human consumption			Proportion of DRG deposited in tissue available for human consumption		
	Animal age (months)			Animal age (months)		
	0–12	13–24	>24	0–12	13–24	>24
No-No-No	0	0.301	0.002	0	0.301	0
No-No-Yes	0	0	0	0	0.3	0
No-Yes-No	0	0.651	0.501	0	0.651	0.5
No-Yes-Yes	0	0	0	0	0.65	0.5
Yes-No-No	0	0.301	0.001	0	0.301	0
Yes-No-Yes	0	0	0	0	0.3	0
Yes-Yes-No	0	0.645	0.462	0	0.651	0.5
Yes-Yes-Yes	0	0.006	0.009	0	0.65	0.5

such as steers and heifers, might be sold with the vertebrae attached (e.g., T-bone steaks), and in those particular circumstances DRG can reach the consumer. However, even if DRG reaches the consumer, it is unlikely to be consumed unless the bone is aggressively cleaned. In other regions of the vertebral column, DRG will remain attached to the bone because they are unlikely to be removed by standard deboning operations. The vertebrae and DRG from young animals are likely to be rendered. For older animals (e.g., bulls and cows) that are deboned by hand, DRG will not reach the consumer and will instead be rendered.

If the spinal column is processed using AMR, the DRG are likely to contaminate the AMR product. For young animals, only a fraction of the vertebral column and DRG will be processed using AMR because parts of the backbone are contained in high-value bone-in cuts of meat. For older animals, such as bulls or cows, all vertebrae are likely to be processed using AMR. If the facility does not process the spinal column using AMR, other technology, such as vibration or handheld knives (e.g., Whizzard knives), are used to recover the remaining meat attached to the bones. Because of the location of the DRG and the presentation of the backbones on the boning table, these knives are unlikely to incorporate DRG or spinal cord into meat or ground beef.

8. Postmortem Inspection

Organs and tissues from cattle passing AM inspection are inspected post-mortem (PM) to ensure fitness for human consumption. FSIS regulations require that the whole animal be condemned when certain diseases are suspected, while for other diseases and conditions, only some tissues are excluded from use in human food. Because BSE infection does not cause lesions that would be visible during PM inspection, the base case assumes that BSE status has no influence on the probability of failing this inspection.

On the other hand, signs indicative of other diseases and problems can result in the condemnation of tissues during PM inspection regardless of the presence of BSE. The base case assumes that the condemnation probability depends on the type of tissue and whether there are emboli present in the tissue. Table 7 summarizes these probabilities.

C. Rendering and Feed Production

The rendering process recovers useful materials like fat, tallow, and protein by cooking the animal remains, separating the products, and further processing and purifying the resulting meat and bone meal (MBM). MBM is a rendering product rich in protein that can be used as a feed

Table 7 Postmortem Tissue Condemnation Probabilities

Emboil present	Organs	Condemnation probability
No	Blood, muscle, bone, trigeminal ganglia	2%
	Brain, spinal, cord, DRG	10%
	Heart, liver, kidney, ileum, eyes	20%
	Lung	100%
Yes	Blood, muscle, bone, trigeminal ganglia	2%
	DRG	10%
	Heart, liver, kidney, ileum, eyes	20%
	Brain, spinal cord, lung	100%

supplement, among other uses. If the remains of an infected animal, including either a sheep with scrapie or a bovine with BSE, are made into MBM that is then fed to cattle, additional animals can become infected.

Current regulations in the United States (35) prohibit the feeding of mammalian-derived protein to other ruminants with some exceptions. The feed ban does not restrict the use in ruminant feed of porcine protein, equine protein, ruminant blood, ruminant milk, plate waste, or gelatin. Other sources of protein, primarily of vegetable origin (e.g., soy), are also widely used to supplement livestock rations. The extent of compliance with the feed ban in rendering and feed formulation influences the extent of possible cattle exposure to infectivity from a rendered diseased animal (see Appendix A for information on misleading probabilities and Appendix B for information on misfeeding probabilities). Infectivity can also be eliminated as a result of using ruminant-derived materials in ways that do not lead to any potential exposure among U.S. cattle (e.g., export). Figure 8 illustrates our characterization of how materials flow through rendering plants, feed formulation plants, and to the farm.

1. Rendering Inactivation

Rendering can reduce the amount of BSE infectivity in material by subjecting it to heat and pressure. Different rendering systems (e.g., continuous, batch, and vacuum) inactivate BSE or scrapie infectivity to different degrees (36–38). For each rendering technology, Table 8 quantifies for the base case the assumed reduction in infectivity achieved and the proportion of animals rendered. Additional information for the development of these assumptions was provided by D.W. Harlan (personal communication, Taylor Byproducts) and D.K. Mullane (personal communication, Taylor Byproducts).

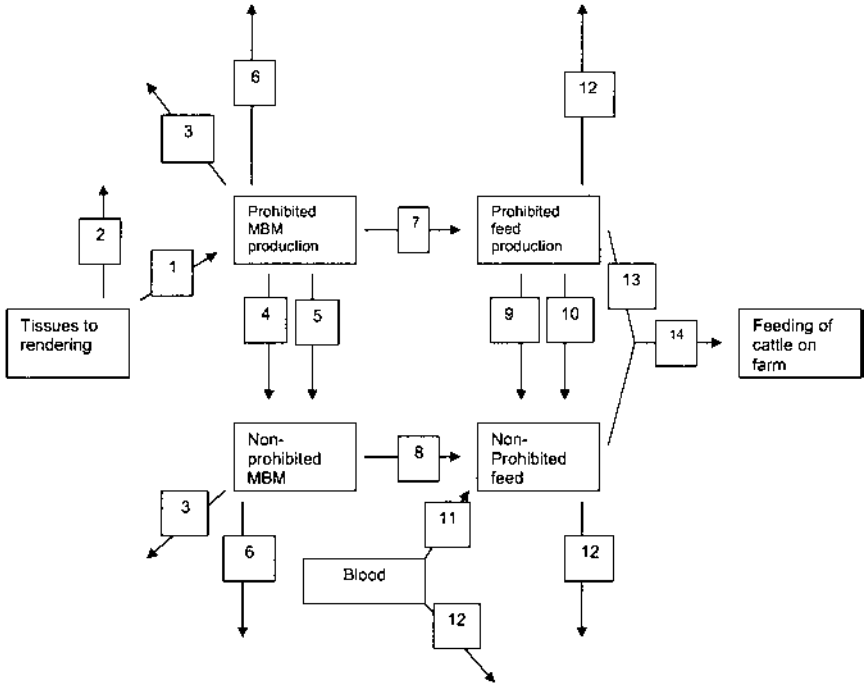

Figure 8 Disposition of infectivity.

2. Meat and Bone Meal Production

U.S. regulations recognize three types of rendering facilities, designated here as nonprohibited, prohibited, and mixed. A nonprohibited plant processes only porcine, equine, or poultry (nonruminant species) and produces animal-based protein products that can be used legally in cattle feed. A

Table 8 Infectivity Inactivation Achieved and Proportion of Cattle Processed by Different Types of Rendering Systems

Technology	Infectivity inactivation achieved (log base 10)	Proportion of cattle rendered
Batch	3.1 logs	5%
Continuous/fat added	2 logs	45%
Continuous/no fat added	1 log	45%
Vacuum	0 logs	5%

prohibited rendering plant may process ruminant, mink, or other raw materials, and produces prohibited MBM that may not be used in cattle feed. Mixed plants produce both nonprohibited and prohibited MBM. These plants must use separate production lines or a common line with specified cleanout procedures. The base case assumes that 94.9999% of cattle remains are sent to prohibited rendering plants, 5% are sent to mixed plants and 0.0001% are sent (incorrectly) to nonprohibited plants.

The base case assumes that cattle infectivity can reach bovines in several ways. Material from a prohibited rendering plant can be mislabeled and used in the formulation of the cattle feed. Mislabeling can also occur in a mixed plant. A mixed plant can contaminate nonprohibited MBM by using incorrect source material or by failing to completely flush and clean shared processing machinery. A nonprohibited plant might contaminate their MBM by using prohibited source material although such an event is unlikely. Even if nonprohibited MBM is contaminated, the potential for bovine exposure is reduced by the fact that much of this MBM goes to uses other than cattle feed. Table 9 describes the disposition of MBM infectivity based on the flow diagram in Fig. 8.

3. Feed Production

FDA feed ban regulations restrict use of prohibited MBM to feed manufacturers that produce only prohibited feed, or to manufacturers that produce both prohibited and nonprohibited feed (mixed producers), so long as they adhere to procedures that minimize the risk of contamination. The base case assumes that at mixed facilities, prohibited feed can contaminate nonprohibited feed. In addition, the base case assumes that prohibited feed can be mislabeled in facilities producing both prohibited and nonprohibited feed.

When recycled animal tissue (i.e., blood meal or MBM) is used as a supplement to animal feed, the material can be divided into feed portions consumed by many cattle. The base case assumes that infectivity in the blood meal from a single animal is divided among 89 cattle. This estimate is based on the assumption that blood meal from a single slaughtered bovine is mixed into a single batch containing 4000 pounds of blood meal, that the 4000-pound batch is consumed by a group of cattle over a 30-day period (e.g., cattle on a single farm to which the 4000 pounds is delivered), and that each cattle consumes 1.5 pounds per day (7). That is, 4000 pounds $\div$ (30 days $\times$ 1.5 pounds/bovine-day) yields approximately 89 bovines.

Similarly, the base case assumes that protein from a single slaughtered animal is typically divided among 89 cattle. This estimate reflects

Table 9 The Flow of Infectivity Through the Rendering Process

Figure 8 flow reference number	Label[a]	Refers to:
1	To P MBM	Infectivity that is sent to rendering for the production of P (prohibited) MBM
2	SRM elimination	Infectivity that is eliminated from potential use in animal feed or human food due to a specified risk material (SRM) ban; the base case assumes there is *no* SRM ban
3	Render elimination	Infectivity that is removed as a result of inactivation by rendering
4	Contamination of NP MBM	Infectivity from P MBM that contaminates NP (nonprohibited) MBM in mixed rendering facilities; the base case assumes that contamination occurs in these facilities with 14% probability[b] and when it does occur, 0.1% of the infectivity in the P MBM is transfered to the NP MBM[c]
5	Mislabel P MBM	Infectivity in P MBM is mislabeled as NP MBM; the base case assumes that this occurs with 5% probability[d]
6	Out after render	Infectivity in rendered material is not used for livestock feed

[a] Entries in the "Label" column are defined in Table 16.
[b] This assumption is based on information from 21 CFR Part 589, 1997. U.S. Food and Drug Administration. Substances Prohibited from Use in Animal Food Feed; Animal Proteins Prohibited in Ruminant Feed.
[c] This assumption is based on information from D.W. Harlan (personal contamination, Taylor Byproducts) and B. Prichett (personal communication, U.S. FDA Center for Veterinary Medicine).
[d] This assumption is based on calculations detailed in Appendix A.

the assumption that material from a single slaughtered bovine is mixed into a feed batch containing 4000 pounds consumed by a single group of animals, that cattle feed consumption is 30 pounds per day, and that 5% of the feed consists of animal protein (7). Additional cattle can become exposed to BSE from a single slaughtered animal owing to contamination during either the rendering process (Section II.C.2) or feed production

Table 10 The Flow of Cattle Infectivity Through the Feed Production and Use Processes

Figure 8 flow reference number	Label[a]	Refers to:
7	To P feed	Infectivity in P (prohibited) MBM that goes to production of P livestock feed
8	To NP feed	Infectivity in NP (nonprohibited) MBM used to produce NP feed
9	Contamination of NP feed	Infectivity from P feed that contaminates NP feed in mixed feed mills; the base case assumes that contamination occurs in these facilities with 16% probability[b] and when it does occur, 0.1% of the infectivity in the P feed is transferred to the NP feed[c]
10	Mislabeled NP feed	Infectivity in P feed mislabeled as NP feed; the base case assumes that this occurs with 5% probability[d]
11	To blood	Infectivity reaching cattle feed through use of blood meal
12	Out after feed prod	Infectivity in livestock feed not used for cattle

[a] Entries in the "Label" column are defined in Table 16.
[b] This assumption is based on information from 21 CFR Part 589, 1997. U.S. Food and Drug Administration. Substances Prohibited from Use in Animal Food or Feed; Animal Proteins Prohibited in Ruminant Feed.
[c] This assumption is based on information from D.W. Harlan (personal Communication, Taylor Byproducts) and B. Pritchett (personal communications, U.S. FDA Center for Veterinary Medicine).
[d] This assumption is based on calculations detailed in Appendix A.

process. Table 10 describes the flow of cattle infectivity through the feed production process.

4. On Farm Feeding

The practice of administrating correctly labeled prohibited feed (i.e., feed labeled "DO NOT FEED TO RUMINANTS") to cattle is referred to as "misfeeding." The base case assumes that correctly labeled prohibited feed is administered to cattle with a probability of 1.6%. Appendix B details the calculations used to develop this assumption. Table 11 details the relevant flows in Fig. 8.

Table 11 The Flow of Cattle Infectivity on the Farm

Figure 8 flow reference number	Label[a]	Refers to:
13	Misfed	Infectivity in nonbovine livestock feed administered to cattle
14	To cattle	Total infectivity in feed and blood meal administered to cattle

[a] Entries in the "Label" column are defined in Table 16.

Table 12 Potential Human Exposure

Organs/tissues	Description/assumptions for materials passing postmortem inspection
Brain	Brain is considered to be a variety meat and reaches the consumers labeled as such. The base case assumes that 1% of all cattle brains are potentially available for direct human consumption.
Spinal cord	Beef spinal cord is considered to be a variety meat and reaches consumers labeled as such. The base case assumes that 1% of the spinal cord is potentially available for human consumption.
Blood	The base case assumes that 5% of the cattle blood is potentially available for human consumption in meat food products, including sausages, blood pudding, etc., and that the infectivity in blood is limited to the potential contribution from brain and spinal cord emboli.
Distal ileum	Distal ileum is considered to be a variety meat and reaches the consumers labeled as beef intestines. The base case assumes that 1% of the distal ileums are potentially available for human consumption. In the United States, distal ileum does not reach consumers as natural sausage casings.

(continued on next page)

Table 12 (continued)

Organs/tissues	Description/assumptions for materials passing postmortem inspection
Contaminated organ meat	Brain and spinal cord are responsible for infectivity in organ meat when air-injected pneumatic stunning is used (not reflected in the base case). Liver, heart, and kidney are sold as variety meat. The base case assumes that 60% of the liver, 50% of the heart, and 25% of the kidneys are potentially available for human consumption.
Eyes	Bovine eyes are considered a variety meat and reach consumers labeled as beef eyes. The base case assumes that 1% of eyes are recovered and potentially available for human consumption.
Contaminated muscle meat	The base case assumes that spinal cord can contaminate edible meat during the splitting process. It is further assumed that other processes, such as steaming or washing, do not reduce this contamination.
AMR	Total infectivity in AMR product is the sum of the contributions from spinal cord and DRG contamination. The amount of spinal cord contaminating AMR product depends on whether the spinal cord is removed, as required by FSIS regulation for plants using AMR, and on whether the carcass in missplit.
Beef on bone	Total infectivity in beef on bone is the sum of the contributions from spinal cord contained in these cuts of meat and DRG attached to these bones. Spinal cord has the potential to reach consumers if it is not removed from the spinal column and if it remains attached to the backbone as bone-in steak. The base case assumes that about 30% of the backbones from steers and heifers are sold bone-in and that because these cuts do not undergo AMR processing, they retain the spinal cord. Although regulations do not require removal of the spinal cord from the backbones that do not undergo AMR (FSIS Directive 7160.2, 1997), many slaughterhouses remove it anyway (R. Brewer, personal communication, Associate Deputy Assistant Secretary, Office of Industrial Technologies, USDA FSIS. Washington, DC.). Note that even if the spinal cord or DRG on beef on bone reaches consumers, this material is not likely to be eaten.
Trigeminal ganglia	The base case assumes that the trigeminal ganglia (TG) does not contaminate cheek meat because it is located at the base of the cranium. Sensitivity analysis investigates the impact of assuming that TG contaminates cheek meat 1% of the time and that when such contamination occurs, it amount to 1/1000 of the infectivity in TG.

D. Potential Human Exposure

The base case assumes that humans can be exposed to BSE infectivity either by directly consuming infected cattle organs, such as brain, spinal cord, eyes, and distal ileum, or by consuming contaminated products, including meat or processed meat containing spinal cord or DRG, or organs containing CNS emboli, such as liver, heart, or kidneys. Table 12 details the assumed availability of these tissues. These estimates are based on information from N. Bauer (personal communication, Veterinary Scientific Liaison. USDA FSIS. Washington, DC); R. Brewer (personal communication, Associate Deputy Assistant Secretary, Office of Industrial Technologies, USDA FSIS. Washington, DC).

E. Disposition of Cattle That Die Prior to Slaughter

The base case assumes that 85% of cattle that die prior to being sent to slaughter are sent to rendering, while the remaining 15% are disposed of in a manner that poses no exposure risk to other cattle or to humans (personal communication, D. Franco, National Renderers Association; D.W. Harlan, Taylor Byproducts; L. Detwiler, USDA APHIS).

III. SCENARIOS EVALUATED

This section describes three sets of scenarios evaluated using our model. Section III.A describes the introduction of infected cattle into the United States, assuming contemporary conditions, government regulations, and prevailing agricultural practices in the United States. Section III.B describes the sensitivity analysis conducted to determine how changing various assumptions influences the model's predictions. Finally, Section III.C evaluates the model's ability to reproduce the spread of BSE in Switzerland following its introduction there in the 1980s. That evaluation serves as an indicator of the model's plausibility.

A. Hypothetical Introduction of BSE into the United States

The assumptions in the base case correspond to contemporary conditions in the United States, including all risk management actions taken by government and industry. Because BSE has not been found in the United States, the base case is evaluated by assuming the import of 10 BSE-infected animals. Such an introduction is considered unlikely because of the ban on importing ruminants from countries known to have BSE. However, this

approach allows characterization of the way in which infectivity could spread to animals or humans should the disease be introduced. To evaluate the robustness of the U.S. agriculture system against the introduction of BSE, we simulated the impact of introducing 1, 5, 20, 50, 100, 200, or 500 infected cattle.

B. Sensitivity Analysis

We evaluated the relative importance of 15 sources of uncertainty by determining how each individually influences model predictions for two cumulative outcomes over a simulated 20-year period—the total number of cattle that become infected after the introduction of 10 infected animals at the beginning of the period, and the amount of BSE infectivity (quantified in terms of the number of cattle oral ID_{50}s) in food produced for human consumption over that period. In particular, we ran the base case simulation 1000 times and recorded the arithmetic mean values for each of these two outcomes.

We then altered each of 15 sets of assumptions, one at a time, setting all of the other assumptions to their base case values. Each assumption was alternatively set equal to each of its bounding values. The "best case" value refers generally to the bounding value for an assumption expected to result in the smallest predicted risk of BSE spreading, whereas the "worst case" value refers generally to that bounding value for an assumption expected to result in the largest predicted risk of BSE spreading. For each alternative value, we again ran the simulation 1000 times and recorded the mean values of the two outcomes described above.

Alternative values for these 15 sets of assumptions fall into five categories, each of which is detailed in the following sections:

Section III.B.1—Maternal BSE transmission rate.

Section III.B.2—Slaughter process assumptions, including (a) the quantity of infectivity in an animal with clinical BSE, (b) the probability that AM inspection will detect an animal with clinical signs of BSE, (c) the type of stunners used, (d) the probability that the spinal cord is removed.

Section III.B.3—Rendering and feed production process assumptions, including (a) the extent to which rendering reduces infectivity, (b) the probability that prohibited MBM will contaminate nonprohibited MBM, (c) the magnitude of the contamination when this occurs, (d) the probability that prohibited MBM will be mislabeled as nonprohibited, (e) the probability that prohibited feed will contaminate nonprohibited feed, (f) the magnitude of the contamina-

tion when it occurs, (g) the probability that prohibited feed will be mislabeled as nonprohibited, (h) the probability that correctly labeled prohibited feed will be incorrectly administered to bovines.

Section III.B.4—Food inspection, including, in particular, the fraction of tissue in each tissue group recovered for human consumption.

Section III.B.5—Farm practices, including the fraction of all animals that die on the farm that are sent to rendering.

Before detailing the values used in this analysis, we note that because information characterizing the range of plausible values for these parameters is unavailable, we relied on our own judgment to identify reasonable bounds. The lack of information also made it impossible to characterize probability distributions for these quantities. Nonetheless, because the purpose of the sensitivity analysis was to qualitatively distinguish between those assumptions that contribute substantial uncertainty and those assumptions that do not, this limitation does not defeat the purpose of the analysis.

1. Maternal BSE Transmission Assumptions

We evaluated the assumption that during the last one-sixth of the BSE incubation period, an infected bovine will transmit disease to a calf it gives birth to with 10% probability (base case). The best case value for this assumption was assumed to be 0% (i.e., mother-to-calf transmission does not occur), and the worst case value for this assumption was assumed to be 13%.

2. Slaughter Process Assumptions

For assumptions related to the slaughter process that have been evaluated as part of the sensitivity analysis, Table 13 details base case, best case, and worst case values.

3. Render and Feed Production Process Assumptions

For assumptions related to the rendering process, the feed production process, and on-farm feed practices that have been evaluated as part of the sensitivity analysis, Table 14 details base case, best case, and worst case values.

4. Proportion of Tissues Recovered for Human Consumption

Table 15 details the base case, best case, and worst case assumptions regarding the proportion of tissue recovered for human consumption. Note

Table 13 Base Case, Best Case, and Worst Case Values for Slaughter Process Assumptions

Assumption	Base case	Best case	Worst case
Cattle oral ID_{50}s in carcass of a full-blown BSE case	10,000	5,000	20,000
Antemortem inspection clinical BSE detection rates	90%	99%	50%
Proportion of cattle stunned using an air-injection pneumatic stunner	0%	0%	15%
Probability that the spinal cord is removed			
Plants using AMR	98%	99.9%	80%
Plants not using AMR	50%	99%	10%

that none of these sets of assumptions reflect any regulations not currently enacted (e.g., a specified risk material ban.)

5. Proportion of Animals That Die on Farm That Are Rendered

The base case assumes that 85% of the animals that die on the farm (i.e., before they are sent to slaughter) are rendered. The best case assumes that this proportion is 60%, while the worst case assumes that it is 99%.

C. Evaluation of the Swiss BSE Outbreak

Because there has never been a controlled experiment to quantify the impact of introducing BSE into a country, a true validation of the simulation model described in this chapter is not possible. Instead, this section describes an evaluation of the model's plausibility that involves modeling the small BSE outbreak observed in Switzerland following the introduction of BSE from the United Kingdom. Working with experts in Switzerland, we identified appropriate parameter values to characterize Switzerland's cattle population dynamics, conditions, agricultural practices, and procedures. The Switzerland scenario reflects changing conditions over time. In addition to specifying conditions at the beginning of the simulation (1986), the scenario also reflects changes to these conditions in 1990, 1993, 1996, 1998, and 2001. Except where noted, the information in this section on agricultural practices, including rendering and feeding, and regulatory changes comes as personal communications from D. Heim, Swiss Federal Veterinary Office, Bern-Liebefeld, Switzerland.

Table 14 Base Case, Best Case, and Worst Case Values for Render Process, Feed Production Process, and On-Farm Feed Practice Assumptions

Assumption	Base case	Best case	Worst case
Proportion of animals rendered using various technologies			
Batch (3.1-log reduction)	5%	5%	5%
Continuous/fat added (2.0-log reductions)	45%	85%	20%
Continuous/no fat added (1.0-log reduction)	45%	5%	70%
Vacuum (no reduction)	5%	5%	5%
Rendering—contamination of nonprohibited MBM by prohibited MBM in mixed facilities			
Probability for a particular prohibited packet	14%	5%	25%
Magnitude of contamination[a]	0.1%	0.01%	1.0%
Rendering—probability that prohibited MBM will be mislabeled as nonprohibited MBM when produced by either a mixed or prohibited rendering plant	5%	2%	10%
Feed production—contamination of nonprohibited MBM by prohibited MBM in mixed facilities			
Probability	16%	5%	16%
Magnitude of contamination[a]	0.1%	0.01%	1.0%
Feed production—probability that prohibited feed produced by a mixed-feed production plant will be mislabeled as nonprohibited feed	5%	2%	33%
Probability that correctly labeled prohibited feed will be incorrectly administered to cattle	1.6%	0.1%	15%

[a] Refers to the proportion of the prohibited material that ends up in the nonprohibited packet when contamination occurs.

Table 15 Base Case, Best Case, and Worst Case Values for the Proportion of Tissues Recovered from Cattle for Human Consumption

Tissue	Base case	Best case	Worst case
AMR meat	0.98	0.98	0.98
Blood	0.05	0.025	0.3
Bone (in-bone cuts of meat)	0.98	0.98	0.98
Brain	0.01	0.001	0.02
Dorsal root ganglia	0	0	0
Eyes	0.001	0	0.002
Ileum	0.01	0.001	0.02
Heart	0.5	0.3	0.6
Kidney	0.25	0.15	0.35
Liver	0.6	0.4	0.7
Lung	0	0	0
Muscle	0.98	0.98	0.98
Spinal cord	0.01	0.001	0.02
Trigeminal ganglia	0	0	0

1. 1986

The Switzerland scenario begins in 1986, the year we assume that 67 newly infected female dairy cattle were incubating BSE [see Fig. 7 in Doherr et al. (39)]. Thirty of these cattle are assumed to be 25 months of age and the remaining 37 are assumed to be 26 months of age.

At the same time, the Switzerland scenario assumes that feed containing 4000 cattle oral ID_{50}s was imported. The assumption is based on information that three tons of MBM were imported from the United Kingdom between 1985 and 1989 (Part II, Section 3.12 in Ref. 40). We assume that during that period, MBM from Britain was contaminated with BSE. In particular, we assume that the three tons of MBM imported from Britain represented rendered protein from three cattle, each of which harbored between 800 and 2000 cattle oral ID_{50}s. We assume that the 3 tons of MBM were used to supplement feed at a concentration of 5% and was therefore distributed as part of a total of 60 tons of feed. Assuming that cattle consume 30 pounds of feed a day (3% of their weight) and that farms purchase feed in lots sufficient to last them 30 days, the 60 tons (120,000 pounds) of feed would be divided among 133 cattle [i.e., 120,000 pounds ÷ (30 pounds/cow-day × 30 days)].

Differences between the base case and the Switzerland scenario in 1986 include the following. First, the misfeeding rate is assumed to be 15%, considerably higher than the 1.6% misfeeding rate in the base case. The

assumption of a substantially higher misfeeding rate is based on the observation that a substantial proportion of the farms in Switzerland raise both livestock that can consume prohibited feed and livestock that are restricted to nonprohibited feed. For example, nearly 67% of the poultry in Switzerland are raised on farms that also raise cattle. For hogs, the corresponding proportion is 59%.

Second, the Switzerland scenario assumes that most rendering systems in use in 1986 in Switzerland used batch processing technology, which normally reduces infectivity by a factor of 1000 (i.e., 3 logs). However, because use in Switzerland typically did not conform to the 133°C/20 min/3 bars of pressure minimum treatment standard, we assume that the majority of rendering facilities achieved only 2 logs infectivity inactivation.

Finally, the Switzerland scenario reflects the absence of a feed ban in 1986.

2. 1990

In December 1990, Switzerland enacted a feed ban. However, the structure of the MBM and feed production industries provided opportunities for failures to this ban. In particular, a substantial portion of the prohibited feed was produced by mixed feed producers. We assume that these producers mislabeled or failed to properly label 10% of their prohibited feed and that contamination occurred during production of 20% of the prohibited feed. We also note that a ban on the use of specified risk materials (SRMs) in human food increased the flow of this material into MBM and ultimately into animal feed (Part II, Section 2.2 in Ref. 40).

3. 1993

By 1993, rendering practices improved (Part II, Section 2.2 in Ref. 40). We assume that at that time, all renderers complied with the 133°C/20 min/3 bars of pressure standard, and hence that all rendering achieved a 3.1 logs of infectivity reduction (a factor of approximately 1260).

4. 1996

Farming practice changes in 1996 helped to reduce the spread of BSE infectivity. These change included reduced misfeeding of prohibited rations to cattle (we assume this rate decreased to 0.1%) and an end to the rendering of cattle that died prior to being sent to slaughter. In addition, SRMs were prohibited from use in feed in 1996 (Part II, Section 2.2 in Ref. 40). Tissues and products covered by the SRM ban included brain, spinal cord, dorsal root ganglia, gut, lung, eyes, and AMR meat.

5. 1998

In 1998, slaughter facility practices further improved with an increased effort to remove spinal cords after splitting. We assume the spinal cord was removed 99.9% of the time.

6. 2001

Finally, in January 2001, Switzerland outlawed the practice of feeding MBM to *any* farm animal. This move essentially eliminated the possibility of misfeeding animals. In addition, Switzerland prohibited the feeding of blood meal to cattle.

IV. RESULTS

This section highlights key results of the analyses in this report. Because the simulation model used in this analysis is probabilistic, the results depend on values selected for random quantities (e.g., whether a particular packet of prohibited MBM is mislabeled). To characterize the range of plausible results associated with a particular scenario (i.e., with a particular set of assumptions), we have simulated each scenario 1000 times. The tabular summaries of the simulation results report all quantities to two significant digits. In some of the figures, nonzero values that round to zero are displayed as ".00" to distinguish them from values that are exactly zero (displayed as "0").

The range of results generated by these simulations is characterized by a set of summary statistics for each outcome quantity (e.g., total number of infected cattle, total number of clinical cattle, total number of cattle oral ID_{50s} to humans, etc.). These statistics include the 5th, 25th, 50th, 75th, and 95th percentiles and arithmetic average.

We use box and whisker plots to illustrate some of the results. These figures plot the interquartile range (i.e., the range extending from the 25th percentile to the 75th percentile) as a box. The horizontal lines within each box designate distribution medians. Extending from the ends of each box are whiskers that designate the 5th and the 95th percentile values. Values that are less than the 5th percentile or greater than the 95th percentile are individually displayed as an "x."

For each simulation run we present both tabular and graphic summaries of the output. Each format summarizes different sorts of information. The tabular summary presents cumulative values for the entire simulated period. Many of the graphs illustrate the evolution of

the simulated environment over time, using box and whisker plots to characterize the range of plausible values for various quantities, and bar graphs to illustrate the probability that the quantities exceed zero.

Tabular Output Summaries: Table 16 defines the entries in the tabular summaries of the simulation runs.

Graphic Output Summaries: For each run, we prepared six pairs of graphs (i.e., 12 graphs in all). The second graph in each pair (the "range of values" graph) describes the distribution for all recorded values. However, because most graphs use a log scale, they do not clearly illustrate the probability that the quantity described equals zero. For that reason, we also present graphs (labeled "probability of value exceeding zero") that clearly illustrate these probabilities. The pairs of graphs illustrate the following information. (See Figs. 9–14 for the base case results discussed in Section IV.A.)

Number of cattle infected (Fig. 9, a and b)—For each year of the simulation period, these graphs illustrate how likely it is that at least one bovine is infected (probability of value exceeding zero) and the estimated total number of cattle infected (range of values).

Number of cattle clinical (Fig. 10, a and b)—For each year of the simulation period, these graphs illustrate how likely it is that at least one bovine has clinical signs (probability of value exceeding zero) and the estimated total number of cattle with clinical signs (range of values).

ID_{50}s to cattle (Fig. 11, a and b)—For each year of the simulation period, these graphs illustrate how likely it is that cattle consumed any feed or blood meal contaminated with BSE (probability of value exceeding zero) and the estimated total amount of BSE infectivity consumed by cattle (range of values).

Disposition of ID_{50}s (Fig. 12, a and b)—For the entire simulation period, these graphs illustrate the probability that the cumulative number of ID_{50}s exceeded zero far each component of the flow diagram illustrated in Fig. 8 (probability that quantity exceeded zero) and the estimated range of values for the cumulative quantity of ID_{50}s for each component of that flow diagram (range of values).

ID_{50}s to humans (Fig. 13, a and b)—For each year of the simulation period, these graphs illustrate how likely it is that humans were potentially exposed to any food contaminated with BSE (probability of value exceeding zero) and the estimated total amount of BSE infectivity in food potentially available for human consumption (range of values).

Table 16 Definitions for Entries in the Simulation Tabular Summaries

Table label	Description
Epidemic statistics	
Total infected	Total number of animals infected with BSE during the simulation
Total infected w/o imports	Number of new infected animals in addition to those imported during the simulation
Total clinical	Number of infected animals that remain alive long enough to develop clinically detectable signs of BSE during the simulation
Probability N infected > 0	Probability that at least one animal is infected at the end of the simulation
Mode of infection	
Maternal	Number of animals infected via transmission from mother to calf during the simulation
Spontaneous	Number of animals infected during the simulation via spontaneous infections (exceeds zero only for the scenario that assumes spontaneous infections are possible)
Protein	Number of animals infected due to consumption of infectivity in MBM during the simulation
Blood	Number of animals infected due to consumption use of blood as a feed supplement during the simulation
Exogenous	Number of animals infected by infectivity introduced exogenously during the simulation (e.g., via introduction of scrapie into the feed supply, etc.)
Mode of death for infected animals	
Slaughter	Number of infected animals slaughtered during the simulation
Die on farm—render	Number of infected animals that die on the farm that are rendered during the simulation
Die on farm—no render	Number of infected animals during the simulation that die on the farm that are buried on the farm or otherwise diverted from applications that can result in the exposure of additional animals to infectivity in the animal disposed

Table 16 (continued)

Table label	Description
Disposition of ID_{50s}	
From slaughter	Number of ID_{50s} from animals sent to slaughter during the simulation
From death on farm	Number of ID_{50s} in animals that die on the farm and are rendered during the simulation
Eliminated by SRM ban	Number of ID_{50}s during the simulation removed from material that cattle or humans may consume due to implementation of a UK-like specified risk material ban
To prohibited MBM	Number of ID_{50s} during the simulation sent to rendering plants designated for production of prohibited MBM
To NP MBM—misdirected	Number of ID_{50}s during the simulation incorrectly sent to rendering plants designated for production of nonprohibited MBM
To NP MBM—contamination	Number of ID_{50s} during the simulation entering the supply of nonprohibited MBM due to contamination in mixed rendering facilities
To NP MBM—mislabeling	Number of ID_{50}s during the simulation entering the supply of nonprohibited MBM due to mislabeling of prohibited material as nonprohibited
Eliminated by rendering	Number of ID_{50}s during the simulation eliminated in the rendering process
Out after rendering	Number of ID_{50}s during the simulation in MBM diverted to uses for which there is no potential for either human or cattle exposure in the United States (e.g., export)
To prohibited feed	Number of ID_{50}s during the simulation sentto feed plants designated for the production of prohibited feed
To NP feed—misdirected	Number of ID_{50}s during the simulation in prohibited MBM that is used to make nonprohibited MBM
To NP feed—contamination	Number of ID_{50}s during the simulation entering the supply of nonprohibited feed due to contamination in mixed feed mills

(*continued on next page*)

Table 16 (continued)

Table label	Description
To NP feed—mislabeling	Number of ID_{50s} during the simulation entering the supply of nonprohibited feed due to mislabeling of prohibited material as nonprohibited
To blood	Number of ID_{50s} in blood meal during the simulation
Out after feed production	Number of ID_{50s} during the simulation in feed diverted to uses for which there is no potential for either human or cattle exposure in the United States (e.g., pet food)
Misfed to cattle	Number of ID_{50}s during the simulation in prohibited feed is inappropriately administered to cattle on the farm
Total to cattle	Number of ID_{50}s in feed and blood meal administered to cattle during the simulation
Total to humans	Number of ID_{50}s potentially available for human consumption during the simulation
Potential sources of human exposure to transmissible agent	
Brain	Number of ID_{50}s in cattle brain potentially available for human consumption during the simulation
Spinal cord	Number of ID_{50}s in cattle spinal cord potentially available for human consumption during the simulation
Blood	Number of ID_{50}s in cattle blood potentially available for human consumption during the simulation
Distal ileum	Number of ID_{50}s in cattle distal ileum potentially available for human consumption during the simulation
Contaminated organ meat	Number of ID_{50}s in cattle organ meat potentially available for human consumption during the simulation
Eyes	Number of ID_{50}s in cattle eyes potentially available for human consumption during the simulation

Table 16 (continued)

Table label	Description
Muscle contamination	Number of ID_{50}s in cattle muscle due to contamination potentially available for human exposure during the simulation
AMR	Number of ID_{50}s in AMR product due to contamination potentially available for human consumption during the simulation
Beef on bone	Number of ID_{50}s in cattle beef on bone potentially available for human consumption during the simulation
Trigeminal ganglia	Number of ID_{50}s potentially available for human exposure from consumption of cheek meat tissue associated with the trigeminal ganglia during the simulation

ID_{50}s to humans by tissue (Fig. 14, a and b)—For the entire simulation period, these graphs illustrate the probability that the cumulative infectivity in food potentially available for human consumption exceeded zero for each tissue group (probability of value exceeding zero) and the estimated total amount of infectivity in food available for human consumption by tissue group (range of values).

Before proceeding, we note that many of the simulation results are "right skewed." meaning that the average value often exceeds the median (50th) percentile and can sometimes even exceed the 95th percentile. A right-skewed distribution arises when relatively infrequent events can result in very large (always positive) outcome values. For example, the probability that the brain of a BSE-infected animal will be selected for potential human consumption is very low because there are few sick animals and few brains harvested for human consumption. However, if this event does occur, it makes a substantial quantity of infectivity available for potential human consumption. If this event only occurs one time out of 1000 simulation runs, the arithmetic mean for the number of cattle oral ID_{50}s available for human consumption from brain would exceed this outcome's value for 999 of the 1000 runs (i.e., zero). For this reason, we report key percentile value for each outcome, in addition to the arithmetic mean. The results discussion focuses on mean and median values to characterize the central tendency for each quantity, and the 95th percentile to characterize a quantity's extreme (although not worst possible) value.

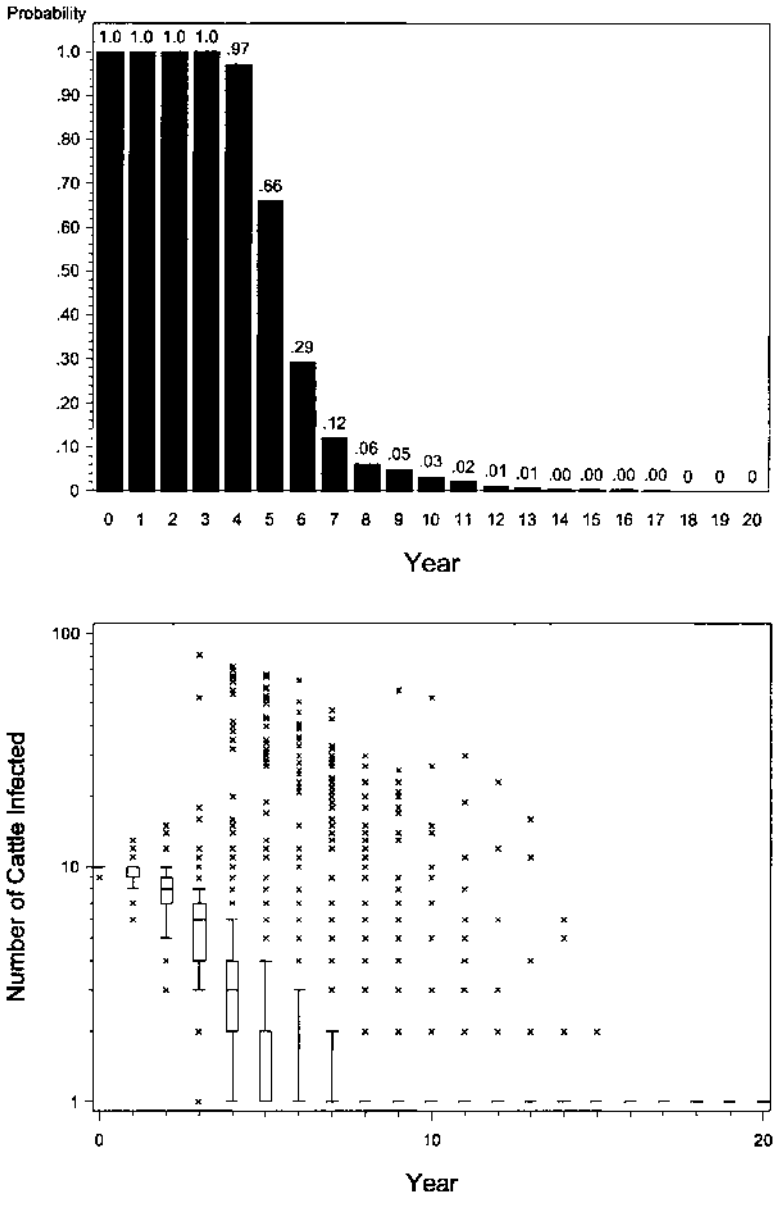

Figure 9 (top) Number of cattle infected—probability of value exceeding zero. (bottom) Number of cattle infected—range of values.

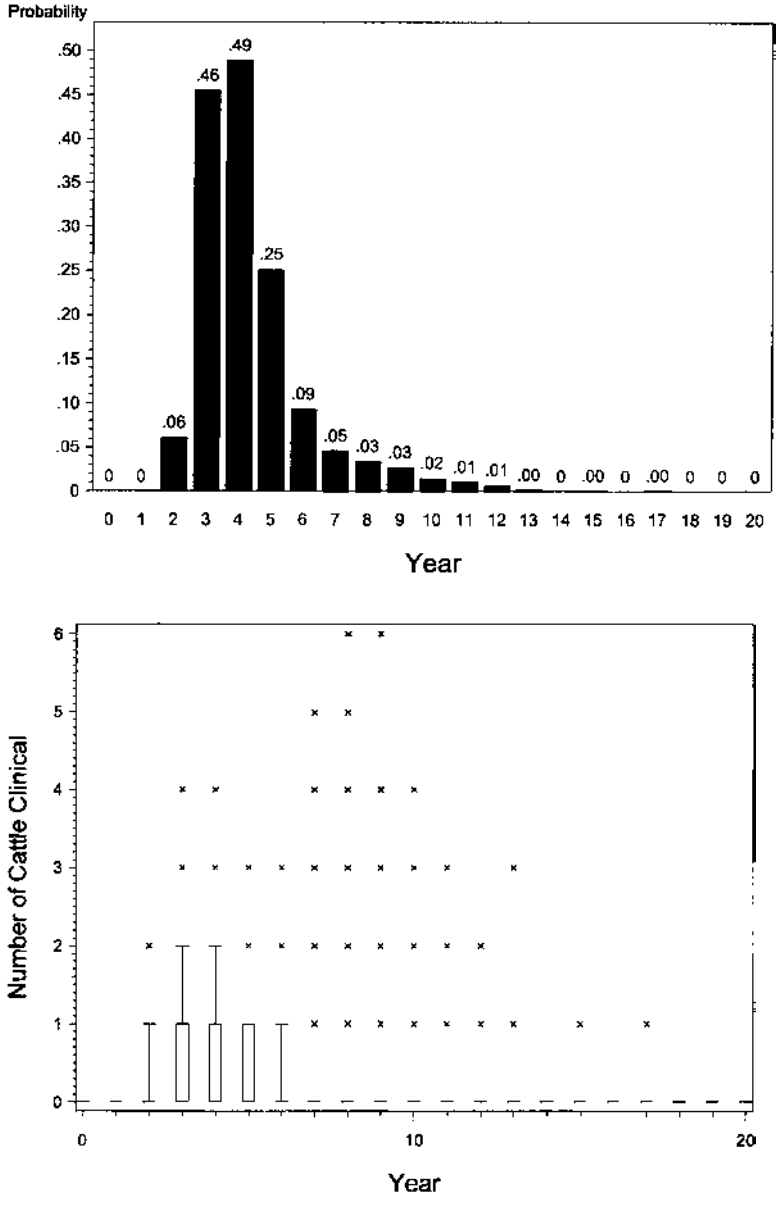

Figure 10 (top) Number of cattle clinical—probability of value exceeding zero. (bottom) Number of cattle clinical—range of values.

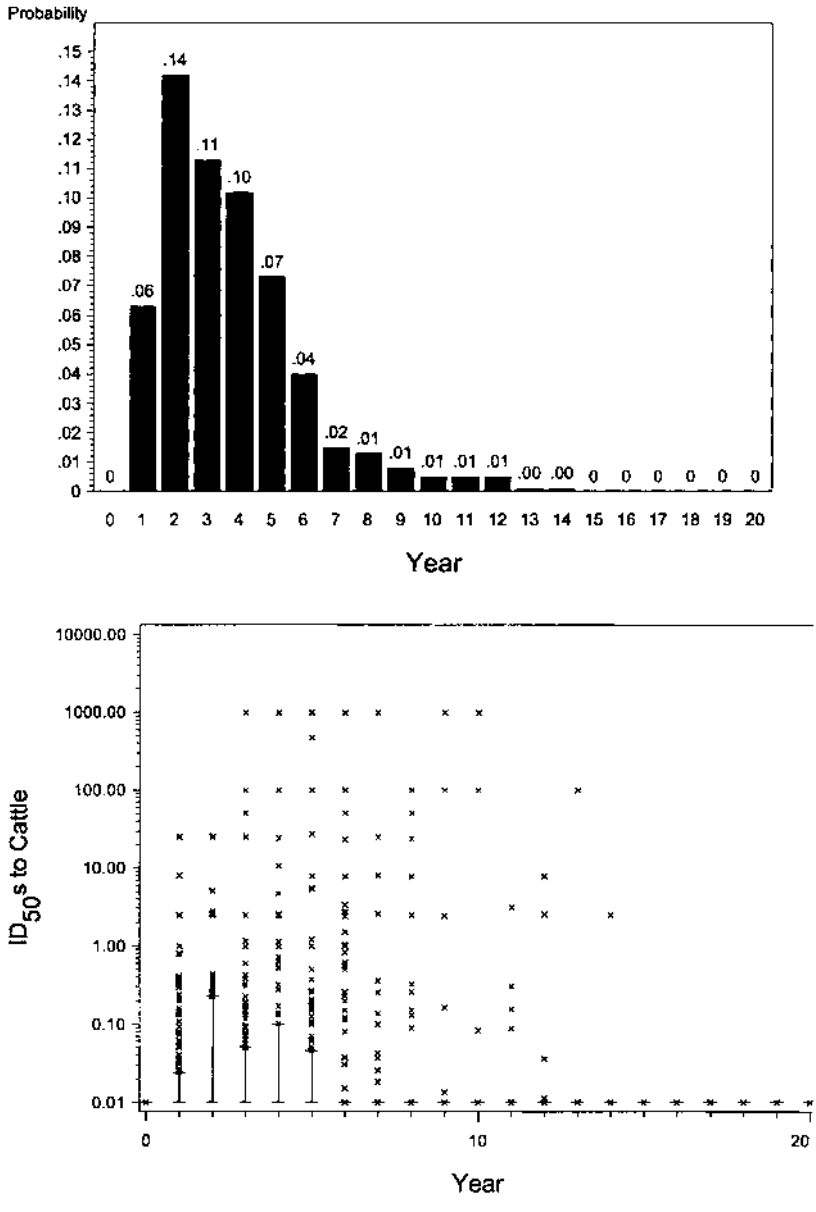

Figure 11 (top) ID_{50}s to cattle—probability of value exceeding zero. (bottom) ID_{50}s to cattle—range of values.

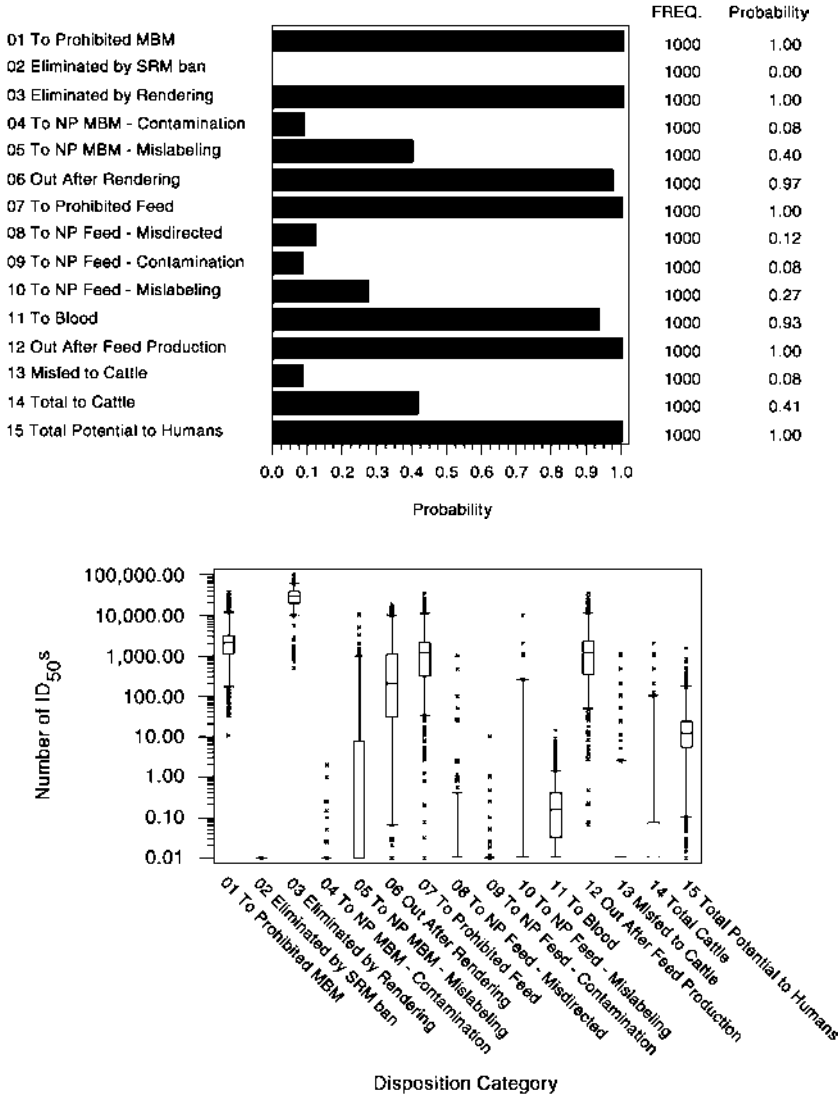

Figure 12 (top) Disposition of ID_{50}s—range of values—probability of value exceeding zero. (bottom) Disposition of ID_{50s}—range of values.

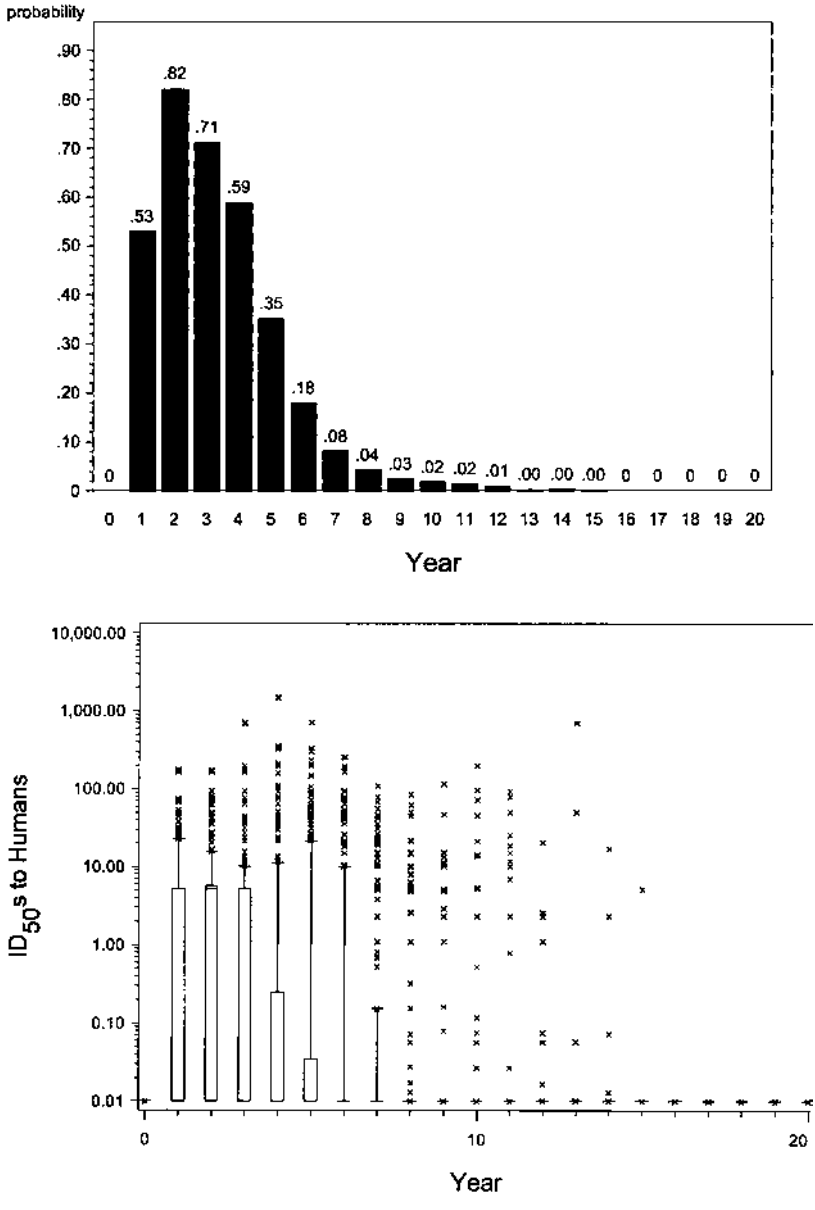

Figure 13 (top) ID_{50}s to humans—probability of value exceeding zero. (bottom) ID_{50}s to humans—range of values.

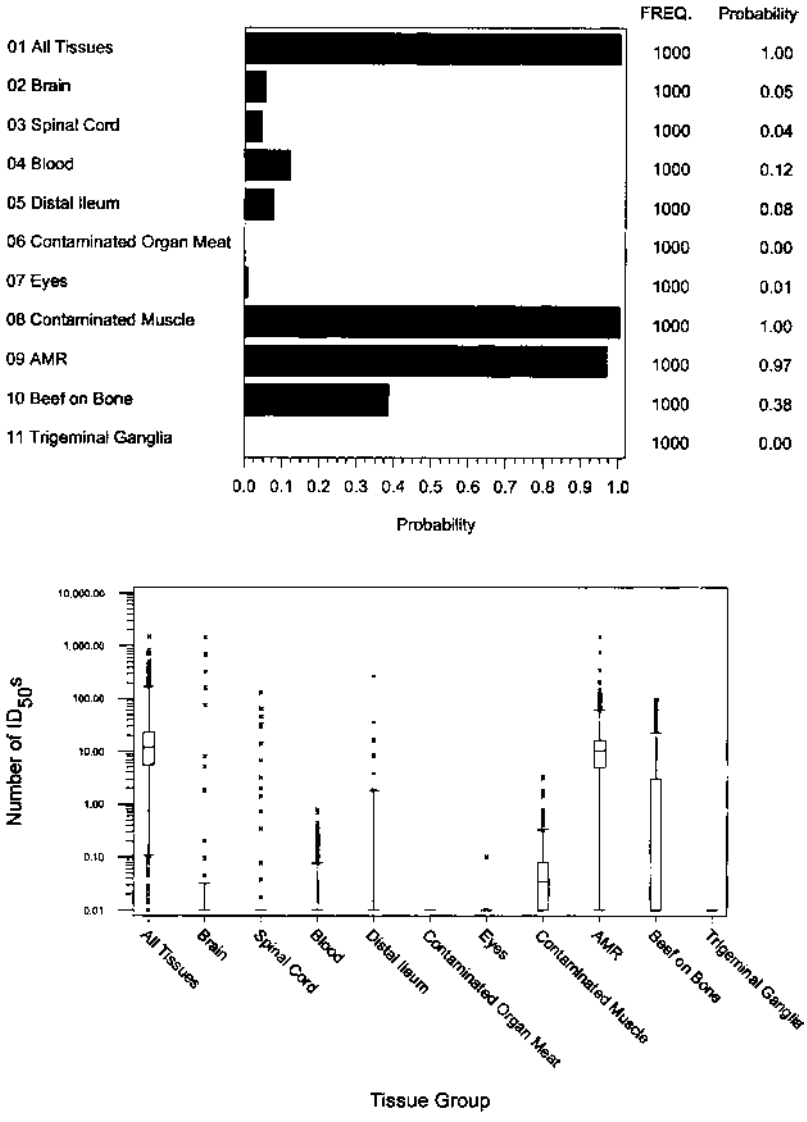

Figure 14 (top) ID_{50}s to humans by tissue—probability of value exceeding zero. (bottom) ID_{50}s to humans by tissue—range of values.

A. Base Case Results

1. Cattle Exposure to BSE

Introduction of 10 animals demonstrates the robustness of U.S. regulations and practices in preventing the establishment of BSE (see Table 17). On average, there are fewer than three new cases of BSE, with a 75–95% chance that there will be no new cases at all over a 20-year period. The extreme case (the 95th percentile of the distribution) predicts 11 new cases. In all cases, the disease is quickly cleared from the United States, with virtually no chance that there are any infected animals 20 years after the import of infected animals (Figs. 9–14).

Increasing the number of infected cattle introduced at the beginning of the simulation increases the number of additional infected and clinical animals, and the time required for BSE to die out. However, in all cases tested (i.e., up to the introduction of 500 infected cattle), the prevalence of BSE declines after its introduction. Moreover, predicted values for the key quantities just mentioned appear to increase linearly with the number of infected cattle introduced. That is, over the domain we evaluated, we found no evidence that the behavior of this dynamic system changes in some qualitative manner when the size of the challenge reaches a critical value. The tendency for the prevalence of disease to decline indicates that the U.S. agricultural system is "stable," meaning that it can effectively limit the spread of BSE if it is introduced and eventually eliminate it.

A second line of analysis, based on estimating the number of new BSE cases generated from each inflected animal, leads to this same conclusion. This value is referred to as the epidemic R_0 value (41). As long as this value is less than 1.0 for any disease, that disease's prevalence will tend to decline over time. We consider the version of the base case in which we introduced 500 infected animals at the beginning of the simulation. At the end of the 20-year simulation period, BSE was eliminated with almost 90% probability, the number of infected cattle decreased to no more than two with 95% probability, and the maximum number of cattle still infected (out of 1000 simulations) amounted to approximately 100. Because the arithmetic mean of the incubation period distribution assumed in the simulation is 52 months, even this last result suggests an R_0 of approximately 0.7. The number of cattle after n incubation periods is approximately Ie^{rn}, where I is the initial number of cattle (500 in this case), $r = R_0 - 1$, and n = number of incubation periods (240 months $\div$ 52 R_0 months).

On the other hand, the value of R_0 may exceed 1.0 if extreme values are assigned to some parameters. As discussed in Section IV.B, the most influential source of uncertainty is the misfeeding rate. If the worst case value of this parameter is assumed, the value of R_0 exceeds 1.0 with a probability of

Table 17 Base Case—Import of 10 Infected Animals

Label	Mean	5th	25th	50th	75th	95th
Epidemic statistics						
Total infected	13	10	10	10	10	21
Total infected w/o imports	2.9	0	0	0	0	11
Total clinical	4.8	2	3	4	5	8
Probability N infected > 0	0					
Mode of infection						
Maternal	0	0	0	0	0	0
Spontaneous	0	0	0	0	0	0
Protein	2.9	0	0	0	0	11
Blood	0.003	0	0	0	0	0
Exogenous	0	0	0	0	0	0
Mode of death						
Slaughter	7.4	3	4	6	7	13
Die on farm—render	4.7	1	3	4	5	8
Die on farm—no render	0.78	0	0	1	1	3
ID_{50} sources						
From slaughter	1,500	260	520	830	1,300	5,800
From death on farm	37,000	10,000	20,000	30,000	40,000	62,000
Disposition of ID_{50}s						
1 To prohibited MBM	3,600	170	1,200	2,100	3,200	12,000
2 Eliminated by SRM ban	0	0	0	0	0	0
3 Eliminated by rendering	35,000	10,000	20,000	30,000	39,000	60,000
4 To NP MBM—contamination	0.022	0	0	0	0	0.0033
5 To NP MBM—mislabeling	190	0	0	0	7.9	1,000
6 Out after rendering	1,300	0.064	30	200	1,100	10,000
7 To prohibited feed	2,500	33	300	1,200	2,200	11,000
8 To NP feed—misdirected	6.2	0	0	0	0	0.4
9 To NP feed—contamination	0.031	0	0	0	0	0.0025
10 To NP feed—mislabeling	70	0	0	0	0.0066	260
11 To blood	0.38	0	0.032	0.16	0.4	1.4
12 Out after feed production	2,500	49	340	1,200	2,200	11,000
13 Misfed to cattle	17	0	0	0	0	2.6
14 Total to cattle	37	0	0	0	0.075	100
15 Total potential to humans	35	0.11	5.5	12	24	170
Potential human exposure						
Brain	9.2	0	0	0	0	0.032
Spinal cord	1.7	0	0	0	0	0
Blood	0.012	0	0	0	0	0.077
Distal ileum	0.75	0	0	0	0	1.8
Contaminated organ meat	0	0	0	0	0	0
Eyes	0.00042	0	0	0	0	0
Contaminated muscle meat	0.092	0.00072	0.0022	0.036	0.08	0.32
AMR	20	0.0062	5.1	10	16	61
Beef on bone	3.8	0	0	0	3.1	23
Trigeminal ganglia	0	0	0	0	0	0

approximately 25%. The second most influential source of uncertainty is the feed mislabeling probability. Assuming the worst case value for this parameter yields an R_0 value exceeding unity with less than 5% probability.

2. Human BSE Exposure

The simulation predicts an average of 35 cattle oral ID_{50}s potentially available for human consumption during the 20-year period after the import of the infected animals, with a 95th percentile value of 170 cattle oral ID_{50}s. Potential sources of human exposure include brain (26% of the total on average), contaminated AMR product (67%), beef on bone (11%), intestine (2%), and spinal cord (5%).

We note that the total exposure estimates reported here are likely to overstate true human exposure because they represent the amount of infectivity in food presented for human consumption but do not take into account waste or actual consumption rates. For example, the reported quantity for potential exposure to ID_{50}s in beef on bone reflects the presence of spinal cord and dorsal root ganglia in cuts like T-bone steaks. The spinal cord may never be consumed but is reflected in our estimates because it is available for consumption. Similarly, not all bovine brain removed for human consumption is actually eaten by humans. Some is not purchased at the retail level (e.g., due to spoilage) and some is not consumed even when purchased. These issues are also relevant to the other tissue categories.

B. Sensitivity Analyses

Figures 15 (number of new BSE cases) and 16 (human exposure to BSE) summarize the sensitivity analysis results. In these two figures, the horizontal axis lists the individual parameters analyzed, while the vertical axis quantifies the associated range of outcome values. Note that the alphanumeric designations preceding the name of each quantity listed along the horizontal axis refer to the listing of the parameters in the introduction to Section III.B. The first character (a digit) indicates the subsection in which that parameter is discussed, and the second character (a letter) designates the parameter in that section (omitted if that subsection describes only one parameter). For example, "2c Stunner" refers to the third parameter ("c") in Section III.B.2 ("2"). A horizontal line above the horizontal axis designates the arithmetic mean value for the outcome quantity computed when all parameters were set equal to their base case value. The range of values associated with each parameter's best and worst case values is represented by the small horizontal lines at the extreme ends of a vertical line above the label for each parameter.

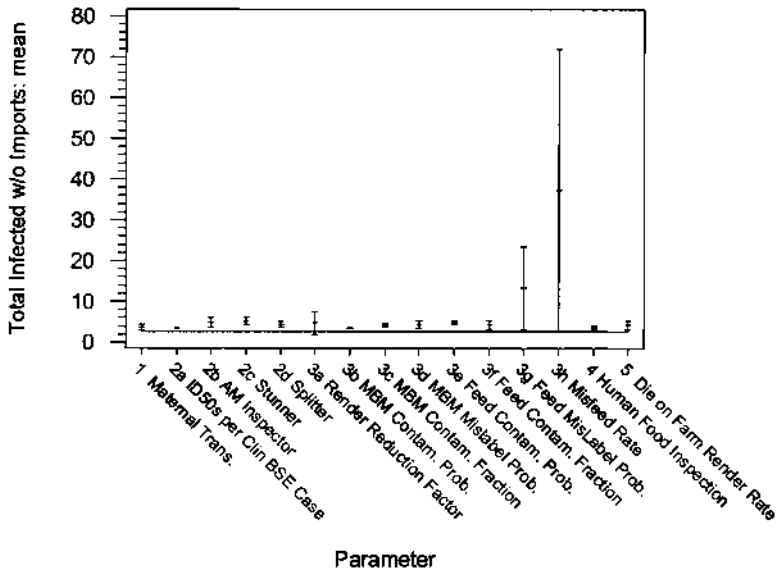

Figure 15 Sensitivity analysis—number of cattle infected.

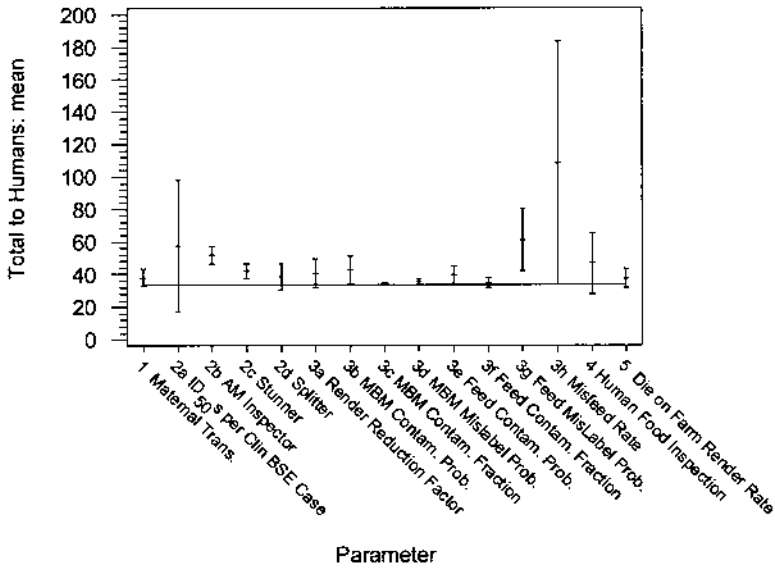

Figure 16 Sensitivity analysis—potential human exposure.

Figure 15 indicates that the parameters that have the greatest influence on the mean number of new BSE cases are directly associated with feed ban compliance. The most influential parameter is the misfeeding rate, which represents the proportion of feed formulated for other species and containing prohibited MBM illegally administered to cattle. The second most influential parameter is the probability that prohibited feed will be mislabeled (i.e., lack the required warning labels). Other parameters evaluated had only a very small influence on the total number of new BSE cases.

Figure 16 illustrates the impact of parameter uncertainty on the mean number of cattle oral ID_{50}s potentially available for human consumption. Both the misfeeding and mislabeling rates are again prominent, but two other parameters are influential as well. These other parameters are the proportion of animals with clinical BSE signs identified and eliminated by the AM inspector, and the assumed number of cattle oral ID_{50}s in a full-blown clinical BSE case. Predicted human exposure to BSE is also sensitive to assumptions about contamination during the carcass splitting process. Note that even the worst case estimates in Figure 16 amount to relatively limited potential exposure over a 20-year period after the introduction of 10 infected cattle.

We note that the results indicate that conducting 1000 runs for each set of parameter values was sufficient to identify those assumptions making the most important contributions to uncertainty. Although 1000 runs was not sufficient to distinguish between the base case predictions and predictions corresponding to the best and worst case values for "unimportant" parameters (e.g., parameters for which the best and worst case values for the number of new infected cattle differed by no more than five), 1000 runs was sufficient to identify those parameters that contributed substantial uncertainty. For example, the maximum theoretical value of the 95% upper confidence limit (UCL) on the number of additional infected cattle in the base case (calculated using Chebychev's bound) is approximately 20 (see Appendix C). Similar bounds limit the corresponding 95% UCL values predicted when worst case value are assigned to 13 of the 15 parameters analyzed. Because the Chebychev inequality provides a theoretical bound, the true 95% UCL values are likely to be substantially less. For the remaining two parameters, the worst case values yielded 25 (3g—Feed Mislabel Prob.) or 67 (3h—Misfeed Rate) additional infected cattle.

C. Switzerland

The 50th percentile results for our simulation of Switzerland predicts that 580 cattle will become infected (in addition to the 67 assumed infected at the beginning of the simulation), and that of all infected cattle, 166 will

develop clinical signs before being sent to slaughter or dying from causes unrelated to BSE. The Swiss reported identifying 324 cattle with clinical signs between 1990 and 2000, or approximately twice as many as our 50th percentile prediction (although almost identical to our 95th percentile prediction of 320 clinical animals). The time course of the outbreak predicted by the simulation is similar to the observed time course, with the number of cases increasing during the early 1990s and then falling after 1995, although simulation results show a peak in the early 1990s prior to a second peak in 1995.

The modest differences between the predicted annual incidence of clinical BSE cases and the empirically reported incidence of clinical BSE cases may be due to incorrect specification of a number of assumptions, such as the number of infected animals imported, the amount of contaminated feed introduced, or the timing of these introductions, among other factors. For example, it is likely that spreading out the introduction of infected cattle and contaminated feed over 1–2 years would decrease the pominence of the predicted early 1990s incidence peak, or eliminate it completely (the actual simulation introduced all infected animals in month 0 of the simulation and all contaminated feed in month 1). Increasing the number of cattle exposed to the 4000 ID_{50}s in contaminated feed by spreading out its introduction over 3 months increased the predicted number of clinical cases from 166 to 400 animals. Increasing the number of exposed animals substantially increased the number of infected animals in this case because the vast majority (if not all) of the exposed cattle in the original Switzerland simulation received more than 2.0 susceptibility-adjusted ID_{50}s. As a result, much of the infectivity they were exposed to was "wasted" in the sense that it could have been diverted to other animals without decreasing the probability of infection among the exposed animals in the original Switzerland scenario.

Although the similarity of our predictions and the observations from Switzerland provide some confidence that the model's structure and approach are reasonable, it is important to note that this is not a true validation and, in fact, the model's predictions could be close to reported observations for the "wrong" reasons. However, given the absence of data suitable for validating the model, the results of the Switzerland scenario are encouraging.

D. Summary

This model was developed to help evaluate the potential for BSE to become a major animal health or public health threat in the United States. Based on the simulation model and assumptions developed for this anal-

ysis, inferences can be drawn about the robustness of regulations and practices in the United States, and data or research can be identified that would increase confidence in the model's predictions. In addition, the simulation can be used to characterize the effectiveness of additional risk management strategies.

We recognize that the identification of a single case of BSE in the United States would have important ramifications for public opinion, trade, and other areas. Yet this analysis demonstrates that even if BSE were somehow to arise in the United States, few additional animals would become infected, little infectivity would be available for potential human exposure, and the prevalence of disease would decrease over time, leading to its eventual eradication. In short, the United States appears resistant to a BSE challenge, primarily because of the FDA feed ban, which greatly reduces the chance that an infected animal will infect other animals. However, the effectiveness of the feed ban is somewhat uncertain because compliance rates are not precisely known.

Potential sources of human exposure to BSE infectivity can be divided into two categories: specific high-risk tissues and contamination of low-risk tissues. Although not widely popular in the United States, both brain and spinal cord are consumed by some members of the population. If BSE were present in the United States, these tissues would be an obvious source of exposure. Our analysis indicates that the most important means by which low-risk tissue can become contaminated is the use of AMR technology, which can leave spinal cord or DRG in the recovered meat. Our analysis further indicates that missplitting of the spinal column and the resulting incomplete removal of the spinal cord is largely responsible for contamination of AMR meat. In addition, we assume that even in the absence of missplitting, some amount of DRG is extracted whenever vertabrae are processed by AMR. Aerosolization of the spinal cord during splitting contributes substantially less contamination even though it occurs every time an infected animal is processed.

Despite the potential for the consumption of high risk-tissues and the contamination of low-risk tissues, our results indicate that only small amounts of infectivity are available for human consumption. The import of one infected animal yields in an average of 2.7 cattle oral ID_{50}s for potential human exposure over a 20-year period, while the import of 10 infected cattle results in an average of 35 cattle oral ID_{50}s in this period. These results can be put into context by comparing them to potential exposure in the United Kingdom where it is estimated almost one million cattle were infected over a 15–20-year period. If the UK population was potentially exposed to only one cattle oral ID_{50} from each of these animals, potential human exposure in the United Kingdom would dwarf our projec-

tions for the United States. At this time, just over 100 cases of variant Creutzfeldt-Jakob disease (the human TSE linked to BSE) have been identified in the United Kingdom, although projections range from a few hundred to tens of thousands of eventual cases. If the number of cattle oral ID_{50}s available for human consumption is a good indicator of possible disease risk, it is unlikely the UK experience would be duplicated in the United States.

There are a number of model assumptions that cannot be verified with confidence, some of which influence the conclusions drawn. With regard to estimating the spread of BSE among cattle, the most influential sources of uncertainty are related to compliance with the FDA feed ban. Within this category, the most important source of uncertainty is the misfeeding rate on farms. Misfeeding prohibited feed (containing ruminant protein) to cattle on farms that raise both cattle and either pigs or chickens completely compromises the feed ban. This practice is the focus of efforts to understand how animals born after the implementation of feed bans in Europe have become infected with BSE. Uncertainty with respect to compliance rates can be reduced with field work and data collection. A second source of uncertainty associated with the feed ban is the proportion of feed produced that is mislabeled (i.e., the absence of proper labels identifying prohibited feed as not to be administered to ruminants).

Improving estimates of compliance with the feed ban would also improve estimates of potential human exposure to BSE-contaminated meat. Other important sources of uncertainty influencing estimates of human exposure include the number of ID_{50}s per clinical case of BSE and the proportion of clinical animals that would be correctly identified by AM inspectors. While the first of these two factors may be amenable to research, it is not clear how estimates for the second factor could be improved.

In addition to the scenarios described here, this model has been used to evaluate several risk management options (3). The insights provided by the model demonstrate that steps very early in the rendering and feed production process can avoid the need for other, more obvious, measures. Specifically, removing most of the infectivity from rendered product can protect human and animal health even if the feed ban is not 100% effective. Prohibiting the rendering of dead animals, which may have died of BSE and hence have high levels of infectivity, or disposing of all specified risk materials reduces potential new cases of BSE by more than 75%. Reducing the misfeeding rate, a key parameter identified in our sensitivity analysis, is not important if the infectivity in prohibited MBM is greatly reduced or eliminated. Of course, it must be recognized that even in the absence of these measures, animal health risks and human exposure are both small, with the import of 10 infected cattle leading to an average of fewer than three additional cases of BSE and potential human exposure to 35 cattle oral ID_{50}s.

As we strive to learn from BSE and limit the extent of the disease, the model developed for this analysis has many potential uses because of its flexibility. For example, if appropriate data are available, its parameters can be modified so that other countries or regions can be simulated. Specific scenarios of interest can be evaluated, including risk management actions under consideration. The model can also be used to evaluate hypotheses about sources and factors influencing the spread of BSE. We hope this model will find a place among the useful tools for understanding and controlling BSE.

APPENDIX A

This appendix describes how we estimated the probabilities that a prohibited or mixed renderer or feed producer will mislabel prohibited product as nonprohibited. Because this information is not directly available, we used a simple mass-balance approach to characterize the set of plausible mislabeling rates. In particular, we estimated the total amount of mammalian protein used to make cattle feed and assumed that the mislabeled MBM and feed makes up the difference between this total and the amount of pure porcine protein available. Figure A1 summarizes the relationships assumed.

Material from ruminants and other prohibited species can either be labeled as prohibited MBM or mislabeled as nonprohibited MBM. The prohibited MBM can be exported, used to produce pet food, or used to produce prohibited feed. The prohibited feed may also be exported or mislabeled as nonprohibited feed. Of the total porcine MBM produced, some fraction is exported or used for nonlivestock feed. The rest is used to make nonprohibited feed. Nonprohibited feed made from porcine MBM supplies a portion of the protein needed by cattle. Other sources of protein include nonmammalian animal protein, like poultry feather meal, and vegetable protein, primarily from soy.

We estimated that 4.6 billion pounds of prohibited MBM and 1.5 billion pounds of porcine MBM are produced each year (42). Of the bypass protein consumed by cattle, it is estimated that 4.4% is from porcine (D.W. Harlan, personal communication, Taylor Byproducts). We estimated total bypass protein consumption by cattle (9.7 billion pounds) from the cattle population estimates and consumption rate estimates detailed in Sections 3.9 and 3.13 of Appendix 1 in Cohen et al. (3). Hence, total consumption of porcine protein by cattle is $4.4\% \times 9.7$ billion pounds, or 430 million pounds per year.

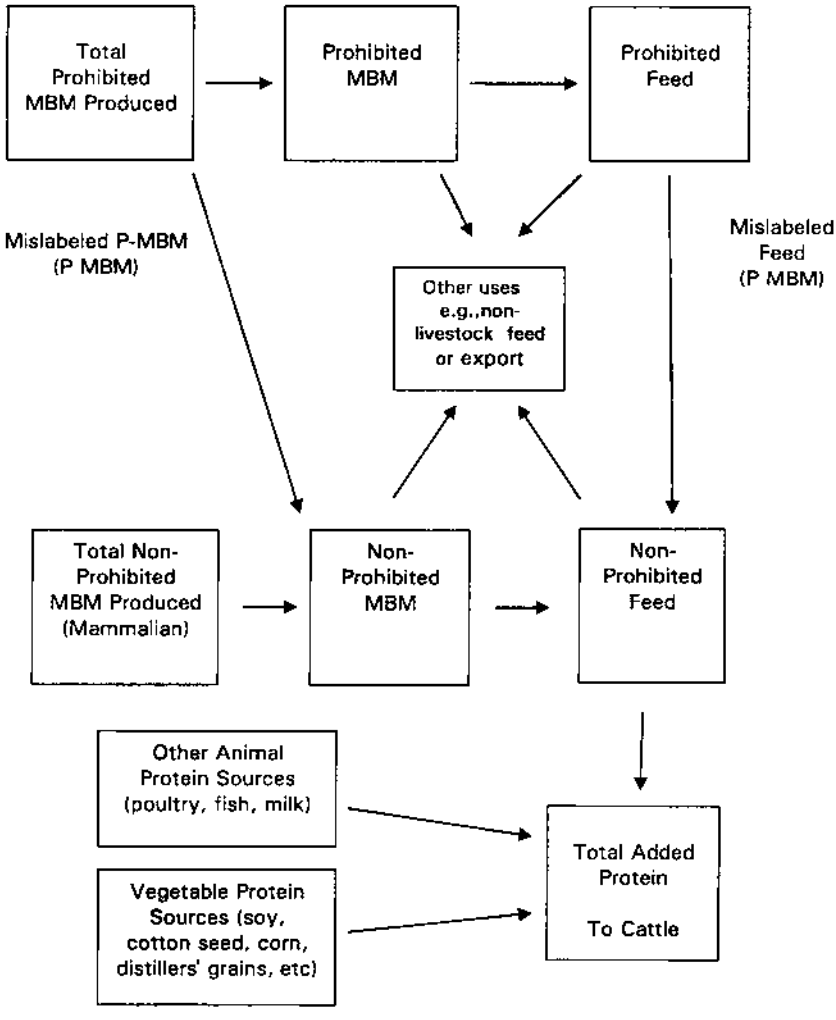

Figure A1 Mass balance approach used to calculate mislabeling rates.

We assume that the ostensible supply of 430 million pounds of non-prohibited protein also includes mislabeled material. In particular, we calculated the feed and MBM mislabel probabilities from the relationship T1 + T2 + T3 = 428 million pounds, where T1 is the amount of protein from prohibited feed that has been mislabeled as nonprohibited feed, T2 is the amount of protein from prohibited MBM that has been mislabeled as

nonprohibited MBM and ultimately used to produce nonprohibited feed, and T3 is the amount of protein from nonprohibited MBM. These three quantities are computed from the following relationships:

$$T_1 = KP \times (1 - PrMislabel_{MBM}) \times (1 - A) \times (1 - B) \times PrMislabel_{Feed} \times (1 - C)$$

$$T_2 = KP \times PrMislabel_{MBM} \times (1 - D) \times (1 - C)$$

$$T_3 = KNP \times (1 - C) \times (1 - D)$$

Here,

$PrMislabel_{Feed}$ = fraction of prohibited feed that is mislabeled as nonprohibited feed
$PrMislabel_{MBM}$ = fraction of nonprohibited MBM that is mislabeled as nonprohibited MBM
A = fraction of prohibited MBM diverted to other uses = 0.32
B = fraction of prohibited feed diverted to other uses = 0.02
C = fraction of nonprohibited feed diverted to other uses = 0.75
D = fraction of nonprohibited MBM diverted to other uses = 0.10
KP = total prohibited protein source material = 4.6 billion pounds
KNP = total nonprohibited protein source material = 1.5 billion pounds

Making the appropriate substitutions yields the relationship

$$PrMislabel_{Feed} = \frac{0.0905 - 1.035PrMislabel_{MBM}}{0.76636(1 - PrMislabel_{MBM})}$$

This relationship implies that $PrMislabel_{MBM}$ ranges from 0 (when $PrMislabel_{Feed}$ is approximately 12%) to as much as 8% (when $PrMislabel_{Feed}$ is 0). We chose $PrMislabel_{MBM}$ = 5% and $PrMislabel_{Feed}$ = 5% as central estimates for these quantities.

APPENDIX B

This appendix describes how we estimated the probability that properly labeled prohibited feed is fed to cattle. We assumed that this probability depends on whether the farm to which prohibited feed is sent also raises cattle. In particular, we assumed that the probability that a randomly chosen "packet" of prohibited feed is administered to cattle equals the product of: (1) the proportion of farms with animals consuming prohibited

feed that also have cattle, and (2) the probability that such an operation incorrectly administers prohibited feed to cattle. We assumed that animals consuming prohibited feed include hogs and pigs, egg-laying hens and pullets, broilers, and turkeys. Our computations ignore that fact that farms have different numbers of animals. There are insufficient data to take this variation into account.

We used Bayes law to calculate the probability that dairy animals are present on a farm given the presence of animals that can consume prohibited feed, designated "Prob(Dairy|Prohibited)." In particular,

$$\text{Prob(Dairy|Prohibited)} = \frac{\text{Prob(Prohibited|Dairy)} \times \text{Prob(Dairy)}}{\text{Prob(Prohibited)}},$$

where Prob(Prohibited|Dairy) is the probability that a farm raises animals consuming prohibited feed given the presence of dairy cattle, Prob(Dairy) is the proportion of farms that raise dairy cows, and Prob(Prohibited) is the proportion of farms that raise animals consuming prohibited feed. Note that this computation is limited to dairy animals because we were unable to identify data to estimate Prob(Prohibited|all cattle) or Prob(Prohibited|beef).

We estimated the value of the quantity Prob(Prohibited|Dairy) as the sum of Prob(Chicken|Dairy) and Prob(Hogs|Dairy). These two proportions are 7.5% and 3.9%, respectively ((Section E, part 1b in Ref. 43), yielding a sum of 11.4%. The value of Prob(Dairy) was calculated by dividing the total number of milk cow inventory farms (116,874) (Table 1 in Ref. 44) by the number of total farms (1,911,859) Table 1 in Ref. 44), yielding 6.1%. The value of Prob(Prohibited) was calculated by dividing the total number of hog/pig farms (109,754) (Table 1 in Ref. 44), egg-laying hens/pullets farms (69,761) (Table 22 in Ref. 44), broiler chicken farms (23, 937) (Table 1 in Ref. 44), and turkey farms (6,031) (Table 23 in Ref. 44) by the total number of farms (1,911,959) (Table 1 in Ref. 44), yielding 11.0%. Plugging these values into the preceding equation yields Prob(Dairy|Prohibited) = 6.4%.

Next we assumed that beef operations are distinct from dairy operations and Prob(Beef|Prohibited) is equal to the value of Prob(Dairy|Prohibited) scaled by the ratio of the number of beef operations to the number of dairy operations. As a result, we assumed

$$\text{Prob(AllCattle|Prohibited)} = \text{Prob(Dairy|Prohibited)} * \left(1 + \frac{N_{\text{Beef}}}{N_{\text{Dairy}}}\right)$$

where Prob(AllCattle|Prohibited) is the probability of any cattle raised on a farm that also raises animals that consume prohibited feed, and N_{Beef} and N_{Dairy} are the number of beef operations and dairy operations in the United

States, respectively. Using the USDA's values of $N_{Dairy} = 116{,}874$ and $N_{Beef} = 804{,}595$ (44) yields Prob(AllCattle|Prohibited) = 50%.

Finally, we assumed that one-thirtieth of the prohibited feed sent to farms that raise both cattle and animals consuming prohibited feed is administered to cattle. As a result, the misfeeding rate is approximately 1.7%.

APPENDIX C

Chebychev's inequality states that the expected number of infected cattle, designated here as X, exceeds the bound designated a with no more than probability $E(\overline{X}^2)/a^2$. The numerator of this fraction can be calculated from the relationship $E(\overline{X}^2) = Var(\overline{X}) + [E(\overline{X})]^2$. Although the value of $\overline{X}$ for the 1000 simulation runs of the base case was 2.9, the fact that this value is less than the lower bound associated with many of the parameters evaluated as part of the sensitivity analysis (see Fig. 2) indicates that $E(\overline{X})$ is likely to be somewhat greater. We therefore assume that the value of $E(\overline{X})$ is around 4.5 and hence that $[E(\overline{X})]^2$ is around 20. $Var(\overline{X}) = Var(X)/n$, where $Var(X) \approx 200$ based on the results of the 1,000 base case simulation runs. Dividing by $n = 1000$ yields $Var(\overline{X}) \approx 0.2$, which does not substantially alter the value of $E(\overline{X}^2)$. To compute the maximum theoretical value for the 95% upper confidence bound on the arithmetic mean number of additional infected cattle, we use the Chebychev inequality to solve for a. In particular, $0.05 = P\{\overline{X} \geq a) \leq E(\overline{X}^2)/a^2 \approx 20/a^2$, which indicates that $a = 20$.

REFERENCES

1. European Union Scientific Steering Committee. Report on the Assessment of the geographical BSE-risk of USA (July 2000). 2000. Accessed online at: http://europa.eu.int/comm/food/fs/sc/ssc/out137_en.pdf
2. US Department of Agriculture Food Safety Inspection Service. Animal reporting system (ADRS) livestock slaughtered in USDA year 1998. 1998. Accessed online at: http://www.fsis.usda.gov/OPHS/adrsdata/1998adrs/98crm1.htm.
3. Cohen JT, Duggar K, Gray GM, Kreindel S. Evaluation of the potential for bovine spongiform encephalopathy in the United States. Harvard Center for Risk Analysis. Boston, MA. 2001. Accessed online at: http://www.aphis.usda.gov/oa/bse/.
4. Radostits O, Leslie K, Fetrow J. Herd Food Animal Production Medicine, 2nd Ed. Philadelphia, Pennsylvania: WB Saunders Company, 1994.
5. US Department of Agriculture Animal and Plant Health Inspection Service.

Part I: Reference of 1996, dairy management practices. 1996. Accessed online at: http://www.aphis.usda.gov/vs/ceah/cahm/Dairy_Cattle/dr96des1.pdf.
6. US Department of Agriculture Animal and Plant Health Inspection Service. Part II: Reference of 1997 beef cow-calf health management practices. 1997. Accessed online at: http://www.aphis.usda.gov/vs/ceah/cahm/Beef_Cow-Calf/bf97des2.pdf.
7. Ensminger M, Oldfield J, Heinemann W. Feeds and Nutrition. Clovis, California: The Ensminger Publishing Company, USA, 1990.
8. National Research Council. Nutrient Requirements for dairy cattle, Sixth Revised Edition, Update 1989. Washington DC, US, 1989.
9. de Koeijer A, Schreuder B, Heesterbeek H, Oberthur R, Wilesmith J, Jong M. BSE Risk Assessment by Calculating the Basic Reproduction Ratio for the Infection Among Cattle. Institute of Animal Science and Health. Lelystad, the Netherlands. 1999.
10. Kimberlin RH. Transmissible encephalopathies in animals. Can J Vet Res 1990; 54:30–37.
11. Foster JD, McKelvey WA, Mylne MJ, Williams A, Hunter N, Hope J, Fraser H. Studies on maternal transmission of scrapie in sheep by embryo transfer. Vet Rec 1992; 130:341–343.
12. Elsen JM, Amigues Y, Schelcher F, Ducrocq V, Andreoletti O, Eychenne F, Khang JV, Poivey JP, Lantier F, Laplanche JL. Genetic susceptibility and transmission factors in scrapie: detailed analysis of an epidemic in a closed flock of Romanov. Arch Virol 1999; 144:431–445.
13. Wilesmith JW, Wells GA, Ryan JB, Gavier-Widen D, Simmons MM. A cohort study to examine maternally-associated risk factors for bovine spongiform encephalopathy. Vet Rec 1997; 141:239–243.
14. Ferguson NM, Donnelly CA, Woolhouse ME, Anderson RM. A genetic interpretation of heightened risk of BSE in offspring of affected dams. Proc Roy Soc Lond Ser B: Biol Sci 1997; 264:1445–1455.
15. Donnelly CA, Ferguson NM, Ghani AC, Wilesmith JW, Anderson RM. Analysis of dam-calf pairs of BSE cases: confirmation of a maternal risk enhancement. Proc Roy Soc Lond Ser B: Biol Sci 1997; 264:1647–1656.
16. Donnelly CA, Ferguson NM, Ghani AC, Woolhouse ME, Watt CJ, Anderson RM. The epidemiology of BSE in cattle herds in Great Britain. I. Epidemiological processes, demography of cattle and approaches to control by culling. Phil Trans Roy Soc Lond Ser B: Biol Sci 1997; 352:781–801.
17. C Donnelly. Maternal transmission of BSE: interpretation of the data on the offspring of BSE-affected pedigree suckler cows. Vet Rec 1998; 142(21):579–580.
18. G Horn, M Bobrow, M Bruce, M Goedhert, A McLean, J Webster. Review of the origin of BSE. UK Department for Environment, Food, and Rural Affairs, 2001. Accessed online at http://www.defra.gov.uk/animalh/bse/bseorigin.pdf on June 19, 2002.
19. Spongiform Encephalopathy Advisory Committee. SEAC Annual report 1997–98. 1999. London, UK. Accessed online at: http://www.seac.gov.uk/seacrept.pdf.

20. Ferguson NM, Donnelly CA, Woolhouse ME, Anderson RM. The epidemiology of BSE in cattle herds in Great Britain. II. Model construtions and analysis of transmission dynamics. Phil Trans Roy Soc Lond Ser B: Biol Sci 1997; 352:803–838.
21. Wells GA, Hawkins SA, Green RB, Spencer YI, Dexter I, Dawson M. Limited detection of sternal bone marrow infectivity in the clinical phase of experimental bovine spongiform encephalopathy (BSE). Vet Rec 1999; 144:292–294.
22. Wells GA, Hawkins SA, Green RB, Austin AR, Dexter I, Spencer YI, Chaplin MJ, Stack MJ, Dawson M. Preliminary observations on the pathogenesis of experimental bovine spongiform encephalopathy (BSE): an update. Vet Rec 1998; 142:103–106.
23. European Union Scientific Steering Committee. Opinion of the Scientific Steering Committee on the Human Exposure Risk (HER) via food with respect to BSE—Adopted on 10 December 1999. 1999. Accessed online at: http://europa.eu.int/comm/food/fs/sc/ssc/out67_en.html.
24. Spongiform Encephalopathy Advisory Committee. Risk Assessment for the disposal of treated rendering plant ruminant condensate to agricultural land. London, UK, 2000.
25. Gale P, Stanfield G. Towards a quantitative risk assessment for BSE in sewage sludge. J Appl Microbiol 2001; 91:563–569.
26. US Department of Agriculture Food Safety Inspection Service. Livestock and carcass disposition review, 1997.
27. Heim D, Wilesmith JW. Surveillance of BSE. Arch Virol 2000; Suppl:127–33.
28. Miller LD, Davies AJ, Jenny AL, Fekadu M, Whitfield SG. Surveillance for lesions of bovine spongiform encephalopathy in U.S. cattle. Dev Biol Stand 1993; 80:119–121.
29. European Union Scientific Steering Committee. Opinion on the safety of ruminant blood with respect to TSE risks adopted by the SSC at its meeting of 13–14 April 2000. Accessed online at: http://europa.eu.int/comm/food/fs/sc/ssc/out74_en.pdf.
30. Anil MH, Love S, Williams S, Shand A, McKinstry JL, Helps CR, Waterman-Pearson A, Seghatchian J, Harbour DA. Potential contamination of beef carcases with brain tissue at slaughter. Vet Rec 1999; 145:460–462.
31. Garland T, Bauer N, Bailey M Jr. Brain emboli in the lungs of cattle after stunning [letter] [see comments]. Lancet 1996; 348:610.
32. Schmidt GR, Hossner KL, Yemm RS, Gould DH. Potential for disruption of central nervous system tissue in beef cattle by different types of captive bolt stunners. J Food Protect 1999; 62:390–3.
33. Harbour D. Measures to reduce contamination of meat and environment with CNS tissue during slaughter and processing of cattle and sheep. 2001. Accessed online at: http//europa.eu.int/comm/research/press/1998/pr2710en.html.
34. I Sparks Companies. Advanced meat recovery systems—An economic analysis of the proposed USDA regulation. 1999. McLean, VA. Accessed online at: http://www.fsis.usda.gov/OPPDE/rdad/FRPubs/98-027R/SparksCo_AMR_Economic_Analysis.pdf.

35. Food and Drug Administration. Substances prohibited from use in animal food or feed; animal proteins prohibited in ruminant feed; Final Rule-21 CFR Part 589. 1997. Accessed online at: http://www.fda.gov/cvm/index/bse/6597bse.htm.
36. Taylor DM, Woodgate SL, Atkinson MJ. Inactivation of the bovine spongiform encephalopathy agent by rendering procedures. Vet Rec 1995; 137:605–610.
37. Taylor DM, Woodgate SL, Fleetwood AJ, Cawthorne RJ. Effect of rendering procedures on the scrapie agent. Vet Rec 1997; 141:643–649.
38. Schreuder BE, Geertsma RE, van Keulen LJ, van Asten JA, Enthoven P, Oberthur RC, de Koeijer AA, Osterhaus AD. Studies on the efficacy of hyperbaric rendering procedures in inactivating bovine spongiform encephalopathy (BSE) and scrapie agent. Vet Rec 1998; 142:474–480.
39. Doherr MG, Heim D, Vandevelde M, Fatzer R. Modelling the expected numbers of preclinical and clinical cases of bovine spongiform encephalopathy in Switzerland. Vet Rec 1999; 145:155–160.
40. European Union Scientific Steering Committee. Report on the assessment of the geographical BSE-Risk (GBR) of Switzerland. European Commission. 2000. Accessed online at: http://europa.eu.int/comm/food/fs/sc/ssc/out136_en.pdf.
41. Dietz K. Some problems in the theory of infectious disease transmission and control. In: Mollison D, ed. Epidemic Models: Their Structure and Relation to Data. Cambridge, UK: Cambridge University Press, 1995: 3–16.
42. I Sparks Companies. The Economic Impact of the Proposed Regulations Concerning Ruminant-Based Protein Products. National Renderers Associations. Alexandria, VA. 1997.
43. US Department of Agriculture Animal and Plant Health Inspection Services. Part II: Changes in the U.S. dairy industry: 1991–1996. 1996. Accessed online at: http://www.aphis.usda.gov/vs/ceah/cahm/Dairy_Cattle/dr96trnd.pdf.
44. US Department of Agriculture—National Agricultural Statistics Service. 1997 Census of Agriculture Volume 1: Part 51, Chapter 1: United States summary and state data. 1997. Accessed online at http://www.nass.usda.gov/census/census97/volume1/toc97.htm.

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Actions to Prevent Bovine Spongiform Encephalopathy from Entering the United States

Linda A. Detwiler
Animal and Plant Health Inspection Service, U.S. Department of Agriculture, Robbinsville, New Jersey, U.S.A.

Burt Pritchett
Center for Veterinary Medicine, Food and Drug Administration, U.S. Department of Health and Human Services, Rockville, Maryland, U.S.A.

I. INTRODUCTION

For the past decade the U.S. government has actively instituted policies and practices to mitigate the entry of the bovine spongiform encephalopathy (BSE) agent into the United States and to minimize the chance for disease amplification in the national cattle herd, were it to be found here. To date, there is no evidence of BSE in the United States. The U.S. approach to managing the risk of BSE is focused on three primary goals:

Prevent the BSE agent from entering the country and infecting U.S. cattle
Prevent the amplification of BSE throughout the U.S. cattle herd, were it to occur here
Prevent the BSE agent from exposing the U.S. public

The key actions that appear to have been effective in achieving these goals are:

Prohibiting the importation of live ruminants and most ruminant products including meat and bone meal from BSE-affected countries (since 1989) and all of Europe (since 1997)

Prohibiting the feeding of most mammalian tissues to ruminants in the United States beginning in 1997

II. HISTORY

With the official announcement that the United Kingdom had identified a "novel" spongiform encepalopathy of cattle in 1986, the U.S. government began to monitor the science and events regarding the disease that would become bovine spongiform encephalopathy. In the late 1980s U.S. Department of Agriculture's Animal and Plant Health Inspection Service (USDA, APHIS) established a special committee to examine different facets of the disease. The purpose of the committee was to summarize known and unknown information as well as to make recommendations to the agency. The following topics were detailed in a written report from the committee:

1. Import regulations and export certification—outlined current and proposed regulations
2. Surveillance—summarized efforts to date and proposed recommendations to increase and enhance surveillance
3. Comparative risk—compared the animal populations, production practices, rendering practices, and relevant regulations between the United States and the United Kingdom to determine whether significant differences of risk existed between the two countries
4. Rendering policy—analyzed the need for regulations on rendering and the use of rendered products; identified other federal agencies with authority in this area
5. Scrapie—assessed the risk of BSE developing from endemic scrapie and assessed the impact of BSE on the U.S. scrapie program
6. Rendering research—summarized ongoing research and proposed research that would be pertinent for the United States
7. Contingency plans—determined feasible contingency plans for APHIS if BSE were to occur in the United States, giving attention to the possible need for quarantine, depopulation, and carcass disposal

8. Public relations—analyzed strategies and options for managing APHIS's public relations concerning BSE; evaluated problems experienced in the UK

The report was presented to APHIS and other government agencies. These included USDA's Agricultural Research Service (ARS) and Food Safety Inspection Service (FSIS) and the Health and Human Service's Food and Drug Administration (FDA) and National Institutes of Health (NIH).

The special committee became the BSE Working Group, which evolved into the TSE Working Group in the mid-1990s. The TSE Working Group still continues to monitor the changing science and world events, providing recommendations to APHIS. The Group also serves as a liaison with government personnel in other U.S. agencies, Canada, and Mexico.

III. IMPORT PROHIBITIONS

Since 1989, APHIS has prohibited the importation of live ruminants from countries where BSE is known to exist in native cattle. Other products derived from ruminants (for example, fetal bovine serum, meat-and-bone meal, blood meal, offal, fats, and glands) are also prohibited from entry, except under special conditions or under USDA permit for scientific or research purposes. These restrictions were initially applied to the United Kingdom in 1989, and then were applied to each country that subsequently identified native cases of BSE. In 1997 APHIS extended these restrictions to include most of the countries in Europe, owing to concerns about widespread risk factors and inadequate surveillance for BSE. As of December 2000, USDA prohibited all imports of rendered animal protein products, regardless of species, from BSE-restricted countries. This decision followed the recent determination by the European Union that feed of nonruminant origin was potentially cross-contaminated with the BSE agent. The restriction applies to products originating, rendered, processed, or otherwise associated with products from BSE-restricted countries. When BSE was recently identified outside Europe, in native-born cattle in Japan, Israel, and Canada, APHIS restricted imports of ruminants and ruminant-origin products from there also.

The Food and Drug Administration (FDA) has taken several steps to help ensure the safety of medical products, such as human and animal drugs, dietary supplements, human blood, human vaccines, and human medical devices. In 1990 the agency began requiring manufacturers of products derived from bovine sources to document that the animal tissues did not come from a country with cattle that had tested positive for BSE. Beginning in 1992 and

most recently in May 1996, FDA also issued a series of letters advising all manufacturers of FDA-regulated products derived from bovine tissue materials that source materials should not come from cattle that ever resided in a country with BSE.

Regarding ruminants and ruminant products, the primary trading partners for the United States are Canada and Mexico. Hence it is important that the countries of North America coordinate efforts to reduce risks associated with BSE. The import measures as stated above have been taken by all three countries. The United States, Canada, and Mexico continue to work together when considering additional risk reduction measures.

Evidence that additional countries may have introduced BSE-contaminated feed into their livestock systems has prompted the three North American countries to develop a methodology to evaluate BSE risks within a country. The methodology is based on criteria established by the Office of International Epizooties (OIE). Evaluations of countries around the world are currently underway.

IV. DOMESTIC FEED PROHIBITIONS

To prevent the spread of BSE through feed, in June 1997, the FDA published a regulation that prohibits the use of mammalian protein in the manufacture of animal feeds for ruminants [Title 21, *Code of Federal Regulations* (CFR) Part 589]. The regulation excludes in the definition of mammalian protein certain tissues that, according to available scientific data, pose little risk of transmitting BSE. The excluded proteins are permitted in ruminant feed and include milk and milk products, blood and blood products, gelatin, products whose only mammalian protein is of porcine or equine origin, and "plate waste," defined as inspected meat products that have been cooked and offered for human food and then further heat-processed for animal feed.

The regulation, frequently referred to as the "feed ban," requires that all manufacturers of feedstuffs, from renderers to manufacturers of finished feed, label product containing prohibited material with the caution statement "Do Not Feed to Cattle or Other Ruminants." It also requires companies to keep for 1 year records of receipt, processing, and distribution of prohibited material. Finally, the feed ban requires that firms that produce both ruminant feed (or feed ingredients that may be used in ruminant feed) and feed for nonruminants be able to prevent cross-contamination. Two options for preventing cross-contamination are: (1) either maintain separate equipment or facilities or (2) use clean-out procedures, such as flushing, sequencing, or physical cleaning, to prevent unsafe carryover from a batch of nonruminant feed into a batch of ruminant feed.

To help ensure good compliance with the regulation, the FDA, with strong support from the renderers, cattle owners, feed manufacturers, and feed lot owners, launched a compliance and education program, including a rigorous inspection program. The FDA sponsored many educational workshops and other training initiatives aimed at those affected by the feed ban. The inspection program relies heavily on state inspectors, in addition to the FDA's own inspectors, to inspect renderers, protein blenders, feed mills, haulers, distributors, and ruminant feeders for compliance with the regulation.

V. SURVEILLANCE

APHIS has had a comprehensive surveillance program in place in the United States (since 1990) to ensure timely detection and swift response in the event that an introduction of BSE were to occur. This surveillance program incorporates both the location of imports from the United Kingdom or other countries that have detected BSE, and targeted active and passive surveillance for either BSE or any other TSE in cattle.

Samples for BSE surveillance of adult cattle are obtained from:

1. Field cases of cattle exhibiting signs of neurological disease
2. Cattle condemned at slaughter for neurological reasons
3. Rabies-negative cattle submitted to public health laboratories
4. Neurological cases submitted to veterinary diagnostic laboratories and teaching hospitals
5. Sampling of aged cattle that are nonambulatory (i.e., fallen stock)
6. Adult cattle dying on farms from unknown causes

Cattle showing evidence of neurological disease when presented for slaughter are condemned upon antemortem inspection. Samples of brain tissue are submitted for diagnosis to the National Veterinary Services Laboratory.

As of April 30, 2003, over 47,900 brains had been examined for BSE or another form of a transmissible spongiform encephalopathy in cattle. Over 19,900 of these samples have been collected and tested in fiscal year (FY) 2002. The use of immunohistochemistry for the detection of the abnormal form of the prion protein was introduced in 1994. As of 1997 all samples submitted to the National Veterinary Services Laboratory (NVSL) are tested by immunohistochemistry. Immunohistochemistry is used by many countries as the confirmatory test. No evidence of either condition has been found. In FY 2002, the number of samples tested is more than 40 times the international standard set for the U.S. cattle population (Fig. 1).

APHIS has continued to adjust and improve the surveillance system as science world events dictate. APHIS officials recently established a regional

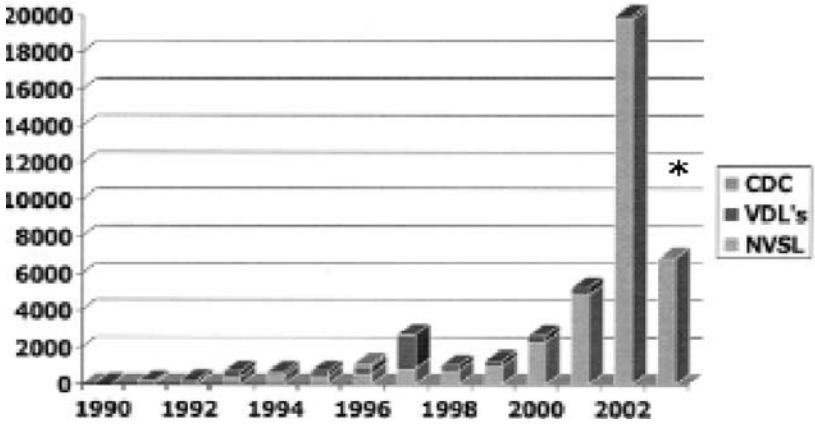

Figure 1 In fiscal year 2002, the number of brain samples tested was more than 40 times the international standard set for the U.S. cattle population. *11,152 through April 30, 2003.

sampling model after they determined that a state-based approach was not the most effective method. (Adult cattle are often not slaughtered in the state in which they are born and raised, and therefore a state-based approach may not allow sufficient sampling.) Based on animal movement patterns, APHIS divided the United States into eight regions where adult cattle would be raised and exit the production system (Fig. 2). Using the adult cattle population in each region, surveillance goals were calculated based on the OIE standards as if each region were an individual country. In 2001, these goals were doubled to be able to detect an even lower level of BSE. These goals had been increased even further for 2002–2003, with a national annual goal of testing at least 12,500 samples.

The goal of testing 12,500 samples was established to detect one BSE-infected animal per million cattle. The prevalence of classic Creutzfeldt-Jakob disease (CJD) in human populations appears to be approximately one in a million worldwide. It has been hypothesized that other spongiform encephalopathies also might occur in the host populations at the same rate (1). This is what has led the USDA to set a prevalence level of one in a million as the target for our BSE surveillance.

The international animal health organization (OIE) has established guidelines for the number of samples that should be tested each year: *http://www.oie.int/eng/normes/mcode/A_00154.htm*. For the United States, OIE recommends a surveillance level of 433 samples per year. However, USDA wanted the extra measure of security that a higher sampling level would pro-

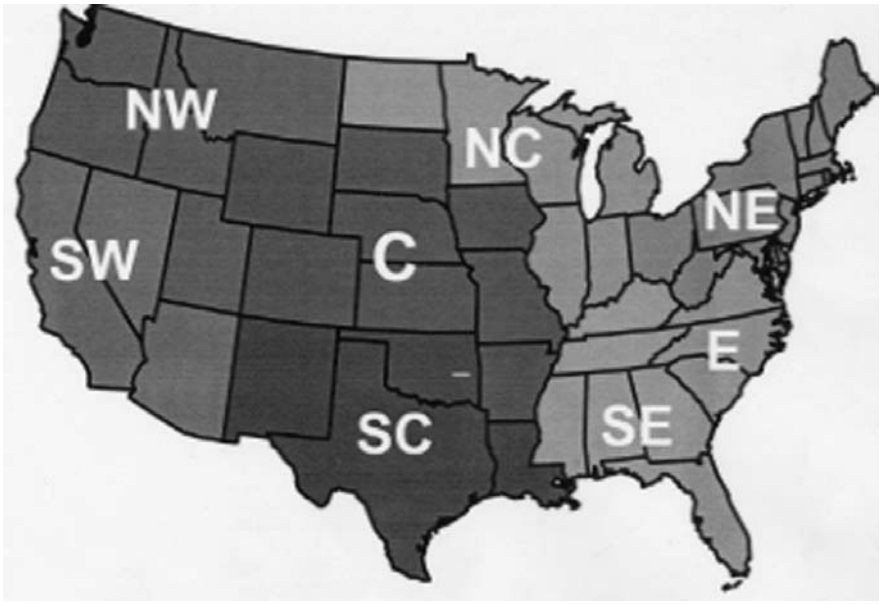

Figure 2 The eight regions of the United States where adult cattle are raised and exit the production system.

vide, and therefore we have maintained surveillance far above the OIE-recommended level since 1994. USDA set out to design a surveillance program that is based on being able to detect if we had one BSE-infected animal in a population of a million. Given that the United States has an adult cattle population of approximately 45 million, if we did have BSE in this country at the one-in-a-million level, we could assume that it would we would have 45 infected animals. To achieve a 95% confidence level in the accuracy of a random sample of adult cattle, we would have to sample and test some 3 million animals.

However, to conduct a more efficient, targeted, and effective survey, USDA's program instead focused on the highest risk population of cattle: adult cattle with central nervous system clinical signs and nonambulatory cattle. Defining nonambulatory cattle as a high-risk population is based on the BSE surveillance experience of European countries that have BSE, since their experience and testing schemes have proven nonambulatory cattle to be an appropriate population for active targeted surveillance. For example, in Switzerland, testing of fallen stock and emergency slaughter cattle (these two populations combined are essentially equivalent to the U.S. nonambulatory cattle population) has revealed a BSE prevalence of 0.2% in 1999

and 0.12% in 2000. In comparison, Switzerland's BSE prevalence in routine healthy slaughter populations was 0.004% in 1999 and 0% in 2000 (2). BSE surveillance in France during 2001 identified 91 cases (19.4%) from the 469 cattle exhibiting central nervous system clinical signs, and 100 BSE cases (0.07%) from the 133,889 nonambulatory cattle tested. French testing of healthy slaughter cattle found 83 BSE cases (0.003%) from the 2,382,225 tested. These data also support the decision to conduct a targeted surveillance scheme rather than a simple random sampling scheme.

The next step is to determine the number of nonambulatory cattle there are in the United States. No one knows the exact number; however, an estimate of 195,000 per year was obtained from a survey conducted of American Association of Bovine Practitioners members (3). An assumption is made that the 45 potential cases of BSE would all be found in the high-risk cattle population. Dividing the potential cases into the high-risk population (45/195,000) gives a prevalence of 0.023%. This is the level of disease that needs to be detected in the high-risk population. Using Cannon and Roe's formula to determine the sample size needed to be tested to detect disease at a prevalence of 0.023%, it is determined that, nationally, a sample size of 12,500 is needed (4). Sampling at this level will not prove that BSE does not occur at a lower prevalence level, but it should allow detection of a case if BSE truly exists at a level of one or more cases per million in the adult cattle population.

Another part of the surveillance program is to locate and monitor all cattle imported from the United Kingdom during the 1980s, before the USDA ban. Any of these cattle found to be still alive were monitored, and APHIS offered to purchase them. Upon purchase, they were destroyed and tested for BSE. No evidence of BSE has been found in any of these imported animals. Currently, three of these UK imports are still alive and are regularly monitored by a federal veterinarian for clinical signs compatible with BSE. In addition, APHIS traced all 46 cattle imported from continental Europe in 1996 and 1997. As with the UK imports, APHIS has offered to purchase these animals. As purchases occur, the cattle are destroyed and tested for BSE. No evidence of BSE has been found. Five of the 46 European imports are still alive as of July 2003, and federal veterinarians are monitoring them. APHIS has also traced cattle imported from Japan during the last decade. The imported Japanese cattle are also under movement restrictions.

VI. EMERGENCY RESPONSE PLANNING

USDA and FDA have drafted emergency response plans to be used in the event that BSE is introduced into the United States. The plans include specific actions that would be taken by the various government agencies to pro-

hibit exposure to the American public and to prevent amplification in the cattle feeding system. Also outlined are specific communication steps to keep the public, stakeholders, and foreign governments informed about the U.S. government's activities related to BSE.

VII. PROTECTING PUBLIC HEALTH

In addition to the FDA guidances regarding sourcing of bovine by-products for pharmaceuticals, biologicals, cosmetics, etc., the FDA has recommended certain precautions involving the human blood supply. In August 2001, the FDA issued new, more cautious draft guidance to prevent potential spread of vCJD by blood, although it is not presently known whether human blood transmits vCJD. Because there is no test to screen blood for the agents of CJD or vCJD, the FDA evaluated potential TSE-related risk factors for donors. The agency now recommends deferring potential blood donors who have lived or traveled in the United Kingdom for 3 months or more cumulative from 1980 to 1996. Also, because a limited number of cases of vCJD have been diagnosed outside the United Kingdom, the FDA recommends deferral of donors who lived in France for 5 years or more since 1980. Deferrals will also affect military personnel stationed in Europe at the same time and anyone who has had a blood transfusion in the United Kingdom since 1980.

VIII. EVALUATION

Diseases with long incubation periods and no preclinical live animal test require vastly different risk management strategies than highly contagious, short-incubation diseases. There needs to be continual evaluation of changes in the science and world events to adjust prevention, control, and response policies. In addition, government agencies of both animal and public health need to coordinate activities.

In the early 1990s, APHIS conducted several risk assessments examining the possibility of BSE emerging in the United States. These risk assessments led to a model to track the spread of BSE, an assessment of risk in the United States at the state and county levels based on scrapie contamination of rendered products, and a comparison of U.S. risk factors for BSE against those in the United Kingdom. These assessments all concluded that the potential risk of BSE emerging in the United States was substantially less than in the United Kingdom.

In April 1998, USDA entered into a cooperative agreement with Harvard University's Center for Risk Analysis, School of Public Health, to ana-

lyze and evaluate the U.S. government's measures to prevent BSE. The risk analysis reviewed current scientific information, assessed the ways that BSE could potentially enter the United States, and evaluated existing regulations and policies to prevent the spread of BSE within the United States, if the disease were to occur. The Harvard risk assessment, released on November 30, 2001, concluded:

> The United States is highly resistant to any introduction of BSE or a similar disease. BSE is extremely unlikely to become established in the United States. . . . Similarly there appears to be no potential for an epidemic of BSE resulting from scrapie, chronic wasting disease, or other cross-species transmission of similar diseases found in the U.S.

These assessments and one conducted by the European Union had similar conclusions. BSE is unlikely to occur in the United States but cannot be fully excluded. Given that we still do not have complete scientific knowledge about a number of the disease characteristics, we cannot say there is zero risk.

In response to the Harvard Risk Assessment the USDA announced several actions to assist in further reductions of risk:

Increase testing for BSE to a target of 12,500.

Publish an options paper in the *Federal Register* that will outline possible regulatory action to limit the risk of BSE exposure if it were to occur in the United States. The options will include prohibiting the use of brain and spinal cord in human food.

Issue a proposed rule prohibiting the use of certain stunning devices used to immobilize cattle during the slaughter process.

Publish an advance notice of proposed rule making to consider disposal options for dead and downer cattle, as they are an important potential pathway for the spread of BSE in the animal chain.

IX. SUMMARY

BSE has not been detected in the United States. It appears as if actions taken as early as the late 1980s have played a major role in reducing risks associated with BSE. This is owed in large part to cooperation between government agencies and various industry groups. Although risk management strategies have been adjusted many times over the last decade, it is important that continual evaluation of changing science and world events be maintained. Furthermore, it is essential that the evaluations result in the necessary regulatory, policy, or technical changes needed to respond to BSE.

ACKNOWLEDGMENT

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REFERENCES

1. Brown P, Will RG, Bradley R, Asher DM, Detwiler L. Bovine spongiform encephalopathy and variant Creutzfeldt-Jakob disease: background, evolution and current concerns. Emerg Infect Dis 2001; 7(1):6–16.
2. Doherr MG, Hett AR, Cohen CH, Fatzer R, Rufenacht J, Zurbriggen A, Heim D. Trends in prevalence of BSE in Switzerland based on fallen stock and slaughter surveillance source. Vet Rec 2002; 150(11):347–348.
3. Hansen Don, Bridges Victoria. A survey description of down-cows and cows with progressive or non-progressive neurological signs compatible with a TSE from veterinary-client herd in 38 states. Bovine Pract 1999; 33(2):179–187.
4. Cannon Rob M, Roe RT. Livestock Disease Surveys: A Field manual for veterinarians. Canberra: Australian Government Publishing Service, 1982.

5

Overview of Transmissible Spongiform Encephalopathy in Cervids in the United States

Sam D. Holland
South Dakota Animal Industry Board, Pierre, South Dakota, U.S.A.

I. RECOGNITION OF CHRONIC WASTING DISEASE IN DEER AND THEN IN ELK

An insidious chronic wasting disease in deer held in captivity at wildlife research facilities in Colorado and Wyoming, recognized since 1967, was first identified as a spontaneous occurring spongiform encephalopathy in 1980 (1). The first reported cases of spongiform encephalopathy in elk occurred in 1979 and were reported in 1982 (2).

The confirmation of bovine spongiform encephalopathy (BSE) in November 1986 and the announcement by British officials in March 1996 of a possible link between BSE and new variant Creutzfeldt-Jakob Disease (nvCJD) sparked new awareness of the transmissible spongiform encephalopathies throughout the world.

In 1991 Williams and Young reported chronic wasting disease (CWD) cases had occurred in a few free-ranging animals, but that cases had occurred primarily in captive mule deer and elk. The same report stated that CWD is not the result of food-borne exposure to the agent, as is now thought to be the accepted method of transmission of BSE. Warnings were given that, in light of international movements of nondomestic ruminants and expansions of game farming, CWD could potentially be of greater importance in the future (3). These words of warning for a large part went

unheeded for several years and both wildlife managers and game farmers are today paying the costs of inaction.

Clues to the potentially devastating effects of CWD on cervid populations should have been obvious. Of 23 elk remaining in one research herd in Colorado between 1986 and 1997, four (17%) developed CWD. In fact, CWD was the sole source of mortality in this research facility between 1986 and 1997—not surprising perhaps, as CWD had been responsible for the loss of 71% of the elk held at this same facility between 1976 and 1985! (4).

CWD has been observed in free-ranging mule deer, white-tailed deer, and elk from Northern Colorado since 1981 (5). The disease was first recognized in free-ranging elk in Wyoming in 1986 and has been considered endemic in northern Colorado and southeast Wyoming for over 20 years. The number of CWD case submissions for CWD in free-ranging cervids in the area has increased from less than one per year in 1981 to greater than one case submission per month in 1987. Confirmation of the disease has recently been observed in animals younger than the 24 months previously considered to be the minimum age for diagnosis (6). Nebraska, the most recent state to confirm CWD in free-ranging cervids, has recently reported CWD in deer under 1 year of age. This finding may necessitate alterations in management methods for the disease.

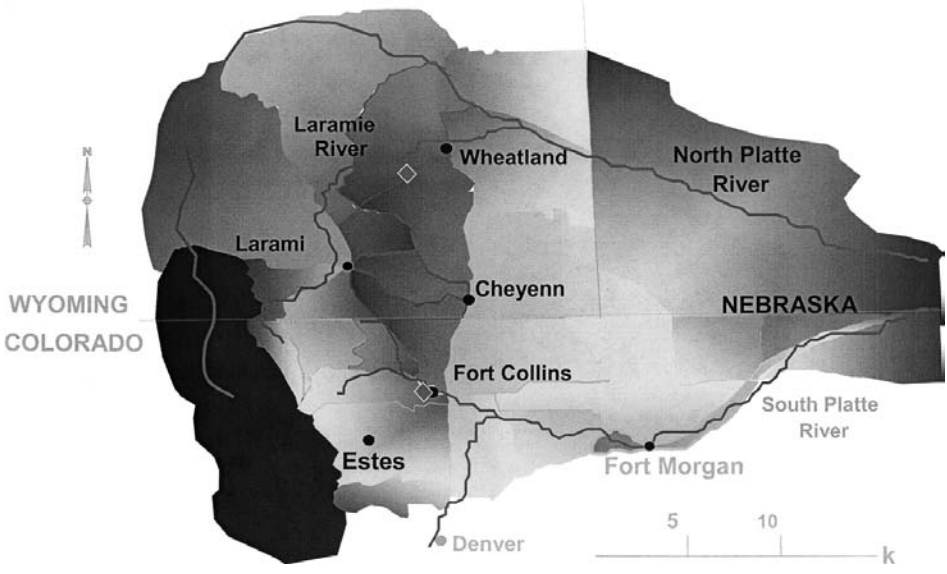

Figure 1 Map of chronic wasting disease in free-ranging deer and elk (1981–2000).

The difficulty in controlling or eradicating CWD, a disease that has insidious onset, for which there is no known antemortem diagnostics, no known method of transmission, no known susceptibility factors, no means of conferring immunity, no known length of incubation, and for which the causative agent appears to be virtually stable in the environment, is astounding. These factors compound difficulties in efforts to eliminate the disease in free-ranging populations. Yet the early warnings of the potential for this disease must be heeded, however late. More is learned each year on the susceptibility, diagnostics, transmissibility, prevalence, and epizoology of the transmissible spongiform encephalopathies (TSEs). Basic principles of disease control must be applied to wildlife populations, leaving all potential methods of control open for consideration, from early detection and surveillance to complete depopulation of selected affected and exposed groups.

The recognition of CWD in free-ranging cervids has included an expanding area from 1981 to 2001, and at present the disease is known to exist in free-ranging animals in a large area of southeast Wyoming, northeast Colorado, and southwest Nebraska (Fig. 1).

II. ATTEMPTS AT CONTROL OF CWD IN FREE-RANGING CERVIDS

Actions taken by state wildlife agencies have been somewhat cautious and limited with no early statements of intent to eliminate the disease. Colorado wildlife officials have taken actions that show gradual ratcheting up of management methods to reduce the incidence and spread of CWD in free-ranging cervids in that state and also to reduce the risk of transmission to farmed cervids. Management actions by Colorado wildlife officials as late as 1996 included public information, prohibiting feeding of elk and deer in the wild, limiting translocation projects by the agency, increased surveillance, and precluding new commercial permits for wildlife in areas where CWD was known to be endemic (5). However, no mention was made of mandatory surveillance for animals harvested or found dead or ill, of considerations for population reductions or eliminations in endemic areas, or of restricting migrations of animals from the endemic areas in attempts to prevent widening of these endemic areas.

In late 2001 Colorado wildlife officials proposed a much more aggressive disease management plan, emphasizing disease control, however still stopping short of any mention of eliminating this disease from cervid populations in that state (7). The newly proposed plans were unprecedented in focusing on disease management as a primary goal in ongoing

management plans for deer populations in areas endemic for CWD. Plans included hunter-reduced populations of deer in endemic areas, targeted monitoring for disease prevalence, and even staff culling of animals when the disease is found in areas of low prevalence within an endemic area. The goal was announced as an effort to reduce the prevalence of the disease to 1% in endemic areas and to prevent it from spreading to unaffected areas. Intentions to adopt similar plans in the future for elk populations were also announced. This is a bold attempt by those responsible to begin a process that hopefully can lead to containment and eventual eradication of CWD in wildlife and certainly will be closely watched by other states' wildlife agencies as they search for potential methods of prevention, control, and eradication of CWD in free-ranging cervids.

III. ATTEMPTS TO REGULATE CAPTIVE CERVIDS

In captive cervids the saga of CWD is only a bit more comforting. In the past three decades the United States has experienced a noticeable increase in entities involved in the raising and marketing of captive cervids and products thereof. It appears to me that a number of socioeconomic factors have contributed to this development. The national economy and standard of living has provided affluence for some and this has led them to develop hobbies of interest in wildlife. Environmental and ecological awareness by some have been incentives to seek to develop or restore cervids in some areas where they have historically been known to exist but have succumbed to civilization over the past couple of centuries. This restocking has been observed particularly within Native American reservations. Others have been stimulated by growing interest in and markets for natural or health food supplements and potions such as the elk antler derivatives both nationally and internationally. Some have been driven by pure desire to study and learn the reproduction, nutrition, habitat requirements, health and disease status, and other ecobiological systems the cervid species experience. Fee-basis hunting on private preserves has been the stimulus for others. The narrowing of margins as traditional agriculture matures has certainly led some to seek alternative means of surviving on their land.

This growing captive cervid element was observed with concern by those responsible for animal health. These concerned parties were primarily at the state level and particularly in the central and western states for two reasons. These states were seeing the greatest amount of captive cervid development and these states also had the interface of significant populations of free-ranging cervids and accompanying resources such as hunting and tourism.

The concerns over this growing, basically unregulated, industry were not without historical justification. The bison industry in the United States had experienced similar growth and had presented risks of disease, competition for habitat, dilution of gene pools through crossbreeding and interbreeding, and public safety, to name a few. In the early 1980s bison from one herd were responsible for disseminating tuberculosis to over 30 herds of bison and cattle in 145 states. Incidentally, the source herd of bison was determined to have acquired tuberculosis through exposure to an infected herd of captive elk prior to 1984. It was not until 1982 that bison were included in federal regulations controlling tuberculosis and brucellosis. Producers involved in the cervid industry have been for the most part supportive of the need for regulatory systems to ensure controlling safeguards for all concerns. Yet no federal regulations have been enacted pertaining to the control or eradication of CWD and no federal regulations restrict the interstate movement of cervids exposed to CWD.

Systems for regulating the cervid industry have been developed in a number of states although there is little consistency as to which agency has been charged with the responsibility among the states, and the extent of the regulatory systems.

IV. REGULATION OF CAPTIVE CERVIDS IN SOUTH DAKOTA

I will provide a synopsis of the experience in South Dakota and then provide a brief outline of the status of CWD in captive cervids in the United States.

Prior to 1989 there were virtually no regulatory provisions involving the captive cervid industry. The growth in entities involved in this industry was creating concern among traditional livestock producers, wildlife officials, and cervid producers over the need to establish regulatory standards for animal health. At this time most other state animal health officials were found to have very little interest in expanding their responsibilities beyond traditional livestock.

As the captive cervid industry became aware of the movements to regulate their industry, the consensus among the industry was to direct such authority to the state animal health official through the South Dakota Animal Industry Board. Resulting legislation was passed in 1989 in the state legislature that broadly expanded the animal health authority of the state veterinarian to include regulatory authority for the health of all animals in the state except humans. This broad legislation provided authority for regulating animal health for not only the captive cervid industry but all other animals including free-ranging animals. Regulations were promulgated

regarding imports and intrastate movements. Requirements were established including official identification; import testing for brucellosis, tuberculosis, blue tongue, and anaplasmosis; intrastate movement requirements for brucellosis and tuberculosis; and a system for certifying and accrediting herds for these two diseases. This system served the animal industry very well, as the state was able to remain untouched by a widespread tuberculosis event in the late 1980s that involved numerous states and Canada. This authority and historical experience was to prove of great value when CWD was determined a concern in the late 1990s.

In the early 1990s concerns arose over those raising nondomestic animals in captivity that involved issues much beyond animal health. Wildlife interests were expressing concerns over fencing that precluded natural migrations of free-ranging elk and deer. Additional concerns included captive cervids that escape and their potential to interbreed with free-ranging cervids and that elk hybrids may dilute the gene pool of native elk. Competition for habitat by escaped animals was a concern. These issues went beyond the cervid industry. In 1993 legislation was passed that gave regulatory authority to the state animal health official to permit the possession of nondomestic mammals, and to restrict entry into the state of any nondomestic animal unless specifically approved by the Animal Industry Board to provide for the safety of other animals and the people of the state. Regulations by the Animal Industry Board were passed in 1993 addressing several species that required a possession permit of each person possessing captive nondomestic animals in the state, including cervids. This regulatory system of permitting possession plus the existing regulatory authority over animal health provided capable methods of addressing the concerns of disease and other risks presented by the cervid industry. Since 1993, as a prerequisite to possessing cervids, permittees were required to have facilities approval. Also, official animal identification, records of movements, compliance with disease testing, and all illnesses, deaths, and escapes were immediately reportable to the Animal Industry Board.

V. SUGGESTED MODEL FOR CWD ERADICATION IN CAPTIVE CERVIDS

A phone call to the South Dakota State Veterinarian in November 1997 from an elk producer in north central South Dakota reported his intent to dispose of an elk cow refractory to treatment for chronic pneumonia. This phone call was to impact the captive cervid industry profoundly! In this phone conversation the producer stated that he was reporting the impending euthanasia and disposal of another elk with chronic pneumonia and

that he was having difficulty keeping these elk alive. The state veterinarian found this quite puzzling, as in his experience with the elk industry it seemed they were a hardy species and had experienced few concerns in the past involving endemic diseases and death losses. The state veterinarian requested the reporting producer have his private veterinarian call and discuss the problem. The practitioner shortly contacted the state veterinarian and it was discerned that several such cases of loss had occurred in this elk herd over the period of two and one-half years this individual had been in the elk business. The state veterinarian then requested that multiple tissue samples be taken in conjunction with a thorough autopsy, and that complete tissue samples including the brain be properly prepared and shipped to the Animal Disease Research and Diagnostic Laboratory at South Dakota State University.

In 1997 CWD was not known to exist in captive cervids and there was very little awareness by producers or regulatory officials of the disease. The South Dakota State Veterinarian had attended U.S. Animal Health Association and Western States Livestock Health Association meetings for 10 years and had attended presentations describing the disease in a localized area of Colorado and Wyoming in free-ranging deer and elk. On more or less a whim, and in an effort to cover all bases in attempting to assist the producer in addressing his herd health concerns, he asked that the state laboratory check the brain for lesions of CWD. On December 1, 1997 it was found that lesions in the brain from the submitted elk were suggestive of CWD but the pathologist involved had not had experience in diagnosing this disease. Tissues were forwarded to the National Veterinary Services Laboratory in Ames, Iowa, where CWD was confirmed on December 12, 1997. Archived tissue specimens later examined revealed that at least two previous submissions from this herd, in which other diagnoses were made, did indeed reveal lesions of CWD.

The circumstances of this, the first diagnosis of CWD in privately owned captive cervids in the United States, should provide insight into a few of the difficulties CWD presents. Owners of very valuable animals with large investments and obvious interests in the health of their animals, practicing veterinarians with experience in a variety of large animal species and their diseases, state animal health officials, and pathologists experienced in diagnostics had all obviously been deceived and unable to recognize a disease that had existed in this species for years!

The confirmation of CWD triggered a cascade of events implemented by the state veterinarian. Epidemiology efforts began and quarantines of all trace source and exposed elk permittees in the state occurred. Notification of the diagnosis was sent to all 50 state animal health officials and to U.S. Department of Agriculture officials. Incidentally, more than one state animal health

official responded by asking, "What is CWD?" Notification of state wildlife and agriculture officials occurred along with livestock industry groups and state and national elk industry officials. Numerous other notifications were made both directly and indirectly and through media releases. A meeting was convened by the state veterinarian less than 30 days after CWD had been confirmed, inviting all potential stakeholders from the state, surrounding states, and nationally. Participants included state and national elk industry officials, state and federal veterinary medical officials, state wildlife officials, national park and state park superintendents, neighboring state animal health officials, nationally known researchers involved in CWD research, state Animal Industry Board members, legislators, and most cervid permittees in the state, among others. The meeting agenda included an overview of the TSEs, an overview of CWD as it was known to exist in the United States, the current status of CWD in the state including results of epidemiology and quarantines issued, and a proposed draft state CWD control program as developed and proposed for adoption by the state veterinarian.

The state veterinarian reported at this meeting that he had contacted various federal animal health and research agencies and had been told that there was no money, no programs, no authority, and very little interest in CWD among these agencies. He also reported that his contacts with several CWD researchers in Colorado and Wyoming, along with visiting Canadian animal health officials and cervid industry representatives, and assistance with information and advice was generous and extremely supportive.

Immediately after this general meeting, the state veterinarian convened a meeting limited to cervid permittees within the state. He outlined for this group the various implications CWD presented to the cervid industry as well as to other industries and various alternatives available in response to the existence of CWD on the cervid industry in South Dakota. Three basic response alternatives were available. The first would be essentially to not respond regulatorily and let the individuals affected by CWD deal with it much as they deal with diseases such as injury, foot rot, pinkeye, ringworm, or other quite innocuous diseases. The second alternative would be to implement a voluntary system of monitoring, surveillance, and certification for producers whereby some degree of confidence in a given producer's herd status could be assured. The third, and the alternative recommended by the state veterinarian, would be a regulated, mandatory system of surveillance, monitoring, herd certification, quarantines, eradication of CWD from the cervids in the state, and prevention of further introduction. After much discussion the cervid industry permittees unanimously agreed to support adoption of the third alternative in South Dakota.

Efforts were then initiated by the state veterinarian to urgently nurse through the state legislature a specific statute that included regulations

specifying CWD control methods to be adopted. This process of going through the state legislature enabled enactment of regulations directly without the 60–90-day time constraints incurred in the customary process of officially adopting regulations. The state legislature approved the statute and the law was signed by the Governor as an emergency act and became effective officially February 5, 1998, less than 60 days after confirmation of CWD in the state.

The South Dakota CWD control and eradication program consists of the following key parts: Definitions of affected herds and of exposed herds, defining exposed as any cervid part of an affected herd or for which epidemiological investigation indicates contact with CWD-affected animals or contact with any animal from a herd affected with CWD in the past 5 years. Monitored herds are defined as those herds complying with the mandatory cervid CWD surveillance identification program. This cervid CWD surveillance identification program requires identification and official laboratory diagnosis including brain tissue, as directed by the state veterinarian, on all deaths of cervids 18 months of age or greater from any cause, including death by slaughter, hunting, illness, or injury.

Epidemiology and quarantine requirements mandate trace-backs be performed for all animals positive for CWD. All herds of origin, all adjacent herds, and all herds having contact with affected animals or exposed animals are quarantined. The duration of the quarantine is 5 years and requires compliance with all provisions of the control and eradication program. Exposed and adjacent herds may be removed from quarantine in less than 5 years, if directed by the state veterinarian, based on epidemiological evidence.

Intrastate movement requirements under the CWD program mandate any intrastate movement be accompanied by an intrastate certificate of veterinary inspection and a telephonically issued permit for movement issued by the state veterinarian. Statements on this certificate for movement must include the fact the herd of origin has been monitored for CWD at least 1 year, all causes of death have been determined with records available, and no epidemiological evidence of CWD is determined. This requirement stopped movement at initiation of the program from any herd other than herds that had been closed for 1 year.

Importation requirements are essentially the same as intrastate requirements, and have since been amended requiring a statement that no members of the herd of origin have been trace-back or trace-forward animals to a CWD-affected herd for the past 5 years.

CWD control in free-ranging cervids is addressed in the regulations by mandating that the Animal Industry Board, in consultation with the Department of Game Fish and Parks, adopt a memorandum of under-

standing concerning the surveillance and control of CWD in free-ranging wildlife in the state.

This control program in South Dakota provided no indemnity to owners of affected herds, and owing to the nature of CWD with its many unknowns, placed a tremendous financial burden on producers affected. Efforts were made to seek indemnity assistance to no avail either locally or nationally. Producers have been extremely cooperative and supportive of the program, and during the period of quarantine considerable participation and cooperation by producers with researchers studying CWD transmission, diagnostics, and susceptibility have occurred. South Dakota also enjoys a state meat inspection program in which cervids are included as amenable to state meat inspection laws. This system was valuable in providing a way for affected herd owners to salvage some value as they went about eliminating their herds. Policy was enacted providing for animals from affected herds to be slaughtered under inspection with all carcasses retained and tissues submitted for CWD examination. Carcasses from negative-tested animals were allowed to be marketed. Any positive-tested animals were required to have the entire carcass, including hide and antlers, disposed of by incineration or by disposal in approved regional landfills.

This option will no longer be available, and under a proposed national program for CWD, all animals from affected herds would be purchased with indemnity and disposed of under specified guidelines.

Four years of experience has demonstrated that a regulatory program such as the South Dakota program can effectively control and eliminate CWD from the captive cervid industry. In South Dakota the industry has experienced going from 10 herds under quarantine containing over 400 animals and confirming over 100 animals positive for CWD to the present with no herds quarantined, and the remaining permittees all having 4 years of mandatory surveillance for the disease (8). Even with the negative stigma CWD has placed on the industry, South Dakota has seen an increase in the number of permittees with captive cervids through this 4-year period.

VI. CURRENT STATUS OF CWD IN CAPTIVE CERVIDS AND POTENTIAL TO ERADICATE

What is the current status of CWD in the captive cervid industry in the United States? The answer may be that no one knows as surveillance has been limited to a few states and progress toward national CWD surveillance and control has been traditionally slow. However, information on the status as known can be taken from information presented at the U.S. Animal Health Association Captive Wildlife and Alternative Livestock

Committee in November 2001 (9). Historically in the United States the captive cervid industry has gone from two herds with less than 300 animals in one state to nearly 2000 animals in three states known to be in affected herds. Cumulatively, five states have reported CWD-affected cervid herds in this 4-year period, containing approximately 2500 animals. Chronologically, CWD was first confirmed in 1997 in South Dakota and involved 10 premises with over 400 animals. Nebraska confirmed CWD in early 1998 on one premise with 150 elk. Oklahoma confirmed CWD in 1998 on one premise with 84 elk, Nebraska confirmed CWD on a second premise in 1999 on two premises with 174 elk. Montana confirmed CWD in late 1999 on two premises involving 102 elk. In 2000 and 2001 CWD was confirmed on a third premise in Nebraska and on seven premises in Colorado. The chronological incidence of CWD identified in captive cervids in the United States is demonstrated geographically (Fig. 2).

Additional concerns were evidenced in August 2001 as Canadian animal health officials reported shipment of 41 CWD-exposed animals to captive cervid producers in the United States over the past several years. Then Colorado officials reported in 2001 that one producer in Colorado with a CWD-affected herd had sold 600 elk to producers in 18 states! The experience in Colorado occurred in spite of some regulatory control over cervids and animal health.

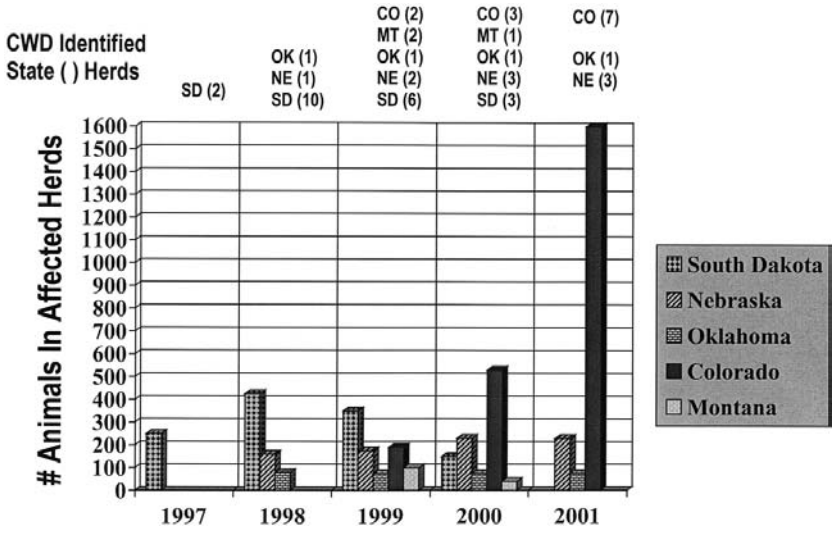

Figure 2 Statistical chart of chronic wasting disease in the United States: herds affected by chronic wasting (November 2001).

The now quite widespread status of CWD among the captive cervid industry may lead one to think that CWD will be the demise of the industry. Although I agree with the possibility if no more is done to address the problem, I do not agree to the industry's inevitable demise. Having proposed a state program as a model in 1998 to the industry and other state and federal animal health officials, and having now observed this model work within one state, I am convinced that control and eradication, though costly and cumbersome, can be effective.

At various animal health meetings in 1998 until the present, there has been limited commitment by officials and industry to timely enact a uniform national CWD control program. However, in the context of CWD occurrences in 2001, it appears there may finally be some movement toward such. Several states have individually enacted CWD control regulations. Those regulatory programs vary from strong mandatory identification, surveillance, movement controls, quarantines, epidemiology, and destruction of affected herds to simply voluntary reporting and surveillance systems. At least one state, Montana, has seen legislation pass that eliminates any expansion or addition to the captive cervid industry. Colorado, after observing the failure of existing regulations, passed more stringent emergency regulations in 2001. Many of these programs address only elk and fail to consider the implications of CWD in other cervids. Knowing the characteristic of CWD makes it imperative that all susceptible species be included in eradication programs.

I firmly believe that a strong, uniform, national program that is broad and inclusive can lead to the eventual eradication of CWD from the captive cervid industry and that it is compelling to enact such a program in the near future to prevent the total demise of the industry.

VII. POTENTIAL TO ERADICATE CWD IN FREE-RANGING CERVIDS

The implications of a TSE being present in free-ranging cervids are greater yet than those for captive cervids. Estimates of free-ranging cervid populations in the United States are as follows: white-tailed deer, 27.4 million; elk, 1.2 million; mule deer, no figures available (10).

The tangible resources that these populations represent are difficult to estimate and intangible resources are beyond comprehension.

Recreational hunting of big game, along with related economic benefits, has been estimated to involve 11.3 million people in the United States, and expenditures by hunters estimated to be $20.6 billion dollars (11).

Beyond the benefits of sport are the vast contributors to state and national park interests for public enjoyment and beyond even these factors are those involved in a balanced ecosystem. TSEs are a definite threat to society's interests and can and should be eliminated at every opportunity. Parameters available to eradicate CWD in free-ranging cervids make the task much more of a challenge than in animals in captivity. While tools such as individual animal identification and inventory accounting are not available, all options must be researched and a determined attempt to eliminate this disease must be made sooner rather than later.

Methods of elimination necessarily involve cooperation between states, between state and federal officials, and between private and public landowners. It appears that, as in the case of CWD eradication in captive cervids, state wildlife officials must assume a vigorous initiative. State wildlife officials hopefully will appropriate resources of staff and funding to recognize the significance of CWD and aggressively act to prevent, prepare, and respond to the risks presented. Animal health and public health must be priority number one in management of wildlife. Immediate assessment of any translocations of cervids must be made with CWD in mind. Statistically and scientifically valid surveillance for CWD must be implemented and continued. Barriers to prevent incidental spread of the disease from areas where it is known to exist must be established. And as with domestic animals and captive nondomestic animals, uniform agreements must be made between state and federal stakeholders to provide for preventing and eradicating this disease from wildlife in the United States. When we face emerging animal health concerns, eradication of disease often seems an insurmountable task. Advancing technology will no doubt lead to more rapid testing—perhaps even on-site-field-type tests. This would provide invaluable assistance to wildlife officials in managing surveillance for CWD on hunter-harvested animals.

Voices of the negative will be heard and easy to listen to. Determination and willingness to use tools existing and implementation of new methods in diagnostics and control will provide a way for those who have the will!

REFERENCES

1. Williams ES, Young S. Chronic wasting disease of captive mule deer: a spongiform encephalopathy. J Wildlife Dis January, 1980; 16(1):89–98.
2. Williams ES, Young S. Spongiform encephalopathy of Rocky Mountain elk. J Wildlife Dis October, 1982; 18(4):465–471.
3. Williams ES, Young S. Spongiform encephalopathies in Cervidae. Rev. sci. tech. Off. int. Epiz. 1992; 11(2):551–567.

4. Miller Michael W, Wild Margaret A, Williams Elizabeth S. Epidemiology of chronic wasting disease in captive Rocky Mountain elk. J Wildlife Dis 1998; 34(3):532–538.
5. Michael W. Miller, DVM, PhD, Wildlife Veterinarian. Chronic wasting disease of deer and elk (Informational publication). Colorado Division of Wildlife, 24 May 1996.
6. Spraker TR, Miller MW, Williams ES, Getzy DM, Adrian WJ, Schoonveld GG, Spowart RA, O'Rourke KI, Miller JM, Merz PA. Spongiform encephalopathy in free-ranging mule deer (*Odocoileus hemionus*), white-tailed deer (*Odocoileus virginianus*) and Rocky Mountain elk (*Cervus elaphus nelsoni*) in northcentral Colorado. J Wildlife Dis 1997; 33(1):1–6.
7. Wildlife report news release from the Colorado Division of Wildlife, Release Date: 11/14/01, Draft Deer Management Plans.
8. South Dakota Animal Industry Board News Release, Sam D. Holland, DVM March 12, 2001.
9. Holland Sam D, DVM. USAHA, Captive Wildlife and Alternative Livestock Committee Presentation. Brief update on CWD, states with programs/need for a national uniform program, Hershey, PA, November 6, 2001.
10. Personal communication, Dr. John Fischer, Southeast Wildlife Disease Cooperative Study.
11. U.S. Department of the Interior, Fish and Wildlife Service and U.S. Department of Commerce, Bureau of the Census. 1996 national survey of fishing, hunting, and wildlife-associated recreation.

6
Bioassays for Prions

Andrew F. Hill, Emmanuel A. Asante, and John Collinge
University College London, London, England

I. INTRODUCTION

The prion diseases include Creutzfeldt-Jakob disease (CJD) and kuru in humans and scrapie and bovine spongiform encephalopathy (BSE) in animals. They are all transmissible to the same species by inoculation with, or dietary exposure to, infected tissues. While scrapie in sheep has been recognized for over 200 years, it is the recent epidemic of BSE (colloquially known as "mad cow" disease) in the United Kingdom and parts of Europe that has centered much public and scientific attention on these neurodegenerative diseases. The occurrence of variant CJD (vCJD) in humans and the experimental confirmation that it is caused by the same prion strain as BSE (1,2) has highlighted the need for intensive study into the pathogenesis of these diseases as well as diagnostic and therapeutic approaches. Although these diseases are relatively rare, affecting around 1 human per million per annum, there is the possibility that a substantial epidemic of vCJD could occur in people exposed to BSE prions through dietary exposure. Here we review the literature on animal models of prion diseases, concentrating on the use of transgenic mouse technology.

II. PROTEIN-ONLY HYPOTHESIS OF PRION REPLICATION

A wealth of scientific evidence suggests that the infectious agent causing these diseases, the prion, is an abnormal isomer (PrP^{Sc}) of a host-encoded protein, the prion protein (PrP^{C}). PrP^{C} is a sialoglycoprotein, with an

approximate molecular mass of 33–35 kDa, anchored to the cell surface via a glycosylphosphatidylinositol (GPI) anchor, and contains two sites for asparagine-linked glycosylation. According to the protein-only hypothesis (3), disease-related prions are composed principally or entirely of PrP^{Sc} (4). It is hypothesised that PrP^{Sc} acts as a conformational template, driving the conversion of PrP^{C} into further PrP^{Sc}. The abnormal isoform is derived from PrP^{C} by a posttranslational process that is thought to involve a conformational rather than covalent change to the protein. Secondary structure analyses of the two isoforms show that PrP^{C} is rich in α-helical structure, whereas PrP^{Sc} is predominantly β-sheet (5). Biochemically, PrP^{Sc} can be distinguished from PrP^{C} by its relative insolubility and partial resistance to protease degradation. Under defined conditions, proteinase K completely digests PrP^{C}, while ~70 N-terminal residues of PrP^{Sc} are digested by this treatment leaving a 27–30-kDa protease-resistant core.

While much research has centered on the pivotal role of PrP in these diseases, the mechanism by which the conversion of PrP^{C} to PrP^{Sc} takes place and results in the distinct pathogenesis of prion disease remains unknown. Understanding the pathogenesis in prion diseases is critical to early diagnosis, development of interventional therapy, and health and safety issues surrounding the potential epidemic of vCJD. This will also involve defining the basis of prion species barriers, which may yield clues as to what makes an animal susceptible or resistant to prion infection.

III. SPECIES BARRIERS

Prion diseases are characterized by their transmissibility to experimental animals. This has been shown by the transmission of kuru to chimpanzees (6), and CJD (7) or GSS (8) to primates. Experimentally, prion diseases are transmitted by intracerebral inoculation but they may also be transmitted via intraperitoneal and oral routes. (See Fig. 1.)

Traditionally, transmission of prion diseases between species is difficult, requiring the exposure of large numbers of animals to obtain illness in only a few individuals. In addition, incubation periods are lengthened in the new host species and often may approach the natural life span of the inoculated animal. These incubation periods shorten on second passage in the new host species and reduce to a stable level on subsequent passages. This phenomenon is known as the "species barrier" (10) and may relate to many factors, such as the strain of prion inoculated, the difference in primary sequence of the prion protein between donor and host species, and the route of inoculation.

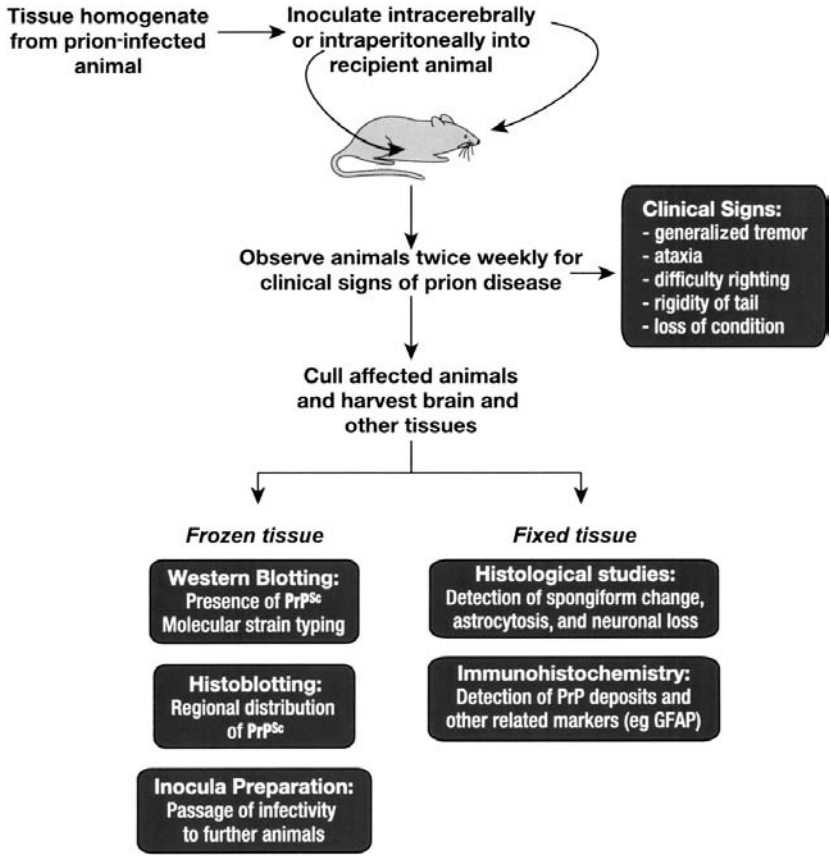

Figure 1 Flowchart describing the bioassay of prions in laboratory mice. Mice inoculated with infectious prion material are examined twice weekly for clinical signs of prion disease (see Ref. 9). Tissues harvested from affected animals are either fixed or frozen and analyzed by the methods listed.

One species barrier that has been extensively examined is that between hamsters and mice. Kimberlin and Marsh described a hamster model of scrapie in which infected hamsters became sick 60–70 days postinoculation, the shortest reported incubation period for rodent models of scrapie (11,12). The hamster prions were found to be pathogenic for hamsters and not mice, suggesting a substantial species barrier between the two species. Advances in transgenic animal technology have proved invaluable in exploring this phenomenon, and abrogation of the species barrier was demonstrated in transgenic mice overexpressing PrP sequences

from the donor species. In-depth discussion of transgenic modeling of the species barrier follows later.

Several parameters are known to influence the transmissibility of prions both across and within species. These include differences in primary structure between the donor and host, which include polymorphisms in the prion protein gene, prion strain type, the route of inoculation (for example peripheral vs. intracerebral), and the dose. Recently, genetic loci other than PrP itself have been shown to influence the susceptibility and incubation period of prion disease in mice inoculated with RML prions (13,14). These results have implications for epidemiological studies predicting numbers of cases in a vCJD epidemic, which have been based on genetic susceptibility at one polymorphic residue (codon 129) in PrP (15). Given the ability of BSE prions to infect a large number of host species, both naturally and experimentally, it now appears clear that prion transmission barriers do not rely solely on one of the parameters mentioned above, namely differences in PrP primary structure, suggesting that studies of these barriers should include genetic, molecular, and clinical assessments.

IV. SPECIES-BARRIER-INDEPENDENT PRION REPLICATION

It has long been recognized that the incubation period of prion diseases can exceed the natural life span of an animal (16). During the pathogenesis of prion diseases, infectious prion titers rise during the prolonged, clinically silent incubation periods prior to the development of clinical symptoms. In rodent models of scrapie, rises in infectious titer are first observed in the spleen and other lymphoreticular tissues before increases are seen in the brain. Therefore, in cases where the incubation period approaches or exceeds the natural life span of the animal, and when there may be no clinical signs of infection, the brain and other tissues can harbor significant levels of infectious prions. Whether such cases result in clinical symptoms should the animal have lived longer makes the distinction in terminology between pre- and subclinical disease difficult to assess. However, carrier states are seen in other infectious diseases, and the definition of subclinical prion disease could refer to animals that are replicating prions but do not show any clinical symptoms within a normal life span.

Subclinical prion diseases have been observed experimentally since early experiments attempting to transmit sheep and goat scrapie to rodents. These studies included the transmission of scrapie from Suffolk sheep via the intragastric route into mice (17). No clinical signs of scrapie were observed after 13 months in the mice; however, some histological lesions were present. In reviewing these experiments, Zlotnik refers to passaging brain and spleen

pools from these "subclinical cases" into additional mice, which developed symptoms some 5–6.5 months after intracerebral inoculation, and it is from these experiments that the ME7 strain of mouse scrapie was derived (17).

Evidence that the incubation period for scrapie can be longer than the life span of the animal was explored using a strain of mouse-adapted scrapie (22A) with an extended incubation period when inoculated intraperitoneally into a particular mouse strain (16). When C57BL6 mice were inoculated in this manner with a dilution series of infectious brain tissue extracts, no clinical signs of scrapie were observed from a 10^{-2}–10^{-6} dilution. However, spleen extracts taken 600 days postinoculation, from clinically normal animals, demonstrated significant infectious titer, as measured by intracerebral inoculation into the VM strain of mice, which are more sensitive to this strain of scrapie. Infectivity was not present in spleen extracts taken from the C57BL6 mice after 100 days, suggesting that the infectious material recovered after the prolonged incubation period is unlikely to be remains of the original inoculum. Hence, the presence of infectivity in the spleen, at least in rodent models, may be used as an indicator that the animal may develop prion disease if the life span is sufficiently long to allow the development of the disease.

Initial studies into the pathogenicity of hamster 263K prions in mice involved intracerebral injection with hamster brain homogenate. Following a 280-day observation period, at which time no clinical signs of scrapie were observed, spleen and brain extracts were prepared and inoculated into additional mice, which showed no clinical signs at up to 480 days postinoculation (18). In additional experiments it was demonstrated that some mice inoculated with 263K prions could reinfect hamsters; this was attributed to the presence of the initial inoculum as material from mice in second- and third-passage transmissions were not able to reinfect hamsters (18).

Recent studies using the transmission barrier between mice and hamsters with 263K hamster prions have provided further compelling evidence that subclinical forms of prion disease exist. In one study, upon injecting wild-type mice with 10^7 ID_{50} (where ID_{50} represents the half-maximal infectious dose) units of 263K hamster prions, no clinical signs of scrapie were observed up to 782 days postinoculation (19). This is consistent with early studies on the pathogenesis of 263K prions in wild-type mice (18). Brain and spleen tissue extracts from the clinically normal 263K-inoculated mice (taken between 204 and 782 days postinoculation) were intracerebrally injected into additonal mice and hamsters. All hamsters inoculated with the mouse-passaged 263K material from both brain and spleen became clinically sick with scrapie, whereas mice inoculated with the same material were not affected. In addition, *Prn-p*$^{0/0}$ mice, which are resistant to infection with prions, were also inoculated with 263K. Spleen

and brain extracts from these mice were bioassayed in additional mice and hamsters to determine whether expression of endogenous mouse PrP was required for the persistence or replication of the original inocula. Infrequent transmissions to hamsters with variable incubation periods resulted from these transmissions, suggesting there may be low-level persistence of the original 263K inoculum in the *Prn-p*$^{0/0}$ mice. The brain and spleen tissues that resulted in these transmissions from the 263K-inoculated *Prn-p*$^{0/0}$ mice were in early time-points (112 and 217 days postinoculation) and the incubation periods in the recipient hamsters were similar in both cases, which is consistent with the possibility that these resulted from persistence of the original 263K inoculum. (There was therefore no replication in the *Prn-p*$^{0/0}$ mice in the absence of PrP^C).

However, the transmissions from 263K-inoculated wild-type mice into hamsters resulted in a drop in incubation period from 313 ± 112 days (mean ± SEM) for inocula from mice killed at 204 days postinoculation, to 128 ± 13 days for inocula from mice killed at 782 days. The authors estimated the titer of infectivity (calculated from the long incubation period seen in the hamsters) for the clinically normal mouse-passaged 263K prions to be about 10^2 ID_{50} per gram of tissue, which is much lower than that seen in clinically sick animals in the terminal stages of disease (19). Based on this, it was proposed that the positive transmissions to hamsters from clinically normal 263K-inoculated mice result from persistence of the original inoculum. However, the reduction in the incubation period and SEM as the observation period in the 263K-inoculated wild-type mice increased is consistent with a replication model, whereby the 263K prions are replicating mouse PrP, albeit at a low level. This may explain why transmission of 263K-inoculated mouse material to additional mice did not result in any clinical disease in this case.

While the above study provided evidence for the existence of subclinical prion disease in a species previously thought to be resistant, the issue of persistence of the original inoculum as opposed to prion replication in these transmissions remained unresolved. Another study, which included more detailed molecular and neuropathological assessment in clinically normal animals, has since provided convincing evidence that in mice inoculated with hamster prions, replication of infectivity is indeed occurring (20). In this set of experiments, CD-1 wild-type mice were inoculated with approximately 8.5×10^6 LD_{50} (where LD_{50} represents half the maximal lethal dose) of hamster Sc237 prions [purported to be the same strain as 263K (21)] or with saline alone. Animals were examined at frequent intervals for signs of clinical disease and continued to live until they died of natural causes. No clinical signs of scrapie were observed in any of the animals. There was no significant difference in the ages at which animals in either

inoculation group died [408–828 days postinoculation for the Sc237-inoculated mice ($n = 18$) and 436–849 days for the saline-inoculated mice ($n = 8$)]. In all animals, brain tissue was examined for the presence of protease-resistant PrP^{Sc} by Western blotting with two different anti-PrP antibodies; one is specific for hamster PrP, the other detects both hamster and mouse PrP. Using both antibodies revealed that all animals surviving after 659 days postinoculation with Sc237 prions showed readily detectable levels of mouse PrP^{Sc}. None of the animals displayed any detectable levels of hamster PrP^{Sc} using the hamster-PrP-specific antibody.

Neuropathological examination of the brains from some of the Sc237-inoculated mice showed histological features of spongiform encephalopathy and the presence of PrP amyloid plaques, consistent with typical prion disease—age-matched saline-inoculated control mice showed none of these signs. Second-passage transmissions of infectivity were performed by inoculation of further CD-1 wild-type mice, Tga20 mice (transgenic mice that overexpress murine PrP and have shortened incubation periods), and hamsters.

Two Sc237-inoculated mice were chosen for passage—one was PrP^{Sc} positive, the other PrP^{Sc} negative; in addition, two saline-inoculated CD-1 mice were also passaged as a negative control. The PrP^{Sc}-positive inocula produced clinical prion disease in both mice and hamsters, whereas the PrP^{Sc}-negative inocula were pathogenic only to hamsters. No clinical signs were observed in the saline-inoculated animals at up to 650 days postinoculation. Strikingly the incubation periods in the mice inoculated with the PrP^{Sc}-positive inocula were highly consistent and, coupled with the 100% attack rate, suggest that infection occurred in the absence of a transmission barrier. Intriguingly, the incubation period in the Tga20 mice, which succumb to infection with RML mouse prions in 60 days (22), was very prolonged (122 ± 7 days). End-point dilution of RML mouse prions in Tga20 mice results in an incubation period of around 109 days (23). The fact that all inoculated Tga20 mice became clinically sick, and with a consistent incubation period, may suggest transmission in the absence of a species barrier. A similar effect was observed in the transmission properties of the PrP^{Sc}-positive inoculum into hamsters—these all became sick with an incubation period of 127 ± 9 days, whereas inoculation with high-titer Sc237 prions produces disease in around 60 days. In both Tga20 mice and hamsters the incubation periods are consistent, and with 100% of the animals succumbing to disease, this argues against the lengthy incubation period resulting from a low titer or persistence of the original inoculum. Instead it supports the replication model, which in this case appears to have produced novel infectivity for both mice and hamsters from an original strain of prion, which has a limited host range.

To further demonstrate the generation of novel infectivity in the mice inoculated with Sc237 prions, end-point dilution experiments were performed, in both mice and hamsters, to quantify the infectious titer in the brain tissues of these animals. The prion titers in the brains of these mice, when assayed in hamsters, were calculated to be approximately 10^8 LD_{50} units per gram of tissue, which is considerably more than what was originally inoculated ($\sim 8.5 \times 10^6$ LD_{50}). While persistence of the original inoculum cannot be formally excluded owing to the insensitive nature of the bioassays employed, the clear demonstration of an increase in infectious titer by end-point titration of infectious material from both mice and hamsters over the original amount of inocula used in the first passage is consistent with replication of infectious prions over persistence.

The kinetics of prion replication in hamster scrapie strain 263K-infected mice has been studied by transmitting 23 clinically normal animals at various time points into both mice and hamsters (24). Western blotting was used with hamster- and mouse-specific antibodies at various time points postinoculation to characterize the species of PrP^{Sc} present in the brains of the inoculated mice. Interestingly, hamster PrP^{Sc} could be detected in the mice brains at 2 h postinoculation, and then became undetectable during all of the time points tested up to 782 days. That the brain tissue at the 2-h time-point was positive and then became negative throughout the remainder of the time course is consistent with it being the presence of the initial inoculum, which then cleared from the brain. Using an antibody specific to mouse PrP, PrP^{Sc} was observed in 263K-inoculated mice from day 310 onward. When brain extracts from these mice were repassaged in hamsters, it was demonstrated that as the observation period of the subclinically infected mice increased, so did the infectious titer when material from these mice was repassaged in hamsters. The 263K-inoculated mice sacrificed at 2 h, and 5 days postinoculation were shown to have significantly higher levels of hamster infectivity than mice tested at 140–390 days. This is consistent with persistence of the original 263K inoculum in the mouse brain at these early time-points following intracerebral inoculation. At extended periods from 463 to 782 days postinoculation, however, brain material from 263K-inoculated mice was able to produce disease in hamsters with 100% attack rate and decreased incubation periods. However, as was seen in the previous study (20), the incubation period of mouse-passaged 263K prions in hamsters is lengthened, even in the longest subclinically infected mice, which would have the highest titer, from 70 days for hamster 263K to around 120 days for mouse-passaged 263K prions.

Again, this suggests that passage of hamster 263K (or Sc237) prions in mice produces infectivity with novel transmission properties leading to a lengthy incubation period in hamsters. That the mice inoculated with

hamster prions produced high levels of infectivity pathogenic for both hamster and mice without the development of clinical symptoms in both of these studies clearly demonstrate the existence of subclinical carrier states in prion diseases. To investigate the molecular mechanisms involved in the pathogenesis of the new strain of prions propagated in the subclinically affected mice it will be of interest to clone the strain(s) present in these cases.

Subclinical prion diseases are not only restricted to instances involving crossing species barriers. In studies investigating the role of the lymphoreticular system in the pathogenesis of prion disease it was demonstrated that B-cell-deficient mice appeared resistant to peripheral prion infection (25). These mice are susceptible to prion infection when inoculated intracerebrally, exhibiting incubation periods similar to those seen in wild-type control animals. Further investigation into the apparent resistance of the peripherally challenged immunodeficient animals to prions showed that while they developed no clinical signs of scrapie, they accumulated PrP^{Sc} in their brains (26). Inoculation of wild-type mice intracerebrally with brain extracts from these animals revealed that the subclinically affected animals harbored significant infectious titers, which in some cases exceeded those seen in terminally sick wild-type control animals.

The influence of PrP gene polymorphisms on susceptibility and incubation time is well established. Other lines of evidence indicate that PrP amino acid differences are not the only genetic influence on incubation time. Studies based on relatively small sample sizes had suggested the presence of quantitative trait loci (QTL) that may influence prion disease incubation time on chromosome 17 (27) and on chromosomes 1 and 11 (28). A larger study aimed at identifying similar QTL was initiated by generating an F2 intercross between two strains of mice, CAST/Ei and NZW/OlaHsd, that have significantly different incubation times (13). The authors analyzed incubation times for a total of 1009 F2 animals and demonstrated unequivocally that genetic loci other than PrP open reading frame (ORF) can have a major effect on prion disease incubation time in mice. Furthermore eight QTL on three chromosomes were identified that explained 82% of the variance (13). The conclusion from this study was that, subject to refinement of the identified regions of linkage, this study provides hope that human alleles that predispose to a shorter incubation time for BSE in humans may be identified. A further important conclusion from this QTL study is that genetic models for predicting the possible size of the vCJD epidemic have largely been based on epidemiological studies and the assumption that only methionine-homozygous individuals are susceptible to vCJD. Such models do not take the major effects of the identified multiple QTL on incubation time into account and are therefore likely to be invalid.

V. PRION STRAINS

While there are several lines of evidence to support the protein-only hypothesis of prion propagation, the existence of distinct "strains" or isolates that can be stably passaged in inbred mice of the same genotype has been a challenge to accommodate in this model. Prion strains can be distinguished by their different incubation periods and patterns of neuropathology when passaged in inbred mice. Three hypotheses have been proposed to explain the existence of prion strains. The first is the virus or virino hypothesis, which assumes a mutation in an agent-specific DNA or RNA genome. However, no direct evidence for such an agent-specific genome has been produced. The second is the protein-only hypothesis where the protein itself encodes the information required to determine the strain phenotype. The third hypothesis tries to bridge the gap between the virus and protein-only hypotheses. Weissmann's unified hypothesis (29) states that while the protein is sufficient for infectivity, some small nucleic acid confers strain-specific properties on the protein.

Evidence that strain specificity is encoded by PrP itself, and not a nucleic acid, was provided by studies on two distinct strains of transmissible mink encephalopathy (TME). Termed hyper (HY) and drowsy (DY), these strains can be serially passaged in hamsters. The strains can be distinguished by physicochemical properties of the PrP^{Sc} deposited in the brains of the infected hamsters. After treatment with proteinase K, strain-specific migration patterns can be seen on Western blots, with DY PrP^{Sc} being more protease-sensitive than HY PrP^{Sc} and producing different banding patterns (30). The banding patterns are caused by different N-terminal cleavage sites for proteinase K, which suggests the strains represent different PrP^{Sc} conformations, a theory supported by infrared spectroscopic studies (31). Maintenance of these TME strains has also been demonstrated in an in vitro conversion model when hamster PrP^{C} is mixed with HY or DY PrP^{Sc}, which also supports the view that prion strains involve different PrP conformers (32,33).

Several human PrP^{Sc} types have recently been identified that are associated with different clinicopathological phenotypes of CJD (34–37). These types are distinguished by different fragment sizes seen after limited proteinase K digestion, suggesting different conformations of PrP^{Sc}. These types can be further classified by the ratio of the three PrP bands seen after protease digestion, representing di-, mono-, and unglycosylated fragments of PrP^{Sc}. PrP^{Sc} conformation and glycosylation are therefore plausible candidate as forming the molecular basis of prion strain diversity. However, it is crucial to then determine whether such biochemical properties fulfill the biological characteristics of strains; that is, they are maintained upon

transmission to experimental animals of both the same and different species. This was confirmed with studies using transgenic mice overexpressing human PrP [which show no species barrier to infection with human prions (34)] that demonstrated that both the fragment sizes and glycoform ratios of PrP^{Sc} can be maintained upon transmission (1,34). Additionally, transmission of human CJD prions and BSE to wild-type mice also results in maintenance of the fragment size and glycoform ratio of the inocula.

Sporadic CJD is associated with PrP^{Sc} type 1–3, while type 4 human PrP^{Sc} is uniquely associated with vCJD and characterized by glycoform ratios that are distinct from those observed in classic CJD (34). The glycoform ratios in vCJD are similar to those seen in BSE and cases of natural or experimental BSE transmission to a variety of species. The PrP^{Sc} types formed in wild-type mice inoculated with either BSE or vCJD are virtually identical (1). Coupled with other parameters of transmission, such as the incubation period, numbers of animals succumbing to illness, and traditional lesion profiling methods, the identical molecular type provides compelling evidence that these two diseases are caused by the same prion strain (1,2). Presumably there are strain-, species-, and tissue-specific effects on PrP glycosylation such that, as with classic biological strain typing, comparison of isolates on the same background may be necessary to reveal the strain characteristics most clearly. This is also shown in the glycoform ratios of classical CJD in humans and the transmission of these types to the same lines of wild-type mice. Thus, there are also host-specific factors involved in the molecular basis of prion strains and these could include both the primary PrP structure and host-specific glycosylation. These data strongly support the "protein only" hypothesis of infectivity and suggest that strain variation is encoded by a combination of PrP conformation and glycosylation. Further evidence to suggest that strain-specific information is encoded in the conformation of PrP^{Sc} comes from transmission of two inherited forms of human prion disease to transgenic mice expressing a chimeric mouse/human prion gene. Here it was shown that the conformation of the inocula (as measured by fragment size after protease cleavage) was maintained upon transmission to the transgenic animal. Furthermore, the incubation periods and patterns of neuropathology were found to differ between the two inocula, which illustrates that the chimeric PrP that is converted in these transgenic mice may adopt two distinct conformations that lead to different disease profiles (38).

As PrP glycosylation occurs before conversion to PrP^{Sc}, the different glycoform ratios may represent selection of particular PrP^{C} glycoforms by PrP^{Sc} of different conformations with inoculated prions preferentially recruiting and converting particular glycoforms of PrP^{C}. This effect can be illustrated in the transmission of sporadic CJD and BSE to wild-type mice

where two different glycoform patterns and fragment sizes can be generated from the same pool of PrP^C. According to such a hypothesis, PrP^{Sc} conformation would be the primary determinant of strain type with glycosylation being involved as a secondary process. However, since it is known that different cell types may glycosylate proteins differently, PrP^{Sc} glycosylation patterns may provide a substrate for the neuropathological targeting that distinguishes different prion strains. Particular PrP^{Sc} glycoforms may replicate most favourably in neuronal populations with a similar PrP glycoform expressed on the cell surface. Such targeting could also explain the different incubation periods, which also discriminate strains, targeting of more critical brain regions, or regions with higher levels of PrP expression, producing shorter incubation periods. Further supportive evidence for the involvement of PrP glycosylation in prion strain propagation has come from the study of transgenic mice expressing PrP^C with mutations interfering with N-linked glycosylation (39). These mutations led to aberrant distribution of PrP^C, which also affected the ability of these mice to be infected with prions.

Mutation of the first glycosylation consensus sequence resulted in a restricted pattern of PrP^C expression that did not support PrP^{Sc} replication. Interestingly, mutation of the second glycosylation site resulted in a wider expression pattern of PrP^C that supported prion replication; however, the incubation period for infection with hamster prions in these mice was more than 500 days. Differences in the relative spatial expression of the glycoform mutants, however, compromised the use of this model in directly evaluating the precise role of glycosylation as a determinant of strain variation. Indeed, it has been established that there may be considerable heterogeneity among the carbohydrate trees attached to PrP^C (selected from over 400 distinct structures) (40) and as a consequence it will be an extremely complex task to determine whether particular carbohydrates are preferentially involved in the replication of distinct prion strains. Evidence that this may be the case is exemplified by the apparent superimposition of strain- and tissue-specific effects on PrP glycosylation seen in vCJD tonsil PrP^{Sc}, which differs in the proportion of the PrP glycoforms from that seen in vCJD brain (41). Transmission of PrP^{Sc} from peripheral tissues will be useful in trying to assess the contribution of glycosylation in conferring strain-specific properties.

VI. BIOASSAYS IN WILD-TYPE MICE

Incubation period has traditionally been defined as the time from experimental inoculation of scrapie prions to diagnosis of neurological dysfunction based on predetermined neurological signs. It is a quantitative trait

known to be influenced by factors including route of infection, dose, prion agent strain, levels of cellular PrP expression, and genetic susceptibility. According to this definition, the presence of clinical signs is imperative before an animal inoculated with prions will be classified as affected. A direct consequence of this current working definition of incubation period is that, for instance, hamster prions have been considered incapable of affecting mice and vice versa, with this apparent difficulty in transmission between the two species being attributed to the "species barrier." However, as previously discussed, recent more detailed studies of the hamster-mouse species barrier have shown that mice inoculated with hamster prions may replicate or harbor the infectious agent without showing any clinical signs (19,20).

The traditional definition has been used to characterize prion strains and was initially thought to be controlled by a distinct locus variously called *Sinc* or *Prn-i*. The two PrP alleles that are found in short-and long-incubation-time mice based on ME7 inoculation are designated $Prnp^{a}$ and $Prnp^{b}$, respectively. These two alleles differ by two amino acid residues at dimorphic positions 108 (leucine-phenylalanine) and 189 (threonine-valine) (42), with both of the PrP-B mutations (108F 189V) found only in inbred mouse strains that have long incubation times (42,43). Early attempts by backcross of NZW × I/LnJ to confirm that *Prn-i* might be congruent with *Prn-p* were not successful. Individual mice with discordant scrapie incubation time and *Prn-p* genotype were observed, suggesting meiotic recombination between *Prn-i* and *Prn-p* (9,44).

Bioassay in transgenic mice was used in an attempt to clarify the relationship between *Prn-i* and *Prn-p* transgenics (45). The long-incubation-time allele of *Prn-i* is dominant, so it was expected that expressing $Prn\text{-}p^{b}$ in short-incubation mice ($Prn\text{-}p^{a}$) would lead to prolonged incubation times. Contrary to expectation, mice from four independent Tg($Prn\text{-}p^{b}$) lines showed shorter scrapie incubation times than nontransgenic control animals (45). These results, originally described as paradoxical, were dictated by gene dosage of the $Prn\text{-}p^{b}$ transgene, thus masking genuine congruence of *Prn-p*/*Prn-i*. Another research group used gene targeting to try to resolve these difficulties surrounding the identity of *Sinc*/*Prn-i* and *Prn-p* (46). They constructed coisogenic 129/Ola mice, which differed only by targeted codon 108 and 189, using a two-step double replacement gene-targeting strategy. The 129/Ola mice expressing the gene-targeted PrP-B allotype instead of PrP-A had dramatically shortened incubation times when infected with the mouse-adapted BSE strain 301V, similar to the short incubation times of VM/Dk ($Sinc^{p7}/^{p7}$) mice (46). Whereas mice homozygous for the targeted allele (Prnp-A, 108F 189V) had an average incubation time of 133 days, the wild-type 129/Ola mice (Prnp-A) had an incubation time of 244 days. Thus data from gene-targeting studies provided evidence that the PrP gene itself is

responsible for differences in incubation periods. It is perhaps worth noting that although these authors used 301V (mouse-passaged BSE) in their study, the original characterization of the short (*Prnp*^{a}) and long (*Prnp*^{b}) incubation-time alleles have been based on the ME7 strain (47). That only two amino acid differences at codon 108 and 189 can cause such a dramatic alteration in incubation periods demonstrates that *Sinc*/*Prn-i* and the PrP gene are congruent. It remains to be seen, however, whether ME7 inoculation into these mice would lead to the same or a different set of results to that observed for 301V.

VII. STRAIN MUTATION

Transmission of type 1 CJD (which are all MM codon 129 genotype) to Tg(HuPrP)152/Prnp$^{o/o}$ mice overexpressing wild-type human prion protein (encoding valine at codon 129) resulted consistently in a type 2 banding pattern (34). The phenomenon of strain mutation has long been recognized in conventional strain-typing studies. For example, when cloned scrapie strain 22A, which is stable in *Sinc*^{p7} genotype, is passaged several times in *Sinc*^{s7} genotype, a new strain, 22F, with altered incubation period characteristics is generated (48). Remarkably, transmission of type 2 CJD (of all three codon-129 genotypes) or type 3 CJD (which were all genotype MV or VV) resulted in production of protease-resistant human PrP in the Tg(HuPrP)152/Prnp$^{o/o}$ mice with an identical banding pattern to the primary inoculum (type 2 or 3, respectively). However, a study of mean incubation periods between inocula of different 129 genotypes did not show any significant difference (34).

Of interest, when vCJD was transmitted to these Tg(HuPrP)152/Prnp$^{o/o}$ mice (carrying valine at polymorphic residue 129), a new strain designated type 5 was produced. The type 5 shares the glycoform ratio of type 4 but the fragment sizes differ from those in the inoculum and were indistinguishable from those in the type 2 pattern (1). The production of a distinct molecular strain type on transmission of vCJD to mice expressing valine 129 human PrP suggests that BSE transmitted to humans of this genotype might produce a similar strain. Such cases may differ in their clinical and pathological phenotype to vCJD, but could be identified by PrP^{Sc} typing. These data suggest that strain type can be as important as PrP primary structure differences between donor and host in species barrier.

Transmission of PrP^{Sc} fragment sizes from two different subtypes of inherited prion disease to transgenic mice expressing a chimeric human mouse PrP has also been reported (38). These transmission studies in mice

carrying valine at codon 129 of the PrP gene resulting in strain mutations (48) need to be repeated in human PrP transgenic mice carrying methionine at codon 129 of the prion gene. This will clarify the effect of codon 129 mismatch on transmission characteristics of type 1 CJD, BSE, and vCJD. Such experiments are ongoing (83).

VIII. BIOASSAYS IN TRANSGENIC MICE

Transgenic mice have contributed enormously in the abrogation of the species barrier, which leads to shortening of incubation time in transmission experiments. The intrinsic properties of the species barrier might result from differences in the primary sequence of PrP of different species. This hypothesis stems from the discovery of the PrP and the subsequent isolation and sequencing of the gene encoding PrP from several species of mammals (49), including Syrian, Armenia, and Chinese hamsters (50). (See Fig. 2.)

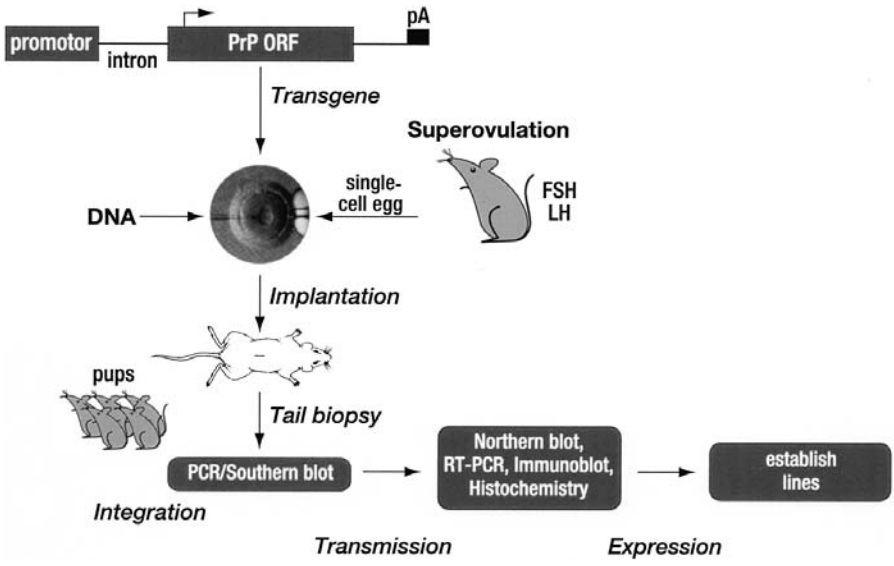

Figure 2 Flowchart summarizing the steps involved in the generation and characterization of transgenic mice for bioassays in prion research. For more details see Refs. 51, 52. PrP ORF = prion gene open reading frame; pA = poly A sequence; FSH = follicle-stimulating hormone; LH = luteinizing hormone; PCR = polymerase chain reaction; RT-PCR = reverse transcription polymerase chain reaction.

A. Hamster PrP Transgenic Mouse Models

The best-known example of species barrier from experimental transmission studies is that between hamster and mouse. In a bid to understand the mechanism of species barrier, wild-type mice were made transgenic for hamster *Prnp* and then challenged with mouse and hamster prions (21). These transgenic mice, which made endogenous levels of mouse PrP^C but differing levels of hamster PrP^C, were used to test the hypothesis that the species barrier for transmission of disease is due to differences in the primary structure of PrP. These mice became susceptible to hamster prions and developed disease with a range of scrapie incubation times inversely proportional to the level of expression of hamster PrP^C (21). Thus the barrier to transmission was abrogated by expression of PrP of the same sequence as that of the inoculating prions, and the efficacy of disease progression was facilitated by increasing the expression level of the homologous PrP^C (53). On inoculation of the Tg(SHaPrP) transgenics with mouse prions a prolongation of incubation periods was observed compared with wild-type mice, suggesting that expression of the SHaPrP transgene impeded mouse prion synthesis (53).

To determine the species range of the prions produced in the transgenic mice, bioassay was used in which brain material from the inoculated animal was further passaged into normal mice or hamsters. Interestingly, the sick transgenic mice would only make prions with the properties of the species in the inoculum (54). When the mice were inoculated with SHa-adapted Sc237 prions, only $SHaPrP^{Sc}$ having characteristics of Sc237 were formed. In contrast, Mo-adapted RML prions interacted only with $MoPrP^C$ to produce neuropathological conditions characteristic of RML scrapie. The implication of these observations is that hamster prions, when presented with both mouse and hamster PrP^C, will selectively convert hamster PrP^C to hamster PrP^{Sc}.

Conversely, mouse prion will also selectively convert mouse PrP^C in these transgenic lines, suggesting that the specificity of prion-host interaction is determined by the primary structure of PrP^{Sc} in the inoculum, as well as that of PrP^C expressed in the host animal (54). Therefore, the type of PrP^{Sc} made in these transgenic mice was dictated by the type of inoculum, in line with the proposal that efficient interaction of PrP^{Sc} with host-derived PrP^C required homology between the two interacting molecules (54). The inverse relationship between gene expression level and incubation period also shows that PrP gene dosage determines the length of incubation time when there is no species barrier to overcome.

B. Hamster-Mouse PrP Chimeric Mouse Models

The preceding example based on wild-type prion protein is corroborated by results from transgenic mice producing artificial PrP^C. Chimeric transgenic

lines in which the central portion of the mouse gene was replaced with the equivalent region of hamster prion protein gene were generated (55). Two SHa/MoPrP transgenic lines were produced in which the first, Tg(MH2M), contains five amino acid substitutions encoded by SHaPrP, and the other, Tg(MHM2), has two substitutions. The normal SHaPrP and MoPrP genes differ at 16 positions within the open reading frame (ORF). Mice from these two transgenic lines producing chimeric PrP^{C} were then challenged with Syrian hamster (Sc237) or mouse scrapie (RML) prions. Both lines were susceptible to infection with Mo(RML) prions, but only MH2M was susceptible to SHa(Sc237) prions (55). The two amino acid substitutions in the MHM2 were not enough to abrogate the species barrier between mice and hamsters.

When the resulting prions designated MH2M(Sc237) were passaged in mice and hamsters, they were able to infect both Syrian hamsters and mice. More important they showed a distinct preference for infection of mice expressing the homologous MH2M PrP^{C}. This preference for infection of animals expressing a homologous PrP^{C} supports the view that there is a recognition and homophilic interaction of PrP^{Sc} molecules in the inoculum with the PrP^{C} in cells of the host. Of interest, whereas Mo(RML) prions do not ordinarily infect Syrian hamsters, inoculation of brain homogenates from Tg(MH2M) mice into Syrian hamsters had a 100% attack rate, indicating that MH2M(RML) had been formed. The sick hamsters had a unique pattern of PrP^{Sc} accumulation, suggesting that the artificial prions created by the chimeric transgene were a new prion isolate.

C. Human PrP Transgenic Mouse Models

In general, primary transmission from one species to another occurs much less efficiently, if at all, than transmission within the same species. Early attempts to transmit human prions to transgenic mice met with varied success. The Tg(HuPrP)152 transgenic line was made by inserting wild-type human PrP coding sequences encoding valine at codon 129 into cosTet vector (56). When these transgenic mice were inoculated with brain homogenates from cases of GSS and sporadic and iatrogenic CJD cases, only 14 of 169 exhibited scrapie-like symptoms with protracted incubation times of >589 days. A similar percentage of nontransgenic controls became sick with similar incubation times. The authors concluded that additional species-specific factors might be involved in prion replication. However, exceptions to this finding were reported (57). These authors inoculated a panel of human CJD cases into the Tg(HuPrP)152 transgenic mice and showed that one human CJD case, designated PDG170, resulted in eight of nine mice dying from neurological disease, with a mean incubation time of 297 days.

After the apparent lack of susceptibility of the Tg(HuPrP), mice that expressed a chimeric prion protein in which a segment of mouse PrP was replaced with the corresponding human PrP sequence were then constructed (58). Whereas HuPrP differs from MoPrP at 28 of 254 residues, the chimeric PrP designated MHu2MPrP differed from MoPrP by only nine amino acids between residues 96 and 167. When similarly inoculated with CJD prions, all the Tg(MHu2MPrP) mice encoding methionine at codon 129 developed neurological disease ~200 days after inoculation with brain homogenates from three CJD patients, all homozygous for methionine at polymorphic residue 129.

While the chimeric transgene approach (58) was being pursued, the endogenous mouse PrP allele was also removed by breeding the Tg (HuPrP)152 mice to PrP null mice (*Prnp*$^{o/o}$) to produce homozygous Tg (HuPrP)152/Prnp$^{o/o}$ (57). It is clear that breeding out the endogenous mouse PrP alleles has made the Tg(HuPrP)152/Prnp$^{o/o}$ mice highly susceptible to CJD, with all inoculated mice succumbing at short incubation periods usually in the range of 180–220 days (56,57) In contrast, the Tg(MHu2MPrP) mice were rendered only slightly more susceptible when the endogenous mouse PrP gene was removed (56).

The Tg(HuPrP)152/Prnp$^{o/o}$ mice (homozygous for the human transgene and without endogenous mouse PrP) are susceptible to all isolates of classic human CJD, and there is no decrease in incubation period on primary and second passage (57). All of 16 CJD cases encompassing a wide range of clinicopathological phenotypes, all three PrP^{Sc} types reported in sporadic and iatrogenic prion diseases (34), and all *PRNP* genotypes at polymorphic codon 129 have been transmitted to the Tg(HuPrP)152/Prnp$^{o/o}$ transgenic mice (1).

D. Bovine PrP Transgenic Mouse Models

Understanding what constitutes the species barrier has important implications for assessing the risk of BSE transmission to humans. After the success in transmitting human prions to the chimeric Tg(MHu2M) transgenics (58), transgenic mice were created that expressed either an intact bovine/PrP ORF (BoPrP) or a bovine/mouse chimeric ORF (MBo2M) in the cosTet vector (59). On inoculation of Tg(MBo2M)Prnp$^{o/o}$ and Tg(BoPrP)Prnp$^{o/o}$ mice with bovine prions, none of the former group of mice developed neurological dysfunction more than 600 days after inoculation. This was contrary to the authors' expectation as the MBo2M ORF was composed of BoPrP and MoPrP-A sequences, and their previous studies had shown that *Prnp*^{a} mice were susceptible to BSE. The same inoculum was able to transmit disease to control mice including FVB, Tg(BoPrP)833, and Tg(BoPrP)333 mice

expressing MoPrP-A. The Tg(BoPrP)833 and Tg(BoPrP)333 mice were found not to express bovine PrP at levels detectable by Western blotting and were therefore used as *Prnp*$^{a/a}$ controls. When Tg(BoPrP)4125/Prnp$^{o/o}$ mice expressing high levels of BoPrP were inoculated with BSE, they exhibited clinical signs within 250 days. A second line of Tg(BoPrP)4092/Prnp$^{o/o}$ with an intermediate level of BoPrP expression had a longer incubation period with a mean of about 320 days (59).

Primary passage of three separate cases of vCJD killed Tg(BoPrP)4125/Prnp$^{o/o}$ mice with incubation periods of 250–270 days, comparable to that obtained with cattle BSE and secondary passage of BSE from Tg(BoPrP)4125/Prnp$^{o/o}$ mice (60). This is perhaps not surprising, as vCJD has been shown to share both the molecular and biological properties of the BSE strain. These data are in accord with the clear evidence that vCJD is caused by the same agent strain as BSE (1,2,34). However, the bovine-to-human species barrier, which is discussed further later, was not directly tested by Scott and colleagues (60).

The Tg(BoPrP)4125/Prnp$^{o/o}$ mice were also susceptible to natural sheep scrapie deriving from Suffolk sheep in which susceptibility to scrapie is controlled by homozygosity for glutamine (Q) at the PrP codon 171 polymorphism (61). Bovine PrP contains Q at the equivalent position in sheep PrP; thus the ease of transmission of scrapie to Tg(BoPrP) may be due to the homology at this polymorphic residue implicated in susceptibility of sheep to scrapie.

To develop an improved bioassay for the detection of cattle-derived BSE prions, transgenic mice overexpressing bovine (Tgbov XV) or murine/bovine chimeric PrP^{C} (Tgmubo XIII) were generated (62). These authors then studied their susceptibility together with conventional RIII and murine PrP^{C} overexpressing Tga20 mice, by inoculating the four groups of mice with cattle-derived BSE prions. Although overexpression of murine PrP^{C} in Tga20 mice did not lead to a shorter incubation time than conventional RIII mice, the Tgbov XV mice showed markedly reduced incubation times with a mean of about 250 days. Like the chimeric Tg(Bo2M)Prnp$^{o/o}$ (63), the Tgmubo XIII chimeric mice displayed an extremely low susceptibility to BSE as no animal showed clear clinical symptoms even after 800 days. Brains of sick Tgbov XV mice had histopathology and glycoform band patterns typical of BSE. Secondary passage of brain homogenate of sick Tgbov XV did not lead to a lowering of incubation period, indicating that expression of the wild-type bovine sequences alone was sufficient to abrogate the species barrier between bovine and mice.

These authors (63) do offer a possible explanation for the different susceptibilities of the two chimeric transgenic mice, Tg(MHu2M) and Tg(MBo2M). A comparison of the MoPrP-A with MBo2M PrP and

MHu2M PrP translated sequences shows that human residue substitutions in MHu2M extend from 97 to 168, whereas Bo substitutions in MBo2M extend from 97 to 186. This finding raised the possibility that residues 184 and 186, which are not homologous in bovine and mouse PrP and lie at the C-terminal end of the chimeric region, might account for the differences in susceptibility of Tg(MHu2M) and Tg(MBo2M) mice to prion infection (63). Alternatively, residue 203, which is a Val in mouse and human PrP and is Ile in bovine PrP, might be responsible for this difference in susceptibility to prions. In Tg(MBo2M) mice, residue 203 is a Val and thus might prevent conversion of MBo2M PrP^{C} into PrP^{Sc} (63). Until the proposed role of these amino acid differences is experimentally confirmed, the failure of the chimeric transgenic approach with bovine PrP, as demonstrated by two independent research groups, remains to be explained.

E. BSE Transmission to Human PrP Transgenic Mouse Models

The emergence of a variant form of CJD in young people (vCJD) in the United Kingdom raised the possibility that BSE might have crossed the species barrier between humans and cattle. There is compelling evidence that vCJD and BSE are the same strain (1,2,34). What is not so clear is the magnitude of the bovine-to-human-species barrier. Clearly, this cannot be measured directly, as this would require inoculation of humans with BSE. However, transgenic models may offer a way to address this issue, at least in part.

Early studies of the molecular basis of the species barrier suggested that it mainly resided in differences in PrP primary structure between the species from which the inoculum was derived and the inoculated host. The fact that most sporadic and acquired CJD occurred in individuals homozygous at *PRNP* polymorphic codon 129 supported the view that prion propagation proceeded most efficiently when the interacting PrP^{Sc} and PrP^{C} were of identical primary structure (64). However, it has long been recognized that the type of prion strain affects ease of transmission to another species. With BSE prions, the strain component of the barrier seems to predominate, with BSE not only transmitting efficiently to a range of species, but maintaining its transmission characteristics even when passaged through an intermediate species with a distinct PrP gene (65). For example, transmission of CJD prions to conventional mice is difficult, with few if any inoculated mice succumbing after long incubation periods, which is consistent with a substantial species barrier (1,57).

In sharp contrast, transgenic mice expressing only human PrP Tg(HuPrP)152/$Prnp^{o/o}$ are highly susceptible to CJD prions, with 100% attack rate and consistent short incubation periods that are unaltered by

second passage, consistent with a complete lack of species barrier (57). However, variant CJD prions (comprising human PrP of identical structure) transmit much more readily to wild-type mice than do classic CJD prions, whereas transmission to transgenic mice is less efficient than with classic CJD (1). The term "species barrier" does not seem appropriate to describe such effects; "species-strain barrier" or "transmission barrier" may be preferable (66).

The species barrier between cattle BSE and human beings can be modeled in transgenic mice expressing human PrP^{C}, which produce human PrP^{Sc} when challenged with human prions (57). When such mice—expressing human PrP valine 129 (at high concentrations) and mouse PrP—are challenged with BSE, three possibilities may occur: the mice could produce human prions, murine prions, or both. In fact, only mouse-prion replication could be detected. Although there are caveats for this model, especially that propagation of human prions in mouse cells may be less efficient than that of mouse prions, this result would be consistent with the bovine-human barrier being larger than the bovine-mouse barrier for this *PRNP* genotype. In the second phase of these experiments, mice expressing only human PrP were challenged with BSE. Although CJD isolates transmit efficiently to such mice at around 200 days, only infrequent transmissions at more than 500 days were seen with BSE, consistent with a substantial species barrier for this human *PRNP* genotype.

F. Ovine PrP Transgenic Mouse Models

The possibility that BSE may have entered the sheep population through dissemination of BSE-contaminated meat and bone meal remains to be established. Ovine PrP transgenic mice ($AA_{136}RR_{154}QQ_{171}$ genotype) designated Tg(OvPrP4) have been generated that express selectively in their brain the ovine PrP under the control of neuron-specific enolase promoter (67). These transgenic mice were inoculated with brain homogenate from sheep experimentally infected with BSE. The authors reported numerous typical florid plaques, a hallmark of vCJD in humans, in their ovine PrP transgenic mouse model (68). Thus these mice represent a promising tool in the search for BSE in sheep.

G. Effect of Methionine 129 Homozygosity in Human PrP

The *PRNP* valine 129 genotype was studied initially in attempts to produce an animal model of human prion disease, as this genotype was overrepresented among early cases of iatrogenic CJD (69), which suggests increased susceptibility or shorter incubation periods in this genotype. So far, vCJD has affected approximately 100 people in the United Kingdom

and two in France, and to date all these cases have the *PRNP* codon 129 methionine homozygous genotype. A more appropriate model for studying vCJD and BSE transmission characteristics in the human population is transgenic models homozygous for wild-type human PrP with methionine at polymorphic residue 129. These studies have therefore been repeated in mice expressing only human PrP methionine 129 and in heterozygotes and are currently ongoing (83).

H. Bioassay for Decontamination of Surgical Instruments

Prions are not readily destroyed by conventional sterilization procedures and transmission by surgical instruments is well documented. One of the most important public health issues regarding iatrogenic spread of vCJD is the extent of the risk of transmission through tonsillectomy and other neurosurgical procedures. Studies on decontamination of surgical instruments have been carried out on wash solutions or suspensions of the agent, but what is more relevant is the behavior of the surface-bound prion agent in appropriate animal models. Weissmann and colleagues embarked on a prion decontamination study involving bioassay in indicator mice (70). The authors exposed stainless steel wire segments to scrapie agent, washed the wires exhaustively in phosphate-buffered saline (PBS) with or without 10% formaldehyde, and implanted them into the brains of Tga20 indicator mice. Using incubation period as a measure of infectivity, it was shown that the stainless steel wires, decontaminated as above, retained the equivalent of about 10^5 LD_{50} units per wire segment, and that treatment with 10% formaldehyde for 1 h reduced this value by only about 30-fold (70). While this model system confirms reports that steel surgical instruments can retain CJD infectivity even after formaldehyde treatment, it also lends itself to systematic study of the conditions required to effectively inactivate CJD, BSE, and scrapie agent adsorbed to stainless steel surfaces such as those of surgical instruments. Use of suitable mouse models of BSE, CJD, and vCJD should increase the relevance of such studies with respect to public health.

I. Testing of Potential Therapeutic Compounds by Bioassay

There is at present no cure for prion disease, though a number of compounds with different modes of action show promise. While most compounds can and should initially be tested in tissue culture cells in vitro, in vivo tests involving suitable animal models will need to precede any human trials. In vitro studies have also demonstrated that the antibiotic tetracycline, with a well-known clinical profile, (1) binds to amyloid fibrils generated by synthetic peptides corresponding to residues 106–126 and

82–146 of human PrP; (2) hinders assembly of these peptides into amyloid fibrils; (3) reverts the protease resistance of PrP peptide aggregates and PrP^{Sc} extracted from brain tissues of patients with CJD; and (4) prevents neuronal death and astrocyte proliferation induced by PrP peptides (71). Amphotericin B has been shown to delay onset of clinical symptoms in hamsters infected with scrapie agent 263K, although levels of PrP^{Sc} were similar in treated experimental and untreated control groups, suggesting other factors may be involved in the onset of clinical signs (72). Other compounds known to bind PrP^{Sc} include Congo red (73), dextran sulfate, pentosan polysulfate, and other polyanions (74,75). Also β-sheet breaker peptides have been shown to have limited effect in animal models of prion disease (76). Tricyclic compounds with an aliphatic side chain at the middle ring moiety such as quinacrine and chlorpromazine have been shown to be suitable antiprion reagents in vitro (77). These drugs have been used in humans for many years as antimalarial and antipsychotic drugs, respectively, and are known to pass the blood–brain barrier. Hence the authors suggest that these are immediate candidates for the treatment of CJD and other prion diseases.

Immune intervention in the conversion of PrP^{C} to PrP^{Sc} could also be an option for prion disease therapeutic trials. When scrapie-infected neuroblastoma cells (ScN2a) were exposed to the monoclonal antiprion protein (PrP) antibody 6H4, it was observed that not only did the antibody prevent infection, it also cured the chronically scrapie-infected cultures, as judged by the long-term abrogation of PrP^{Sc} accumulation after cessation of treatment (78). Similarly, it was demonstrated that recombinant antibody-binding fragments (Fabs) could inhibit prion propagation in cultured mouse neuroblastoma cells in a dose, dependent manner (79). Prevention of scrapie infection in vivo has also been demonstrated in transgenic mice ($Prnp^{+/o}$-6H4μ) that have high anti-PrP serum concentrations (80). Although this approach is not amenable to human trials, it clearly demonstrates the potential of immunotherapy in inhibiting the prion replication process.

Most of these compounds have shown variable success rates and some may have little practical use because of toxicity and limited bioavailability. Some of them have been examined in rodents but none have been effective when given around the time that neurological signs appear (73). The success of any therapeutic compound in vivo, whatever the mode of action and efficacy, will depend on early diagnosis of prion disease before substantial neuronal damage has occurred. Any candidate therapeutic agent should have an impact on the disease when early diagnostic signs are already apparent. In this respect, reports that behavioral testing can reveal effects in

scrapie-infected mice long before overt clinical signs appear need to be further investigated (81,82).

IX. CONCLUDING REMARKS

Bioassays in both wild-type and transgenic mice have contributed substantially to current understanding of the species barrier and prion strains. However, a great deal remains to be investigated, such as the possibility of multiple BSE strains, the true nature of the infectious agent, and the mechanism of cell death in prion diseases. Bioassays using wild-type animals and specially constructed transgenic mice are likely to play a significant role in investigating the efficacy and prophylactic properties of potential therapeutic compounds.

REFERENCES

1. Hill AF, Desbruslais M, Joiner S, Sidle KCL, Gowland I, Collinge J. The same prion strain causes vCJD and BSE. Nature 1997; 389:448–450.
2. Bruce ME, Will RG, Ironside JW, McConnell I, Drummond D, Suttie A et al. Transmissions to mice indicate that "new variant" CJD is caused by the BSE agent. Nature 1997; 389:498–501.
3. Prusiner SB. Novel proteinaceous infectious particles cause scrapie. Science 1982; 216:136–144.
4. Prusiner SB, Bolton DC, Groth DF, Bowman K, Cochran SP, Mc Kinley MP. Further purification and characterization of scrapie prions. Biochemistry 1982; 21:6942–6950.
5. Caughey BW, Dong A, Bhat KS, Ernst D, Hayes SF, Caughey WS. Secondary structure analysis of the scrapie-associated protein PrP 27–30 in water by infrared spectroscopy. Biochemistry 1991; 30:7672–7680.
6. Gajdusek DC, Gibbs CJ, Alpers M. Experimental transmission of a kuru-like syndrome to chimpanzees. Nature 1966; 209(25):794–796.
7. Gajdusek DC, Gibbs CJ Jr. Transmission of two subacute spongiform encephalopathies of man (kuru and Creutzfeldt-Jakob disease) to New World monkeys. Nature 1971; 230(5296):588–591.
8. Gibbs CJ Jr, Gajdusek DC. Experimental subacute spongiform virus encephalopathies in primates and other laboratory animals. Science 1973; 182: 67–68.
9. Carlson GA, Kingsbury DT, Goodman PA, Coleman S, Marshall ST, DeArmond SJ et al. Linkage of prion protein and scrapie incubation time genes. Cell 1986; 46:503–511.
10. Pattison IH. Experiments with scrapie with special reference to the nature of the agent and the pathology of the disease. In: Gajdusek CJ, Gibbs CJ, Alpers

MP, eds. Slow, Latent and Temperate Virus Infections, NINDB Monograph 2. Washington DC: US Government Printing, 1965: 249–257.
11. Kimberlin RH, Marsh RF. Comparison of scrapie and transmissible mink encephalopathy in hamsters. I. Biochemical studies of brain during development of disease. J Infect Dis 1975; 131:97–103.
12. Marsh RF, Kimberlin RH. Comparison of scrapie and transmissible mink encephalopathy in hamsters. II. Clinical signs, pathology, and pathogenesis. J Infect Dis 1975; 131:104–110.
13. Lloyd SE, Onwuazor ON, Beck JA, Mallinson G, Farrall M, Targonski P et al. Identification of multiple quantitative trait loci linked to prion disease incubation period in mice. Proc Natl Acad Sci USA 2001; 98(11): 6279–6283.
14. Manolakou K, Beaton J, McConnell I, Farquar C, Manson J, Hastie ND et al. Genetic and environmental factors modify bovine spongiform encephalopathy incubation period in mice. Proc Natl Acad Sci USA 2001; 98(13): 7402–7407.
15. Ghani AC, Ferguson NM, Donnelly CA, Anderson RM. Predicted vCJD mortality in Great Britain. Nature 2000; 406:583–584.
16. Dickinson AG, Fraser H, Outram GW. Scrapie incubation time can exceed natural lifespan. Nature 1975; 256:732–733.
17. Zlotnik I. Observations on the experimental transmission of scrapie of various origins to laboratory animals. In: Gajdusek CJ, Gibbs-CJ J, Alpers M, eds. Slow, Latent, and Temperate Virus Infections, NINDB Monograph 2. Washington DC: US Government Printing, 1965:237–248.
18. Kimberlin RH, Walker CA. Evidence that the transmission of one source of scrapie agent to hamsters involves separation of agent strains from a mixture. J Gen Virol 1978; 39:487–496.
19. Race R, Chesebro B. Scrapie infectivity found in resistant species. Nature 1998; 392:770.
20. Hill AF, Joiner S, Linehan J, Desbruslais M, Lantos PL, Collinge J. Species barrier independent prion replication in apparently resistant species. Proc Natl Acad Sci USA 2000; 97(18):10248–10253.
21. Scott M, Foster D, Mirenda C, Serban D, Coufal F, Wälchli M et al. Transgenic mice expressing hamster prion protein produce species-specific scrapie infectivity and amyloid plaques. Cell 1989; 59:847–857.
22. Fischer M, Rulicke T, Raber A, Sailer A, Oesch B, Brandner S et al. Prion protein (PrP) with amino terminal deletions restoring susceptibility of PrP knockout mice to scrapie. EMBO J 1996; 15:1255–1264.
23. Brandner S, Isenmann S, Raeber A, Fischer M, Sailer A, Kobayashi Y et al. Normal host prion protein necessary for scrapie-induced neurotoxicity. Nature 1996; 379(6563):339–343.
24. Race R, Raines A, Raymond GJ, Caughey B, Chesebro B. Long-term subclinical carrier state precedes scrapie replication and adaptation in a resistant species: analogies to bovine spongiform encephalopathy and variant Creutzfeldt-Jakob disease in humans. J Virol 2001; 75(21):10106–10112.

25. Klein MA, Frigg R, Raeber AJ, Flechsig E, Hegyi I, Zinkernagel RM et al. PrP expression in B lymphocytes is not required for prion neuroinvasion. Nat Med 1998; 4(12):1429–1433.
26. Frigg R, Klein MA, Hegyi I, Zinkernagel RM, Aguzzi A. Scrapie pathogenesis in subclinically infected B-cell-deficient mice. J Virol 1999; 73(11):9584–9588.
27. Kingsbury DT, Kasper KC, Stites DP, Watson JD, Hogan RN, Prusiner SB. Genetic control of scrapie and Creutzfeldt-Jakob disease in mice. J Immunol 1983; 131:491–496.
28. Stephenson DA, Chiotti K, Ebeling C, Groth D, DeArmond SJ, Prusiner SB et al. Quantitative trait loci affecting prion incubation time in mice. Genomics 2000; 69(1):47–53.
29. Weissmann C. A "unified theory" of prion propagation. Nature 1991; 352: 679–683.
30. Marsh RF, Bessen RA, Lehmann S, Hartsough GR. Epidemiological and experimental studies on a new incident of transmissible mink encephalopathy. J Gen Virol 1991; 72:589–594.
31. Caughey B, Raymond GJ, Bessen RA. Strain-dependent differences in beta-sheet conformations of abnormal prion protein. J Biol Chem 1998; 273(48): 32230–32235.
32. Bessen RA, Kocisko DA, Raymond GJ, Nandan S, Lansbury PT, Caughey B. Non-genetic propagation of strain-specific properties of scrapie prion protein. Nature 1995; 375:698–700.
33. Bessen RA, Raymond GJ, Caughey B. In situ formation of protease-resistant prion protein in transmissible spongiform encephalopathy-infected brain slices. J Biol Chem 1997; 272:15227–15231.
34. Collinge J, Sidle KCL, Meads J, Ironside J, Hill AF. Molecular analysis of prion strain variation and the aetiology of "new variant" CJD. Nature 1996; 383:685–690.
35. Parchi P, Castellani R, Capellari S, Ghetti B, Young K, Chen SG et al. Molecular basis of phenotypic variability in sporadic Creutzfeldt-Jakob disease. Ann of Neurol 1996; 39(5):669–680.
36. Parchi P, Giese A, Capellari S, Brown P, Schulz-Schaeffer W, Windl O et al. Classification of sporadic Creutzfeldt-Jakob Disease based on molecular and phenotypic analysis of 300 subjects. Ann Neurol 1999; 46(2):224–233.
37. Wadsworth JDF, Hill AF, Joiner S, Jackson GS, Clarke AR, Collinge J. Strain-specific prion-protein conformation determined by metal ions. Nature Cell Biol 1999; 1:55–59.
38. Telling GC, Parchi P, DeArmond SJ, Cortelli P, Montagna P, Gabizon R et al. Evidence for the conformation of the pathologic isoform of the prion protein enciphering and propagating prion diversity. Science 1996; 274: 2079–2082.
39. DeArmond SJ, Sanchez H, Yehiely F, Qiu Y, Ninchak CA, Daggett V et al. Selective neuronal targeting in prion disease. Neuron 1997; 19(6): 1337–1348.

40. Endo T, Groth D, Prusiner SB, Kobata A. Diversity of oligosaccharide structures linked to asparagines of the scrapie prion protein. Biochemistry 1989; 28:8380–8388.
41. Hill AF, Butterworth RJ, Joiner S, Jackson G, Rossor MN, Thomas DJ et al. Investigation of variant Creutzfeldt-Jakob disease and other human prion diseases with tonsil biopsy samples. Lancet 1999; 353(9148):183–189.
42. Westaway D, Goodman PA, Mirenda CA, McKinley MP, Carlson GA, Prusiner SB. Distinct prion proteins in short and long scrapie incubation period mice. Cell 1987; 51:651–662.
43. Carlson GA, Westaway D, Goodman PA, Peterson M, Marshall ST, Prusiner SB. Genetic control of prion incubation period in mice. Ciba Found Symp 1988; 135:84–99.
44. Race RE, Graham K, Ernst D, Caughey B, Chesebro B. Analysis of linkage between scrapie incubation period and the prion protein gene in mice. J Gen Virol 1990; 71:493–497.
45. Westaway D, Mirenda CA, Foster D, Zebarjadian Y, Scott M, Torchia M et al. Paradoxical shortening of scrapie incubation times by expression of prion protein transgenes derived from long incubation period mice. Neuron 1991; 7:59–68.
46. Moore RC, Hope J, McBride PA, McConnell I, Selfridge J, Melton DW et al. Mice with gene targetted prion protein alterations show that *Prnp*, *Sinc* and *Prni* are congruent. Nature Genet 1998; 18:118–125.
47. Dickinson AG, Meikle VM, Fraser H. Identification of a gene which controls the incubation period of some strains of scrapie agent in mice. J Comp Pathol 1968; 78(3):293–299.
48. Bruce ME. Scrapie strain variation and mutation. Br Med Bull 1993; 49: 822–838.
49. Schatzl HM, Da Costa M, Taylor L, Cohen FE, Prusiner SB. Prion protein gene variation among primates. J Mol Biol 1997; 265(2):257.
50. Lowenstein DH, Butler DA, Westaway D, McKinley MP, DeArmond SJ, Prusiner SB. Three hamster species with different scrapie incubation times and neuropathological features encode distinct prion proteins. Mol Cell Biol 1990; 10:1153–1163.
51. Hogan B, Beddington R, Costantini F, Lacy E. In: Nagy A, Gertsenstein M, Vintersten K, Behringer R, eds. Manipulating the Mouse Embryo: A Laboratory Manual. New York: Cold Spring Harbor Laboratory Press, 1994: 1–497.
52. Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning: A Laboratory Manual. 1989. New York, Cold Spring Harbor Laboratory Press.
53. Prusiner SB. Structural biology and genetics of prions. J Neurovirol 1997; 3 Suppl. 1:S21.
54. Prusiner SB, Scott M, Foster D, Pan KM, Groth D, Mirenda C et al. Transgenetic studies implicate interactions between homologous PrP isoforms in scrapie prion replication. Cell 1990; 63:673–686.
55. Scott M, Groth D, Foster D, Torchia M, Yang SL, DeArmond SJ et al. Propagation of prions with artificial properties in transgenic mice expressing chimeric PrP genes. Cell 1993; 73:979–988.

56. Telling GC, Scott M, Mastrianni J, Gabizon R, Torchia M, Cohen FE et al. Prion propagation in mice expressing human and chimeric PrP transgenes implicates the interaction of cellular PrP with another protein. Cell 1995; 83:79–90.
57. Collinge J, Palmer MS, Sidle KCL, Hill AF, Gowland I, Meads J et al. Unaltered susceptibility to BSE in transgenic mice expressing human prion protein. Nature 1995; 378:779–783.
58. Telling GC, Scott M, Hsiao KK, Foster D, Yang S-L, Torchia M et al. Transmission of Creutzfeldt-Jakob disease from humans to transgenic mice expressing chimeric human-mouse prion protein. Proc Natl Acad Sci USA 1994; 91:9936–9940.
59. Scott MR, Safar J, Telling G, Nguyen O, Groth D, Torchia M et al. Identification of a prion protein epitope modulating transmission of bovine spongiform encephalopathy prions to transgenic mice. Proc Natl Acad Sci USA 1997; 94(26):14279–14284.
60. Scott MR, Will R, Ironside J, Nguyen HOB, Tremblay P, DeArmond SJ et al. Compelling transgenetic evidence for transmission of bovine spongiform encephalopathy prions to humans. Proc Natl Acad Sci USA 1999; 96(26): 15137–15142.
61. Hunter N, Moore L, Hosie BD, Dingwall WS, Greig A. Association between natural scrapie and PrP genotype in a flock of Suffolk sheep in Scotland. Vet Rec 1997; 140:59–63.
62. Buschmann A, Pfaff E, Reifenberg K, Müller HM, Groschup MH. Detection of cattle-derived BSE prions using transgenic mice overexpressing bovine PrP^{C}. Arch Virol 2000;75–86.
63. Scott MR, Safar J, Telling G, Nguyen O, Groth D, Torchia M et al. Identification of a prion protein epitope modulating transmission of bovine spongiform encephalopathy prions to transgenic mice. Proc Natl Acad Sci USA 1997; 94:14279–14284.
64. Collinge J. Spongiform encephalopathies. Neuropathol Appl Neurobiol 1991; 17:346.
65. Bruce M, Chree A, McConnell I, Foster J, Pearson G, Fraser H. Transmission of bovine spongiform encephalopathy and scrapie to mice: strain variation and the species barrier. Philos Trans R Soc Lond (Biol) 1994; 343:405–411.
66. Collinge J. Variant Creutzfeldt-Jakob disease. Lancet 1999; 354(9175):317–323.
67. Crozet C, Flamant F, Bencsik A, Aubert D, Samarut J, Baron T. Efficient transmission of two different sheep scrapie isolates in transgenic mice expressing the ovine PrP gene. J Virol 2001; 75(11):5328–5334.
68. Crozet C, Bencsik A, Flamant F, Lezmi S, Samarut J, Baron T. Florid plaques in ovine PrP transgenic mice infected with an experimental ovine BSE. EMBO Rep 2001; 2(10):952–956.
69. Collinge J, Palmer MS, Dryden AJ. Genetic Predisposition to Iatrogenic Creutzfeldt-Jakob disease. Lancet 1991; 337:1441–1442.
70. Zobeley E, Flechsig E, Cozzio A, Masato E, Weissmann C. Infectivity of scrapie prions bound to a stainless steel surface. Mol Med 1999; 5:240–243.

71. Tagliavini F, Forloni G, Colombo L, Rossi G, Girola L, Canciani B et al. Tetracycline affects abnormal properties of synthetic PrP peptides and PrP(Sc) in vitro. J Mol Biol 2000; 300(5):1309–1322.
72. McKenzie D, Kaczkowski J, Marsh R, Aiken J. Amphotericin B delays both scrapie agent replication and PrP-res accumulation early in infection. J Virol 1994; 68:7534–7536.
73. Ingrosso L, Ladogana A, Pocchiari M. Congo red prolongs the incubation period in scrapie-infected hamsters. J Virol 1995; 69:506–508.
74. Ehlers B, Diringer H. Dextran sulphate 500 delays and prevents mouse scrapie by impairment of agent replication in spleen. J Gen Virol 1984; 65:1325–1330.
75. Kimberlin RH, Walker CA. Suppression of scrapie infection in mice by heteropolyanion 23, dextran sulfate, and some other polyanions. Antimicrob Agents Chemother 1986; 30:409–413.
76. Soto C, Kascsack RJ, Saborío GP, Aucouturier P, Wisniewski T, Prelli F et al. Reversion of prion protein conformational changes by synthetic beta-sheet breaker peptides. Lancet 2000; 355:192–197.
77. Korth C, May BC, Cohen FE, Prusiner SB. Acridine and phenothiazine derivatives as pharmacotherapeutics for prion disease. Proc Natl Acad Sci USA 2001; 98(17):9836–9841.
78. Enari M, Flechsig E, Weissmann C. Scrapie prion protein accumulation by scrapie-infected neuroblastoma cells abrogated by exposure to a prion protein antibody. Proc Natl Acad Sci USA 2001; 98(16):9295–9299.
79. Peretz D, Williamson RA, Kaneko K, Vergara J, Leclerc E, Schmitt-Ulms G et al. Antibodies inhibit prion propagation and clear cell cultures of prion infectivity. Nature 2001; 412(6848):739–743.
80. Heppner FL, Musahl C, Arrighi I, Klein MA, Rulicke T, Oesch B et al. Prevention of scrapie pathogenesis by transgenic expression of anti-prion protein antibodies. Science 2001; 294(5540):178–182.
81. Betmouni S, Deacon RMJ, Rawlins JNP, Ferry VH. Behavioral consequences of prion disease targeted to the hippocampus in a mouse model of scrapie. Psychobiology 1999; 27(1):63–71.
82. Guenther K, Deacon RM, Perry VH, Rawlins JN. Early behavioural changes in scrapie-affected mice and the influence of dapsone. Eur J Neurosci 2001; 14(2):401–409.
83. Asante E, Linehan JM, Desbruslais M, Joiner S, Gowland I, Wood AL, et al. BSE prions propagate as either variant CJD-like or sporadic CJD-like prion strains in transgenic mice expressing human prion protein. EMBO J 2002; 21:6358–6366.

7

Cyclic Amplification of Scrapie Prion Protein: Implications for Diagnosis

Claudio Soto
Serono International, S.A., Geneva, Switzerland

I. INTRODUCTION

The development of tests that can effectively identify animals and humans incubating the different forms of TSE is a top priority. PrP^{Sc} is the only validated surrogate marker for the disease and the only known component of the TSE infectious agent. We have recently described a procedure to induce an accelerated prion replication in vitro, based on the cyclic amplification of prion protein misfolding. This procedure, conceptually analogous to DNA amplification by PCR, has tremendous implications for research and diagnosis of TSE.

II. BACKGROUND

Creutzfeldt-Jakob disease (CJD) in humans and scrapie and bovine spongiform encephalopathy (BSE) in animals are some of the diseases that belong to the group of transmissible spongiform encephalopathies (TSE), also known as prion diseases (1). These diseases are characterized by an extremely long incubation period, followed by a brief and invariably fatal clinical disease (2). To date no therapy is available.

Although these diseases are relatively rare in humans, the risk for the transmissibility of BSE to humans through the food chain has taken the attention of the public health authorities and the scientific community (3,4). Variant CJD (vCJD) is a new disease first described in March 1996 (5). In

contrast to typical cases of sporadic CJD (sCJD), this variant form affects young patients (average age 27 years) and has a relatively long duration of illness (median 14 months vs. 4.5 months in traditional CJD). A link between vCJD and BSE was first hypothesized because of the association of these two TSEs in place and time (6). Evidence supporting a link includes identification of pathological features similar to vCJD in brains of macaque monkeys inoculated with BSE, in addition to the demonstration that vCJD is associated with molecular and neuropathological characteristics that distinguish it from other forms of CJD and that resemble those seen in BSE transmitted to a number of other species (6,7). The most recent and powerful evidence comes from studies showing that the transmission characteristics of BSE and vCJD to mice are almost identical, strongly indicating that they are due to the same causative agent (4). Moreover, transgenic mice carrying a human or a bovine gene have now been shown to be susceptible to BSE and vCJD (8,9). Furthermore, no other plausible hypothesis for the occurrence of vCJD has been proposed and intensive CJD surveillance in five European countries with a low exposure to the BSE agent has failed to identify any additional cases. In conclusion, the most likely cause of vCJD is exposure to the BSE agent, probably due to dietary contamination with affected bovine central nervous system tissue. Insufficient information is available at present to make any well-founded prediction about the future number of vCJD cases (3,10).

III. ETIOLOGY

The nature of the transmissible agent has been a matter of passionate controversy (11,12). Initially, the agent was thought to be a slow virus owing to the unusually long incubation period between the time of exposure to the pathogen and the onset of symptoms. Further research, however, has indicated that the TSE agent differs significantly from viruses and other conventional agents in that it seems not to contain nucleic acids (12). Additionally, the physicochemical procedures that inactivate most viruses, such as disrupting nucleic acids, have proved ineffective in decreasing the infectivity of the TSE pathogen. In contrast, the procedures that degrade protein have been found to inactivate the pathogen (12). Accordingly, a new theory regarding the transmissible agent has emerged and recently gained widespread acceptability. This theory proposes that the transmissible agent is neither a virus nor other previously known infectious agent, but rather an unconventional agent consisting only of a protein (12,13). This new class of pathogen was called a "prion," short for "proteinaceous infectious particle" (13). In TSE, prions are composed mainly of a misfolded protein named

PrP^{Sc}, which is a posttranslationally modified version of a normal protein, termed PrP^{C} (14). Chemical differences have not been detected to distinguish these two PrP isoforms (15) and the conversion seems to involve a conformational change whereby the α-helical content of the normal protein diminishes and the amount of β-sheet increases (16). The structural changes are followed by alterations in the biochemical properties: PrP^{C} is soluble in nondenaturing detergents, PrP^{Sc} is insoluble; PrP^{C} is readily digested by proteases while PrP^{Sc} is partially resistant, resulting in the formation of an N-terminally truncated fragment known as PrP^{res} (14,17).

IV. THE DIAGNOSIS PROBLEM

At present there is not an accurate diagnosis for TSE (18,19). The clinical diagnosis of sporadic CJD (sCJD) is currently based on the combination of subacute progressive dementia (less than 2 years), myoclonus, and multifocal neurological dysfunction, associated with a characteristic periodic electroencephalogram (EEG) (19). However, vCJD, most of the iatrogenic forms of CJD, and up to 40% of the sporadic cases do not have the EEG abnormalities (20). On average the accuracy of clinical diagnosis is around 60% for sCJD and highly variable for other prion-related diseases. The clinical diagnosis is more accurate only at the late stage of the disease when clear symptoms have developed (19). Recent data have identified several neuronal, astrocytic, and glial proteins that are elevated in sCJD (21). The protein S-100, neuron-specific isoenzyme, and ubiquitin are significantly increased in the CSF in the early phase of disease with decreasing concentrations over the course of the illness (21). A marker of neuronal death, the 14-3-3 protein, has been proposed to help the diagnosis of sporadic CJD (22). However, it is not useful for vCJD (23), and much less specific in the genetic forms. As the 14-3-3 protein may be present in the cerebrospinal fluid of patients with other conditions, the test is not recommended by the World Health Organization as a general screening for CJD and is reserved to support the clinical diagnosis. When clinical data are combined with the biochemical markers, a higher success in the diagnosis is achieved. However, according to the operational diagnosis currently in use in the European Surveillance of CJD, the definitive diagnosis is established only by neuropathological examination and detection of PrP^{Sc} by either immunohistochemistry, histoblot, or Western blot (18,19). Presymptomatic detection of sCJD or vCJD in living persons is not possible. This problem, in a scenario of a substantial number of people incubating vCJD, raises an enormous concern of iatrogenic propagation of vCJD (24,25). A presymptomatic diagnosis of CJD is also very important

for treatment, because it is likely that potential therapies would require intervention before symptoms appear.

In animal TSEs the situation is no better. Although several tests have been developed to diagnose BSE in postmortem brain tissue (26), there is still no reliable way to identify cattle early after infection (27). Therefore, it is possible that infected animals without symptoms and negative by the biochemical tests enter into the food chain, imposing a risk for human health. All these issues indicate that the development of tests that can effectively detect animals and humans incubating the disease is a top priority (27–29).

PrP^{Sc} is not only the major component of the infectious agent and the most likely cause of TSE, but also the only validated surrogate marker for the disease (12). Detection of PrP^{Sc} in tissues and cells correlates widely with the disease and with the presence of TSE infectivity, and treatments that inactivate or eliminate TSE infectivity also eliminate PrP^{Sc} (1). The identification of PrP^{Sc} in human or animal tissues is considered key for TSE diagnosis.

The problem for a diagnosis based on detecting PrP^{Sc} is that the pathological form of PrP is abundant only on its primary target organ: the brain. However, infectivity studies have shown that prions are also present in minute amounts in peripheral tissues, such as lymphoid organs and blood (24,30–32). Infectious diseases are usually diagnosed biochemically either by the immune reaction elicited in the body in response to the presence of the infectious agent or by amplification of a nucleic acid specific of the agent, using the polymerase chain reaction (PCR). However, in TSE there is no immune reaction, because the infectious agent is a protein that has the same sequence as a natural protein in the body; and PCR is not useful for the simple reason that prions do not contain nucleic acid.

The gold standard and, so far, most sensitive method to detect the infectious agent involves inoculating the material into the brain of experimental animals (mice or hamsters) and subsequently monitoring for the appearance of clinical symptoms (31–34). The high sensitivity of this method is based on the in vivo replication of prions during the incubation phase. Animals are injected with minute (undetectable) quantities of PrP^{Sc}, and by the time the animals show the clinical symptoms, PrP^{Sc} is very abundant in the brain. The biggest practical problem for using the infectivity assay in routine diagnosis is that prion replication during the incubation phase progresses very slowly. Indeed, this period can take from several months to many years before a detectable quantity of PrP^{Sc} has accumulated in the brain. Our strategy for TSE diagnosis is to mimic the in vivo replication of prions in the test tube in an accelerated manner, so that a high level of amplification of PrP^{Sc} can be obtained in few hours. For this purpose we

have recently developed the concept of PMCA (protein misfolding cyclic amplification) (35).

V. RATIONALE BEHIND PMCA

To understand the principles that led to the invention of PMCA, I will first describe the current understanding of the mechanism of prion propagation. Although the molecular mechanism of prion replication is not completely understood, the existing evidence indicates that it involves conversion of the endogenous PrP^{C} into PrP^{Sc} catalyzed by the exogenous PrP^{Sc} (12,14). The notion that host PrP^{C} is involved in the development of infection is supported by experiments in which endogenous PrP gene was knocked out. These animals were both resistant to prion disease and unable to generate new infectious particles (36). In addition, it is clear that during the time between the inoculation with the infectious protein and the appearance of the clinical symptoms, there is a dramatic increase in the amount of PrP^{Sc} (37). These findings suggest that endogenous PrP^{C} is converted to PrP^{Sc} by the action of the infectious form of the PrP molecule. Prion replication is hypothesized to occur when PrP^{Sc} in the infecting inoculum interacts specifically with host PrP^{C}, catalyzing its conversion to the pathogenic form of the protein (38). A physical association between the two isoforms during the infectious process is suggested by the primary sequence specificity in prion transmission (39) and by the reported in vitro generation of PrP^{Sc}-like molecules by mixing purified PrP^{C} with PrP^{Sc} (40).

Investigations with chimeric transgenes showed that PrP^{C} and PrP^{Sc} are likely to interact within a central domain delimited by codons 96 and 169 (41). Studies using several synthetic PrP peptides showed that peptides spanning the region 109–141 (Fig. 1) bind to PrP^{C} and compete with PrP^{Sc} interaction (42,43), providing biochemical confirmation of the conclusions drawn from the results of the transgenetic studies. More detailed biochemical studies should result in further refining of the size of the interactive region as well as definition of critical residues. From molecular genetic studies as well as from the analysis of the requirements for PrP conversion in vitro, the existence of a chaperon-like protein has been postulated, provisionally called protein X, that facilitates $PrP^{C} \rightarrow PrP^{Sc}$ conversion (44,45). However, the nature of this factor is still unknown.

The most plausible model for prion replication is the so-called "nucleation/polymerization" model, which proposes the coexistence of PrP^{C} and PrP^{Sc} in a thermodynamic equilibrium in solution (46–48). The PrP^{Sc} monomer is unstable and becomes stabilized upon aggregation with other PrP^{Sc} molecules. PrP^{Sc} aggregates promote the conversion of PrP^{C} by

Figure 1 Schematic representation of the primary structure of PrP, posttranslational modifications, and mutations associated with familial forms of the disease.

binding to the monomeric PrP^{Sc} and displacing the equilibrium toward the formation of the pathological conformer (49). In this model, the rate-limiting step is the formation of a nucleus that acts as a seed for further stabilization of PrP^{Sc} (46). In the infectious forms of the disease, an exogenous PrP^{Sc} nucleus act as a seed to induce prion replication and hence the disease (47,48). The PrP^{Sc} oligomer is elongated at the ends as new molecules of PrP^{C} are converted and incorporated. This model is supported by mathematical modeling studies (48,50), quantitative data obtained from in vitro conversion experiments (49,51), and the morphological characterization of prion aggregates as unbranched polymers with a relatively constant diameter (52,53). The kinetics of such nucleated prion replication is thus limited by the number of PrP^{Sc} nuclei present in the sample (46,48), which might explain in part the long period of time needed in vivo to generate a concentration of PrP^{Sc} high enough to trigger neurodegeneration.

PMCA consists of cycles of accelerated prion replication (35). Each cycle is composed of two phases (Fig. 2). During the first phase the sample containing minute amounts of PrP^{Sc} and a large excess of PrP^{C} is incubated to induce growing of PrP^{Sc} polymers. In the second phase the sample is sonicated to break down the polymers, multiplying the number of nuclei. In this way, after each cycle the number of "seeds" is increased in an exponential fashion (Fig. 2) (35). The cyclic nature of the system permits the use of as

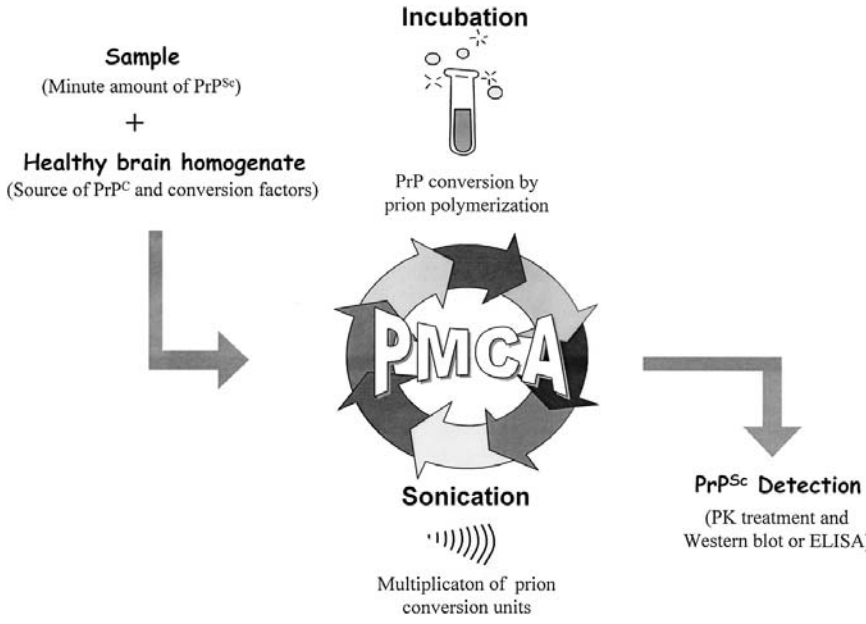

Figure 2 Schematic representation of the PMCA procedure as cycles consisting of phases of growth of polymers and multiplication of converting units.

many cycles as required to reach the amplification state needed for the detection of PrP^{Sc} in a particular sample. PMCA is conceptually analogous to DNA amplification by PCR. In both systems a template grows at the expense of a substrate in a cyclic reaction, which combines phases of growing and multiplication of the template units. It remains to be seen whether PMCA can be applied to amplify the conformation of proteins other than PrP.

VI. PMCA: PROOF-OF-CONCEPT STUDIES

Rodents (mice, hamsters, and guinea pigs) infected with scrapie agent have been used for many years as convenient models for TSE (33). These animal models develop a disease with biochemical, neuropathological, and clinical characteristics typical of scrapie. Animals inoculated with prions develop the disease several months after the inoculation and the exact time depends on the dose of infectious agent injected and the route of administration (33).

We have used hamster brain as a source of the materials needed for PMCA to do the proof-of-concept studies. Brain homogenate extracted

from scrapie-affected animals was used as a source of PrP^{Sc} while brain homogenate from healthy hamsters was used as a source of PrP^{C} and other factors that might be important during prion replication (35). Scrapie brain homogenate was diluted serially into the healthy hamster brain homogenate to mimic samples from various tissues or distinct stages of the disease where different quantities of PrP^{Sc} are expected. Half of these samples were immediately frozen and the other half were subjected to PMCA. The amount of PrP^{C} converted was evaluated by Western blot after treatment of the samples with proteinase K (PK). PK treatment is used routinely in the field to distinguish between the normal and abnormal forms of PrP, which differ in their sensitivity to protease degradation (PrP^{Sc} is partially resistant and PrP^{C} is degraded) (1). The form of PrP that is resistant to PK treatment will be termed from now on PrP^{res}. Without amplification, the signal corresponding to PrP^{res} disappeared after approximately a 600-fold dilution of the sick brain homogenate. However, after 10 cycles of amplification the quantity of PrP^{res} was dramatically higher compared with the equivalent sample without amplification (Fig. 3). The PrP^{res} band was detectable even after >10,000-fold dilution of scrapie brain homogenate (35). Densitometric analysis of the immunoblots indicates that of the total amount of PrP^{res} present in the sample after amplification >98% corresponds to newly generated PrP^{res} (35).

The conversion depends on the presence of PrP^{Sc} since no PrP^{res} was observed when the normal hamster brain homogenate was incubated alone under the same conditions either with or without sonication (Fig. 3). On the

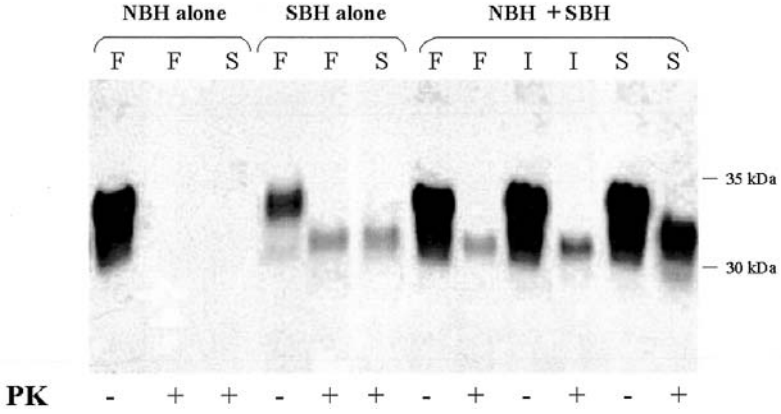

Figure 3 Proof-of-concept amplification experiments using diluted scrapie hamster brain homogenate (SBH) as a source of PrP^{Sc} and healthy hamster brain homogenate (NBH) as a source of PrP^{C}.

other hand, no increase of PrP^{res} reactivity was observed when diluted PrP^{Sc} was incubated in the absence of the PrP^{C} substrate (Fig. 3). The intensity of the PrP^{res} signal after PMCA increases exponentially with the number of cycles of incubation/sonication performed (35). This result suggests that the sensitivity of the detection system can be further improved by increasing the number of amplification cycles. Theoretically, any small amount of material could be detectable if the appropriate number of cycles is performed. However, it is unknown if there is a thermodynamic threshold below which prion replication does not occur. Based on the estimation of PrP^{Sc} reactivity by immunoblot in comparison with the immunoreactivity of known amounts of recombinant hamster PrP, we can estimate that the minimum amount of PrP^{Sc} detectable after 10 PMCA cycles was approximately 6–12 pg, or $0.2–0.4 \times 10^{-15}$ moles. The estimated amount of newly converted PrPres is ~ 250 pg, or 8.3×10^{-15} moles. This detection limit was calculated using low-sensitivity immunoblot assays and therefore it should be further decreased, to reach a similar or even a better sensitivity than the biological assay of infectivity, by using higher-sensitivity detection systems. As stated before, the limit will depend on the number of cycles performed.

We have successfully applied PMCA to a variety of brain samples, including: sporadic and familial human CJD, hamster 263K, mouse ME7, sheep, goat, and BSE samples. These results show that the same principle can be applied to a number of prion strains regardless of their differences, and that PrP^{Sc} in all these samples is capable of converting a large excess of PrP^{C} in vitro. However, the ultrasound strength needed for amplification is clearly different for distinct species/strains of PrP^{Sc}. These findings are probably related to the specific PrP^{Sc} conformation/aggregation state of each strain of prions, which has been proposed to explain the differences in clinical, neuropathological, and biochemical features of distinct strains (54–56). In addition, the incubation time between sonications in each cycle has to be adjusted for optimal amplification when using PrP^{Sc} obtained from different species/strains or from different tissues within the same species. For example, cycles of 1 h of incubation followed by sonication pulses of around 30 watts are appropriate to amplify PrP^{Sc} obtained from hamster 263K, but 3 h of incubation and a lower potency of 10 watts/pulse are optimal for the protein from mouse ME7.

VII. SCIENTIFIC APPLICATIONS OF PMCA

These findings marks the first time that prions have been replicated in vitro with a greater efficiency than the conversion process that occurs in vivo in prion diseases such as BSE and CJD. Also, this is the first time in which the

folding and biochemical properties of a protein have been cyclically amplified in a manner conceptually analogous to the amplification of DNA by PCR. As a result of this research, there is now a sensitive in vitro model to help in understanding the underlying biology of prions, to identify other factors that may be responsible for prion protein conversion, and to discover novel drug targets for TSEs.

A crucial issue in TSE has been the unprecedented nature of the infectious agent (1,11,12), which, according to the prion hypothesis, is composed of a single protein that propagates in the absence of nucleic acid. An important finding supporting the prion hypothesis was the cell-free conversion system reported by Caughey and co-workers, which demonstrated that PrP^{Sc} can indeed induce the conversion of PrP^{C} in vitro (40). The in vitro conversion of PrP^{Sc} has been described in a variety of conditions (57), and has been useful to study the molecular mechanism of PrP conversion (58), the species barrier phenomenon (59), and to identify and evaluate inhibitors of PrP transition (60,61). However, the efficiency of this conversion system is low, requiring excess of PrP^{Sc} to convert minimal amounts of PrP^{C}. This problem has precluded the study of the structural and infectious properties of the newly converted PrP^{res} (62).

Using PMCA, minute amounts of PrP^{Sc} (equivalent to those present at the time of infection) can transform large quantities of PrP^{C} to produce a situation similar to that in the brains of sick animals where the concentration of PrP^{Sc} increases by several orders of magnitude. Therefore, prion replication in vitro by PMCA provides additional support for the prion hypothesis.

Perhaps the most important missing evidence for the protein-only hypothesis is the generation of infectivity in the test tube (11). PMCA provides a great opportunity to evaluate the infectious properties of PrP^{Sc} generated in vitro, because using the optimal conditions, after amplification the total amount of protease-resistant protein is mainly composed of newly produced PrP^{Sc} (>99%). The latter is essential to distinguish the infectivity coming from the inoculum from newly generated infectivity.

Another widely debated issue in the TSE field is the molecular mechanism of species barrier and prion strains (63,64). As a consequence of the transmission of BSE to humans, a great concern has arisen regarding interspecies infectivity and the tissues having a quantity of prions high enough to transmit the disease (32,65). The molecular aspects that underlie the species barrier phenomenon are still not understood. It has been shown that the sequence homology between the infectious PrP^{Sc} and the host prion protein plays a crucial role in determining species barrier (54). It is clear that few amino acid differences between both proteins can modify dramatically

the incubation time and the course of the disease (66,67). So far, the investigation of the species barrier and the tissues carrying infectivity has been done using the biological assay of prion propagation in animals (32,64). However, these studies are time consuming, because it is necessary to wait for several months or even years until the animals develop the clinical symptoms. In addition, the assessment of the species barrier for transmission of prions to humans is compromised by the use of animal models. PMCA might provide a complement to the in vivo studies of the phenomenon of species barrier by combining PrP^{Sc} and PrP^{C} from different sources in distinct quantities and evaluating quantitatively the efficiency of conversion. In addition, the presence of minute quantities of prions in different tissues can be analyzed by attempting to convert brain PrP^{C} with a sample of a determined tissue from an infected individual. These studies should provide the basis for minimizing the risk of prion propagation.

PMCA might also contribute to a better understanding of the mechanism of prion conversion and the identification of additional factors involved. Based on data with transgenic animals, it has been proposed that additional brain factors present in the host are essential for prion propagation (44,68). We reported previously that using highly purified prion proteins (PrP^{Sc} and PrP^{C}) the conversion procedure does not occur under our experimental conditions (45). However, the activity is recovered when the bulk of cellular proteins is reincorporated into the sample (45). This finding provides direct evidence that other factors present in the brain are essential to catalyze prion propagation. PMCA represents an ideal biochemical tool to identify these conversion factors. Historically the enzymatic activity of proteins has been used to facilitate the purification and characterization of new proteins. Up to now no method was available to study the cellular factors involved in the interaction and/or interconversion of PrP^{C} into PrP^{Sc}. Having the appropriate system to identify the elements required for prion conversion may offer new opportunities to study the molecular mechanism of PrP^{Sc} formation and the pathogenesis of TSE. The identification and characterization of these factors will not only increase our knowledge of the molecular mechanism of prion conversion, but may also help to explain similar protein misfolding processes associated with other neurodegenerative diseases, such as Alzheimer's and Parkinson's disease (69).

VIII. PRACTICAL APPLICATIONS OF PMCA

An obvious application of PMCA is in the TSE diagnosis. As stated before, the biggest problem facing a biochemical test to detect PrP^{Sc} presympto-

matically in tissues other than brain is the very low amount of PrP^{Sc}. Most of the efforts to develop a diagnostic system for prion diseases have been focused on the increase of sensitivity of the current detection methods. PMCA offers the opportunity to enhance existing methods by amplifying the amount of PrP^{Sc} in the sample. By combining the strategy of reproducing prions in vitro with any of the high-sensitive detection methods, the early diagnosis of TSE may be achieved. The aim would be not only to detect prions in the brain in early presymptomatic cases, but also to generate a test to diagnose living animals and humans. For this purpose a tissue other than brain is required, and to have an easier noninvasive method, detection of prions in body fluids such as urine or blood is the best option. A blood test for CJD might have many applications, including screening of blood banks, identification of populations at risk, reduction of iatrogenic transmission of CJD and early diagnosis of the disease (27,28).

PMCA might also have applications in the development of novel therapies for TSE. One of the best targets for TSE therapy is the inhibition and reversal of PrP^{C} to PrP^{Sc} conversion (37,70). In drug development it is crucial to have a relevant and robust in vitro assay to screen compounds for activity, before testing them in more time-consuming and expensive in vivo assays. PMCA represents a convenient biochemical tool to identify and evaluate the activity of drug candidates for TSE treatment, because it mimics in vitro the central pathogenic process of the disease. Also, the simplicity of the method and the relatively rapid outcome are important features for this type of study. Moreover, the fact that PMCA can be applied to prion conversion in different species provides the opportunity to validate the use in humans of drugs that have been evaluated in experimental animal models of the disease.

GLOSSARY

Protein misfolding Alteration in the secondary and tertiary structure of a protein, resulting in a protein with altered conformation.

Spongiform degeneration The most typical pathological feature of the brain of individuals affected by TSE and consists in the vacuolation of brain tissue to form literally holes in the brain.

β-sheet structure The β-sheet is one of the most prevalent, repetitive secondary structural motifs in folded proteins. β-sheets are formed of alternating peptide pleated strands linked by hydrogen bonding between the NH and CO groups of the peptidic bond. In β-sheets the β-strands can come from a different region of the same protein or from a different molecule.

Therefore, formation of β-sheets can be stabilized by protein oligomerization or aggregation.

Amyloid A generic term that describes fibrillar aggregates organized in a β-pleated sheet conformation. These aggregates exhibit specific tinctorial properties, including the ability to emit a green birefringent glow after staining with Congo red, and the capacity to bind the fluorochrome thioflavin S. More than a dozen human diseases of different etiology are characterized by the extracellular deposition of amyloid. The amyloid fibrils are usually composed of proteolytic fragments of normal or mutant gene products. So far, more than 16 different proteins have been involved in amyloid deposition in distinct tissues.

Conformational transmission The process by which the tridimensional structure of one protein molecule is transferred to another molecule that acquires the structural and functional properties of the former molecule.

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REFERENCES

1. Prusiner SB. Molecular biology of prion diseases. Science 1991; 252:1515–1522.
2. Roos R, Gajdusek DC, Gibbs CJ Jr. The clinical characteristics of transmissible Creutzfeldt-Jakob disease. Brain 1973; 96:1–20.
3. Cousens SN, Vynnycky E, Zeidler M, Will RG, Smith RG. Predicting the CJD epidemic in humans. Nature 1997; 385:197–198.
4. Bruce ME, Will RG, Ironside JW, McConnell I, Drummond D, Suttie A. Transmissions to mice indicate that new variant CJD is caused by the BSE agent. Nature 1997; 389:498–501.
5. Will RG, Ironside JW, Zeidler M, Cousens SN, Estibeiro K, Alperovitch AP, Poser S, Pocchiari M, Hofman A, Smith PG. A new variant of Creutzfeldt-Jakob disease in the UK. Lancet 1996; 347:925.
6. Bruce ME. "New variant" Creutzfeldt-Jakob disease and bovine spongiform encephalopathy. Nature Med 2000; 6:258–259.
7. Collinge J, Sidle KCL, Meads J, Ironside J, Hill AF. Molecular analysis of prion strain variation and the aetiology of "new variant" CJD. Nature 1996; 383:685–690.
8. Scott MR, Will R, Ironside J, Nguyen HO, Tremblay P, DeArmond SJ,

Prusiner SB. Compelling transgenetic evidence for transmission of bovine spongiform encephalopathy prions to humans. Proc Natl Acad Sci USA 1999; 96: 15137–15142.
9. Hill AF, Desbruslais M, Joiner S, Sidle KC, Gowland I, Collinge J, Doey LJ, Lantos P. The same prion strain causes vCJD and BSE. Nature 1997; 389: 448–450.
10. Hilton DA. vCJD—predicting the future? Neuropathol Appl Neurobiol 2000; 26:405–407.
11. Mestel R. Putting prions to the test. Science 1996; 273:184–189.
12. Prusiner SB. Prions. Proc Natl Acad Sci USA 1998; 95:13363–13383.
13. Prusiner SB. Novel proteinaceous infectious particles cause scrapie. Science 1982; 216:136–144.
14. Cohen FE, Prusiner SB. Pathologic conformations of prion proteins. Annu Rev Biochem 1998; 67:793–819.
15. Stahl N, Baldwin MA, Teplow DB, Hood L, Gibson BW, Burlingame AL, Prusiner SB. Structural studies of the scrapie prion protein using mass spectrometry and amino acid sequencing. Biochemistry 1993; 32:1991–2002.
16. Pan KM, Baldwin M, Njuyen J, Gassett M, Serban A, Groth D, Mehlhorn I, Prusiner SB. Conversion of alpha-helices into β-sheets features in the formation of scrapie prion poteins. Proc Natl Acad Sci USA 1993; 90:10962–10966.
17. Baldwin MA, Cohen FE, Prusiner SB. Prion protein isoforms, a convergence of biological and structural investigations. J Biol Chem 1995; 270:19197–19200.
18. Budka H, Aguzzi A, Brown P, Brucher JM, Bugiani O, Gullotta F, Haltia M, Hauw JJ, Ironside JW, Jellinger K, Kretzschmar HA, Lantos PL, Masullo C, Schlote W, Tateishi J, Weller RO. Neuropathological diagnostic criteria for Creutzfeldt-Jakob disease (CJD) and other human spongiform encephalopathies (prion diseases). Brain Pathol 1995; 5:459–466.
19. Weber T, Otto M, Bodemer M, Zerr I. Diagnosis of Creutzfeld-Jakob disease and related human spongiform encephalopathies. Biomed Pharmacother 1997; 51:381–387.
20. Steinhoff BJ, Räcker S, Herrendorf G, et al. Accuracy and reliability of periodic sharp wave complexes in Creutzfeld-Jakob disease. Arch Neurol 1996; 53:162.
21. Jimi T, Wakayama Y, Shibuya S, et al. High levels of nervous system specific protein in the cerebrospinal fluid in patients with early stage Creutzfeldt-Jakob disease. Clin Chim Acta 1992; 211:37.
22. Hsich G, Kenney K, Gibbs CJ Jr, Lee KH, Harrington MG. The 14-3-3 brain protein in cerebrospinal fluid as a marker for transmissible spongiform encephalopathies. N Engl J Med 1996; 335:924.
23. Will RG, Zeidler M, Stewart GE, Macleod MA, Ironside JW, Cousens SN, Mackenzie J, Estibeiro K, Green AJ, Knight RS. Diagnosis of new variant Creutzfeldt-Jakob disease. Ann Neurol 2000; 47:575–582.
24. Collinge J. Prion diseases of humans and animals: their causes and molecular basis. Annu Rev Neurosci 2001; 24:519–550.
25. Frosh A, Joyce R, Johnson A. Iatrogenic vCJD from surgical instruments. Br Med J 2001; 322:1558–1559.

26. Moynagh J, Schimmer H. Test for BSE evaluated. Nature 1999; 400:105.
27. Schiermeier Q. Testing times for BSE. Nature 2001; 409:658–659.
28. Anonimous. Scientists race to develop a blood test for vCJD. Nature Med 2001; 7:261.
29. Knight R. The diagnosis of prion diseases. Parasitology 1998; 117(Suppl):S3–11.
30. Aguzzi A. Prion diseases, blood and the immune system: concerns and reality. Haematologica 2000; 85:3–10.
31. Brown P, Cervenakova L, Diringer H. Blood infectivity and the prospects for a diagnostic screening test in Creutzfeldt-Jakob disease. J Lab Clin Invest 2001; 137:5–13.
32. Wadsworth JD, Joiner S, Hill AF, Campbell TA, Desbruslais M, Luthert PJ, Collinge J. Tissue distribution of protease resistant prion protein in variant Creutzfeldt-Jakob disease using a highly sensitive immunoblotting assay. Lancet 2001; 358:171–180.
33. Kimberlin RH. Experimental scrapie in the mouse: a review of an important model disease. Sci Prog 1976; 63:461–481.
34. Brown P, CJ Gibbs Jr, Rodgers-Johnson P, Gajdusek DC. Human spongiform encephalopathy: the National Institute of Health series of 300 cases of experimentally transmitted disease. Ann Neurol 1994; 35:513–529.
35. Saborio GP, Permanne B, Soto C. Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding. Nature 2001; 411:810–813.
36. Bueler H, Aguzzi A, Sailer A, Greiner RA, Autenried P, Aguet M, Weissmann C. Mice devoid of PrP are resistant to Scrapie. Cell 1993; 73:1339–1347.
37. Soto C, Saborio GP. Prions: Disease propagation and disease therapy by conformational transmission. Trends Mol Med 2001; 7:109–114.
38. Cohen FE, Pan KM, Huang Z, Baldwin M, Fletterick RJ, Prusiner SB. Structural clues to prion replication. Science 1994; 264:530–531.
39. DeArmond SJ, Prusiner SB. Prion protein transgenes and the neuropathology in prion diseases. Brain Pathol 1995; 5:77–89.
40. Kocisko DA, Come JH, Priola SA, Chesebro B, Raymond GJ, Lansbury PT, Caughey B. Cell-free formation of protease-resistant prion protein. Nature 1994; 370:471–474.
41. Scott MRD, Telling GC, Prusiner SB. Transgenetics and gene targeting in studies of prion diseases. Curr Topics Microbiol Immunol 1996; 207:95–123.
42. Kaneko K, Wille H, Mehlhorn I, Zhang H, Ball H, Cohen FE, Baldwin MA, Prusiner SB. Molecular properties of complexes formed between the prion protein and synthetic peptides. J Mol Biol 1997; 270:574–586.
43. Chabry J, Caughey B, Chesebro B. Specific inhibition of in vitro formation of protease-resistant prion protein by synthetic peptides. J Biol Chem 1998; 273:13203–13207.
44. Telling GC, Scott M, Mastrianni J, Gabizon R, Torchia M, Cohen FE, DeArmond SJ, Prusiner SB. Prion propagation in mice expressing human and chimeric PrP transgenes implicates the interaction of cellular PrP with another protein. Cell 1995; 83:79–90.
45. Saborio GP, Soto C, Kascsak RJ, Levy E, Kascsak R, Harris DA, Frangione

B. Cell-lysate conversion of prion protein into its protease-resistant isoform suggests the participation of a cellular chaperone. Biochem Biophys Res Commun 1999; 258:470–475.
46. Jarrett JT, Lansbury PT Jr. Seeding "one-dimensional crystallization" of amyloid: a pathogenic mechanism in Alzheimer's disease and scrapie? Cell 1993; 73:1055–1058.
47. Harper JD, Lansbury PT Jr. Models of amyloid seeding in Alzheimer's disease and scrapie: mechanistic truths and physiological consequences of the time-dependent solubility of amyloid proteins. Annu Rev Biochem 1997; 66:385–407.
48. Masel J, Jansen VA, Nowak MA. Quantifying the kinetic parameters of prion replication. Biophys Chem 1999; 77:139–152.
49. Caughey B, Kocisko DA, Raymond GJ, Lansbury PT Jr. Aggregates of scrapie-associated prion protein induce the cell-free conversion of protease-sensitive prion protein to the protease-resistance state. Chem Biol 1995; 2:807–817.
50. Masel J, Jansen VA. The measured level of prion infectivity varies in a predictable way according to the aggregation state of the infectious agent. Biochim Biophys Acta 2001; 1535:164–173.
51. Caughey B, Raymond GJ, Kocisko DA, Lansbury PT Jr. Scrapie infectivity correlates with converting activity, protease resistance, and aggregation of scrapie-associated prion protein in guanidine denaturation studies. J Virol 1997; 71:4107–4110.
52. Prusiner SB, McKinley MP, Bowman KA, Bolton DC, Bendheim PE, Groth DF, Glenner GG. Scrapie prions aggregate to form amyloid-like birefringent rods. Cell 1983; 35:349–358.
53. Jeffrey M, Goodbrand IA, Goodsir CM. Pathology of the transmissible spongiform encephalopathies with special emphasis on ultrastructure. Micron 1995; 26:277–298.
54. Telling GC, Parchi P, DeArmond SJ, Cortelli P, Montagna P, Gabizon R, Mastrianni J, Lugaresi E, Gambetti P, Prusiner SB. Evidence for the conformation of the pathologic isoform of the prion protein enciphering and propagating prion diversity. Science 1996; 274:2079–2082.
55. Safar J, Wille H, Itri V, Groth D, Serban H, Torchia M, Cohen FE, Prusiner SB. Eight prion strains have PrP^{Sc} molecules with different conformations. Nature Med 1998; 4:1157–1165.
56. Caughey B, Raymond GJ, Bessen RA. Strain-dependent differences in β-sheet conformation of abnormal prion protein. J Biol Chem 1998; 273:32230–32235.
57. Caughey B, Horiuchi M, Demaimay R, Raymond GJ, Dickson DW. Assays of protease-resistant prion protein and its formation. Meth Enzymol 1999; 309:122–133.
58. Horiuchi M, Caughey B. Prion protein interconversions and the transmissible spongiform encephalopathies. Structure Fold Des 1999; 7:R231–R240.
59. Kocisko DA, Priola SA, Raymond GJ, Chesebro B, Lansbury PT Jr, Caughey

B. Species specificity in the cell-free conversion of prion protein to protease-resistant forms: a model for the scrapie species barrier. Proc Natl Acad Sci USA 1995; 92:3923–3927.
60. Chabry J, Caughey B, Chesebro B. Specific inhibition of in vitro formation of protease-resistant prion protein by synthetic peptides. J Biol Chem 1998; 273:13203–13207.
61. Caughey WS, Raymond LD, Horiuchi M, Caughey B. Inhibition of protease-resistant prion protein formation by porphyrins and phthalocyanines. Proc Natl Acad Sci USA 1998; 95:12117–12122.
62. Aguzzi A, Weissmann C. Prion research: the next frontier. Nature 1997; 389:795–798.
63. Kascsak RJ, Rubenstein R, Carp RI. Evidence for biological and structural diversity among scrapie strains. Curr Topics Microbiol Immunol 1991; 172:139–150.
64. Clarke AR, Jackson GS, Collinge J. The molecular biology of prion propagation. Phil Trans Roy Soc Lond Ser B: Biol Sci 2001; 356:185–95.
65. Hill AF, Joiner S, Linehan J, Desbruslais M, Lantos PL, Collinge J. Species-barrier-independent prion replication in apparently resistant species. Proc Natl Acad Sci USA 2000; 97:10248–10253.
66. DeArmond SJ, Prusiner SB. Transgenetics and neuropathology of prion diseases. Curr Topics Microbiol Immunol 1996; 207:125–146.
67. Asante EA, Collinge J. Transgenic studies of the influence of the PrP structure on TSE diseases. Adv Protein Chem, 2001; 57:273–311.
68. Kaneko K, Zulianello L, Scott M, Cooper CM, Wallace AC, James TL, Cohen FE, Prusiner SB. Evidence for protein X binding to a discontinuous epitope on the cellular prion protein during scrapie prionápropagation. Proc Natl Acad Sci USA 1997; 94:10069–10074.
69. Soto C. Protein misfolding and disease; protein refolding and therapy. FEBS Lett 2001; 498:204–207.
70. Head MW, Ironside JW. Inhibition of prion protein conversion: a therapeutic tool? Trends Microbiol 2000; 6:6–8.

8

The Enfer Transmissible Spongiform Encephalopathies Assay: Chemiluminescent ELISA of Prion Proteins

Riona Sayers
Enfer Scientific, Newbridge, Ireland

I. INTRODUCTION

Bovine spongiform encephalopathy (BSE), or "mad cow disease," is a neurological degenerative, incurable, and fatal disease. Always associated with this disease is the presence of an abnormal prion protein known as protease resistant protein^{Scrapie} (PrP^{sc}) in central nervous tissue (CNS) of the animal. BSE has been linked to an abhorrent disease of humans known as new-variant Creutzfeld-Jakob disease (nvCJD). The detection and identification of the etiological agent associated with BSE have led to the continuing investigation of one of the most fascinating molecular structures that science has encountered in many decades.

The prion protein, a not unusual protein, comprising approximately 250 amino acids found at least in all vertebrates, is believed to be the root cause of BSE and a related group of diseases known as transmissible spongiform encephalopathies (TSE), which includes CJD, kuru, scrapie, and chronic wasting disease among others. The cellular isoform of the prion (PrP^{c}) is a membrane-associated sialoglycoprotein bound to the external surface of cells by a glycosylinositol phospholipid with four hydrophobic sections, which fold in alpha-helical form (1,2). This is not alarmingly spectacular, at first glance, until one looks closer and finds that unlike any other protein ever detected, the prion protein, despite retaining an identical

primary sequence, can exist in two apparently stable forms (3), forms that function in two very different ways, one normal and one disease-causing, thus defying the basic rules of protein structures (4). A number of theories exist as to why this occurs but this is not the appropriate forum for such a discussion. What is an entirely appropriate discussion is why there exists an essential need to continue research into an agent that seems to disobey the basic rules of biology. Fundamental to prion research is the determination of the level of population infectivity and prion frequency that is found around the world. To do this there is a requirement for highly sensitive, rapid testing procedures that can allow accurate detection of the prion protein in its abnormal state. The advent of rapid testing procedures now presents the opportunity to the scientific community to rapidly identify large numbers of both clinical and preclinical cases of BSE. This will allow a significant amount of new knowledge to be gained about BSE, its distribution and modes of transmission. It will allow information on the physical and geographic distribution of BSE to be examined and improved statistical analysis of the incidence of BSE to be carried out. This information will provide a means of safeguarding the consumer from infected meat.

In this chapter, I will outline a brief history of prions and prion diseases and describe the requirement for and fulfillment of criteria for mass screening technology. This will allow better understanding of the distribution of prion diseases worldwide and the role that rapid screening technology has to play in better explaining the biology of prion diseases. The Enfer assay will be discussed in detail, including the principles on which it is based, and how it can contribute to our current knowledge of prion diseases. The later sections of the chapter will describe other areas of research where the Enfer assay has been employed, including its use in other species and varying tissue types. Finally, future prospects for research and development using Enfer technology will be outlined.

II. HISTORY

A. History of Transmissible Degenerate Encephalopathies (TDE)

BSE is one of a group of slow, fatal neurological diseases known as the transmissible degenerate encephalopathies (TDE) (2,5). CJD, Gerstmann-Straussler-Scheinker syndrome (GSS), and scrapie are other familial members of this group (6). The TDE diseases share long incubation periods, transmissibility to experimental hosts, absence of a detectable inflammatory or immunological response, and characteristic pathological changes (5,7,8). Although the molecular nature of the agent(s) that cause TDE has not been

conclusively determined (2), the popular belief at present is that this group of diseases is caused by either a proteinaceous particle, a "prion" (2,7,9), or a virus-like particle known as a "virino" (10). The prion model is more widely accepted than the virino model, although arguments are presented to support both theories.

The prion hypothesis argues that a proteinaceous particle, a prion (PrP^{sc}), is the infectious agent causing TDE and that on entry to the host, it results in a posttranslational conformational modification of PrP^{c} (its normal counterpart within the host) to PrP^{sc} by an unknown mechanism (2,11). The amino acid sequences of PrP^{sc} and PrP^{c} are identical leading to the conclusion that the difference between them is solely conformational (11,12). Strong evidence supporting the prion as the infectious agent is the inability of scrapie infection to replicate in mice in which the PrP gene has been ablated (13). This experiment suggests a highly significant role for the PrP protein in the development of TDE. The virino model also recognizes that PrP^{sc} may play a role in the development of the disease, but suggests that an additional, host-independent component, most likely a nucleic acid, is also present during the development of the disease and that this may trigger the change from PrP^{c} to PrP^{sc}. This additional component has never been identified for any TDE (14,15). It is, however, now widely accepted that a chaperon protein, Protein X, a host-dependent component, although not an inherent part of the prion protein, may play a significant role in assisting the transformation of normal prion proteins into the abnormal disease-causing form (2).

Both models of TDE infection support the fact that PrP^{sc} is a TDE-disease-specific protein and that PrP^{sc} is closely related to the host's normal protein PrP^{c}. They differ only in the components of the infectious agent. Is PrP^{sc} solely responsible for infection or do other agents play a role in the development of the disease? On the basis of existing information, PrP^{sc} can be defined as a marker protein for BSE and detecting its presence in infected tissue is of major importance in determining whether the animal is likely to succumb to BSE.

B. The Prion Protein—PrP

The unusual properties and inferred presence of a protein within the scrapie agent have led to the introduction of the term "prion" to describe and identify the group of infectious pathogens causing TDE (7,16). Though most of the data characterizing the biochemical properties of these infectious pathogens have been obtained from studies of the scrapie agent, the evidence indicates that these properties are common to the entire group of pathogens causing TDE (17). Early evidence for the presence of a protein component

within the scrapie agent was obtained by carrying out protease digestion experiments and reversible chemical modification procedures using diethyl pyrocarbonate (DEP), procedures that are destructive to proteins and resulted in inactivation of the scrapie agent (14,18,19). Further experiments followed and these identified a major protein that was both required for and inseparable from scrapie infectivity (20). This sialoglycoprotein (21) has been designated PrP27–30, protease resistant protein 27–30, and is so called owing to its unusual resistance to protease K digestion, under nondenaturing conditions, and its size microheterogeneity when run on SDS-polyacrylamide gels, showing an apparent relative molecular weight (M_r) of 27–30 kDa (19,22). All evidence suggested PrP27–30 to be a major component of the prion rather than a pathological product of the disease (17,23).

Further investigations were carried out to confirm PrP27–30 as the major component of the scrapie agent using molecular cloning (12,24,25). However, these experiments suggested that PrP27–30 was derived from a larger protein following protease K digestion during the prion purification process (20). The larger protein was predicted to have an apparent molecular weight of 33–35 kDa, and was therefore designated PrP33–35^{sc} (25,26). The existence of this larger protein was finally confirmed by a study that reacted antisera raised against a synthetic prion peptide with purified PrP27–30 and with crude scrapie-infected homogenates that should theoretically contain the predicted precursor protein. The antisera were found to react with both the purified prions and the crude homogenates, and protease K digestion of the homogenates resulted in the production of a protein with a molecular weight of 27–30 kDa that corresponded to PrP27–30. These observations provided solid evidence for the first time that the predicted precursor protein of PrP27–30, PrP33–35^{sc}, existed, and that PrP27–30 and PrP33–35^{sc} shared similar polypeptide epitopes.

This study also highlighted a link between PrP27–30, PrP33–35, and a normal cellular protein. The antisera raised to the synthetic PrP27–30 peptide (PrP-P1) reacted with PrP27–30, PrP33–35^{sc}, and a similar-sized protein isolated from both infected and normal brain tissue. This was named PrP33–35^{c} to distinguish it from PrP33–35^{sc} found only in scrapie-infected brain (20). This finding led to a line of thinking that proposed PrP33–35^{sc} as a posttransitionally modified form of the normal host gene product PrP33–35^{c}, and that this modified form was the agent causing scrapie. To investigate these claims further, a procedure was devised whereby the scrapie agent could be isolated without including the usual protease digestion step. The results of this experiment in scrapie-infected hamsters showed again that a larger protein, HaSp33–37 (PrP33–35^{sc}) was the major protein present in the fraction enriched with the scrapie agent. This protein reacted with antibodies raised to PrP27–30, and a protein identical to PrP27–30 was

produced by digesting HaSp33–37 with protease K. This confirmed a definite link between HaSp33–37, PrP27–30, and the scrapie agent. To confirm the presence of the normal PrP gene product, similar procedures, excluding the use of proteases, were carried out on normal brain tissue. This resulted in the purification of HaCp33–37 ($PrP33–35^{c}$), the predicted product of the host PrP gene in brain tissue (12,20,27). These experiments highlighted a characteristic difference between the normal and abnormal PrP gene products, i.e., that when treated with proteases, $HaSp33–37/PrP33–35^{sc}$ is only partly affected, producing HaSp29–32 on digestion with TCPK-trypsin and PrP27–30 on digestion with protease K with the products of this initial digestion being resistant to further degradation. $HaCp33–37/PrP33–35^{c}$, on the other hand, is not resistant to protease digestion and is fully degraded on digestion with protease K (20,25,27).

This combination of cloning, immunoblotting, and purification experiments has led to a well-supported hypothesis that $HaSp33–37/PrP33–35^{sc}$ is an abnormal form of $HaCp33–37/PrP33–35^{c}$ that is produced only in TSE-infected animals. Table 1 summarizes the findings of these experiments.

Thus, based on the scientific evidence, the theory that a larger form of PrP27–30 is the structural component of the scrapie agent and that this agent is a modified form of the host PrP gene product is well founded (28). The exact function of the host PrP gene product is still not known. However, it is suggested that it may play a role in copper metabolism or transport in the body (29).

Table 1 Comparison Between Normal and Disease-Causing Prion Proteins, PrP^{c} and PrP^{sc}

				Reaction with	
Description	Occur In	M_r (kDa)	Effect of protease digestion	Anti-PrP27–30	Anti-PrP
PrP27–30	Diseased CNS	27–30	Resistant	+	+
$PrP33–35^{sc}$	Diseased CNS	33–35	Converted to PrP27–30	+	+
$PrP33–35^{c}$	Diseased and normal CNS	33–35	Sensitive	+	+

Anti-PrP-P1: antisera raised to PrP.
Anti-PrP27–30: antisera raised to PrP27–30.
M_r: molecular weight.

C. Primary Sequence of the Prion Protein

The PrP gene has been cloned from a variety of species and there is a high degree of structural and organizational homology between the mammalian PrP sequences cloned to date (30). One approach to defining the nature of the differences between PrP^{c} and PrP^{sc}, thereby allowing diagnosis of prion diseases, has been to compare the PrP genes derived from a number of different mammalian species in an effort to define structurally important and evolutionarily conserved domains. However, the high degree of conservation between PrP genes from different species has frustrated this approach (31). Figure 1 outlines the primary sequences of the bovine (32), ovine (33), and rabbit (31) PrP proteins and also highlights the potentially useful amino acid polymorphisms that exist between these proteins. The rabbit is a species that has shown resistance to infection from PrP^{sc} (32) and, therefore, may be structurally distinct from PrPs of species susceptible to infection by prions. It is therefore the species of choice for the production of polyclonal anti-PrP

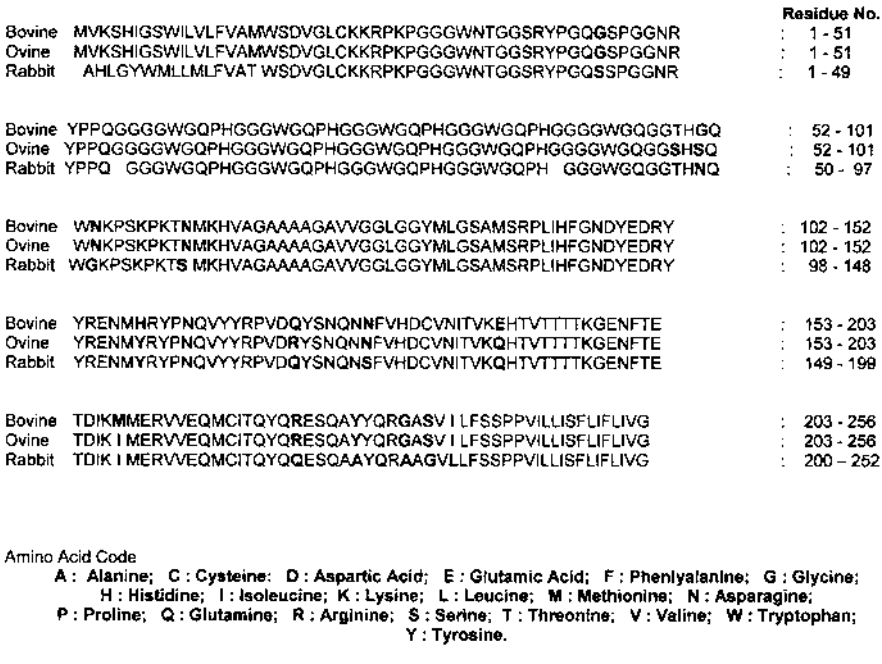


Figure 1 Bovine, ovine, and rabbit PrP prion protein sequences. Residues highlighted in bold are exploitable polymorphisms occurring between each sequence. (Ovine sequence adapted from Ref. 33. Bovine sequence adapted from Ref. 32. Rabbit sequence adapted from Ref. 31.)

antisera, and amino acid sequence comparison of rabbit PrP and ovine/bovine PrP can be used to predict species-specific and cross-reacting epitopes in ovine/bovine PrP protein (35). Identification of single amino acid differences between PrP sequences can therefore highlight potentially useful epitopes for use in TSE diagnosis (31). The importance of one such amino acid difference will become apparent later in this chapter (Sec. IV.A) where the synthetic peptide used to raise antibodies for use in the Enfer ELISA is described in further detail.

D. Prion Diseases—The Current Situation

Owing to the events of recent years involving release of information relating to BSE, there is now a widespread public mistrust of beef in both the European Union (EU) and many non-EU countries. The recent past has seen BSE become a worldwide problem, the first case of BSE outside of Europe being confirmed in Japan in August 2001. Such developments are having a serious negative impact on the beef industry in affected countries and the problem could spread to other major beef-producing nations.

This crisis in the beef industry was initially due to an announcement that there may be a link between BSE and a new, previously unrecognized, variant of CJD, designated nvCJD (37–39). Ten cases of nvCJD were reported in 1996 and all 10 patients showed first symptoms within the previous 3 years. The average age of infected individuals was 26 years with two patients in their late teens and one aged 42 years. The previously known form of CJD encountered up to this (sporadic CJD) is rarely found in individuals under the age of 40 (40). There are now 109 cases of nvCJD reported worldwide. Current public perception is that exposure to BSE-infected beef has resulted in these cases and therefore, the beef consumer in general is, now, afraid of contracting the disease, leading to a decline in beef sales and turmoil in the industry.

This crisis in the beef industry had led to widespread interest across the scientific community in developing rapid screening tests to detect BSE in beef cattle. Such a system would allow testing of large numbers of animals for the disease and help an eradication program to be implemented. Until the advent of rapid testing procedures, existing controls involved clinical examination of the afflicted animal followed by postmortem histopathological or immunohistochemical examination of a fixed section of brain tissue (41–43). Such procedures are still in use and are in fact used as the ultimate confirmation of a diseased animal. However, for the purpose of screening large numbers of animals for the presence of the BSE infectious agent, such procedures would not only be lengthy and costly but would prove highly impractical, indeed impossible, to implement in the high-

throughput meat industry that currently exists. A procedure such as enzyme-linked immunosorbent assay (ELISA) (44) provides the ideal solution to the high-throughput mass screening testing regime required, being a rapid and simple procedure to perform.

III. ANALYTICAL DETECTION OF PRP^{SC}

A. Screening Technology

Even though continued research into the prion protein, its origins, and mode of action is a necessity, one must also recognize that practical steps must be taken to control and prevent prion-associated diseases entering the human food chain. Implementation of a testing system that will allow mass screening of beef carcasses before entry to the human food chain is therefore imperative (2). To develop such a testing system, three testing criteria need to be fulfilled to develop and successfully implement a mass screening testing system for TSEs (45). (See Fig. 2.) These criteria are:

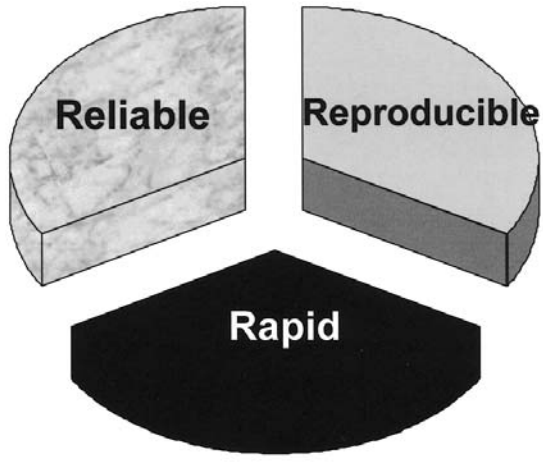
1. Rapidity (rapid)
2. Ability to process high-throughput of samples (reliable)
3. Accuracy/sensitivity and specificity (reproducible)

1. Rapidity

All testing systems available for BSE are postmortem procedures as the prion protein has, as yet, been consistently identified in detectable levels only in CNS tissue. Therefore, for the purpose of screening the beef population for the presence of BSE, all testing must be carried out after slaughter of the animal at a meat-processing plant. The nature of the slaughter process demands that results must be returned within 15 h of slaughter of the last animal on the line because the time required for a beef carcass to chill to 4°C (at which point the carcass can be further processed or shipped) is 15 h. It is essential to have results available before further processing of a carcass to allow full traceability should a suspect positive be identified. In practical terms, should the test procedure not be rapid enough, the slaughtering process will be brought to a standstill and the risk of carcasses being processed without clearance increases.

2. High Throughput

By its very nature, a screening system must be able to process large volumes of samples. The average throughput of a large European slaughter plant is 1000 animals per day. A successful screening system must have the ability to



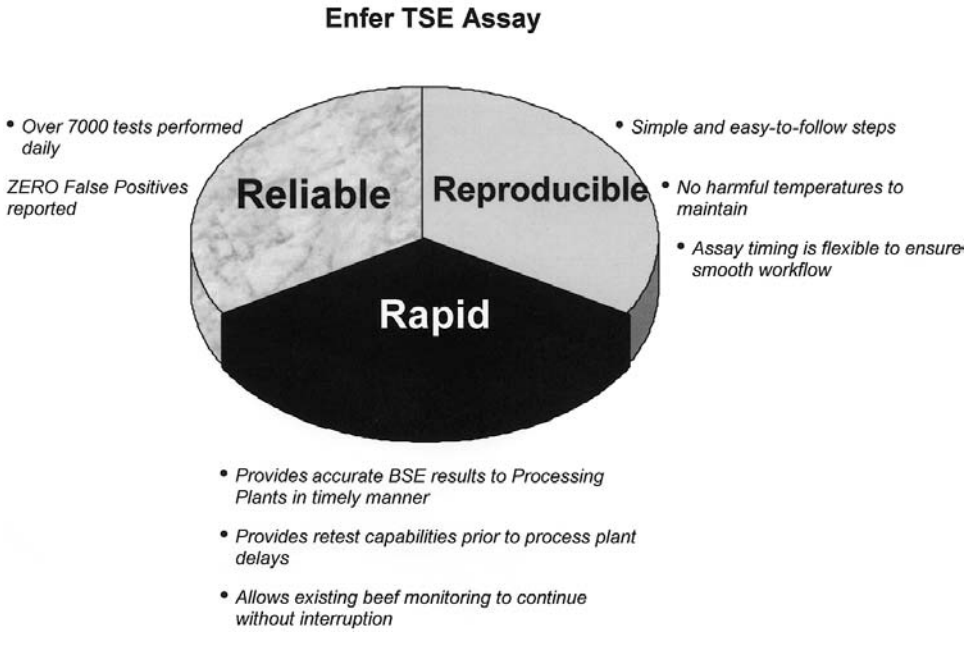


Figure 2 (Top) The three requirements of a successful mass-screening assay. (Bottom) The suitability of the Enfer TSE test as a mass-screening assay.

reliably cope with this level of sample throughput. In many cases, multiples of this volume must be processed if more than a single slaughter site is being serviced by a single laboratory.

3. Accuracy

The sensitivity and specificity of a screening system are the two most crucial aspects of a testing system that need to be considered. A balance must be sought to yield the maximum clinically relevant sensitivity while also maintaining specificity. False positives pose an economical threat from the point of view of consumer confidence and unnecessary animal destruction. More importantly, false negatives cannot be tolerated when one measures the risk to human life from an infected animal entering the food chain.

The success of any screening assay must be measured on its ability to merge these three criteria—rapid, reliable, reproducible—in the most effective way. One must not underestimate the necessity to maintain the discipline of combining all three criteria. A testing system cannot be judged on scientific capability alone but must also satisfy the logistical requirement of supporting the meat-processing industry. Failure to do so will lead to ultimate failure of the testing system in the field.

B. The Enfer TSE Assay—Effective as a Screening Assay?

If one wishes to examine the compliance of the Enfer assay with the criteria for successful screening (Fig. 2), it is best to study the experience of the Enfer assay in Ireland. Here, the Enfer system has been operating under field conditions since the beginning of 1998 and the Enfer laboratory in Ireland is currently the highest-throughput laboratory in the world, routinely testing 6,000–8,000 sample (high throughput) over a 12-h period between 6 P.M. the evening of slaughter and 6 A.M. the following morning (rapid). From January to November 2001, 105 positives were detected in samples from animals displaying no obvious clinical signs of disease. Of this number, 35 animals would have entered the food chain if testing had not been carried out. All suspect positives identified have been confirmed by histopathology, or in the case of autolyzed tissue, confirmation was carried out by immunohistochemistry. The Enfer assay currently operating throughout Europe has never reported a false-positive result. Of a random selection of negative and high-negative samples tested, none have proved positive, indicating that the Enfer assay is accurately and specifically detecting PrP^{sc} (accurate) (Table 2).

The test itself is an uncomplicated technique combining a series of noncritical steps and safe laboratory practices (Fig. 3). The Irish laboratory

Table 2 Field Experience of the Enfer TSE Assay

Date	Country	Total tested to nearest 1000	Total positive	For human consumption	Confirmed (yes/no)
2000–2001	Ireland	500,000	105	35	Yes
2000–2001	Northern Ireland	2,500	54	0	Yes
2001	Denmark	220,000	1	1	Yes
2001	Lithuania	20,000	0	0	—
2001	UK	Testing began November 2001—no data yet available			

The Enfer TSE assay is currently being used across Europe. Data are presented from a selection of testing sites using the Enfer TSE assay. It is important to note that 36 of the positive tissues were taken from animals due to enter the human food chain. These animals were preclincial specimens having passed veterinary inspection at the slaughter plant. Such data highlight the importance of implementing testing regimes for reasons of public health and safety.

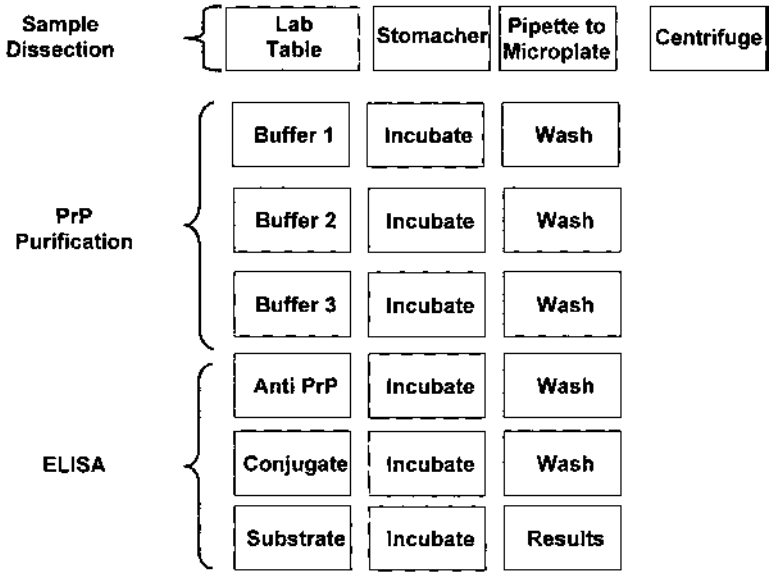


Figure 3 Workflow analysis displaying the simplicity of the Enfer TSE test. The Enfer assay is a simple assay combining a sample preparation method with repeated cycles of buffer addition, incubation, and washing. This procedural simplicity is an essential factor contributing to the robustness of the Enfer assay.

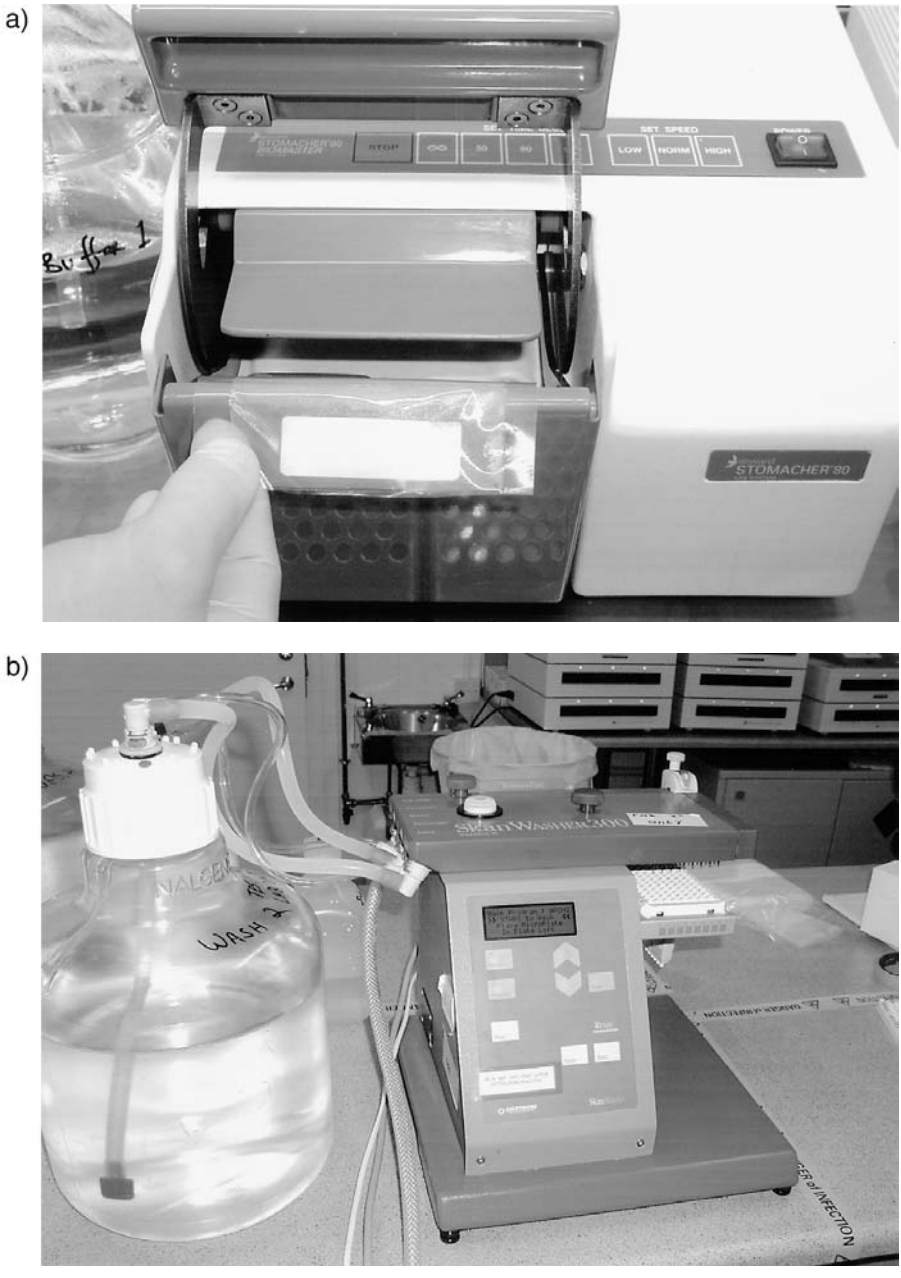


Figure 4 Equipment used for completion of the Enfer assay. (a) Stomacher homogenizer. (b) Ninety-six-well microplate washer. (c) Microplate shaker incubator. (d) Chemiluminometer.

c)

d)

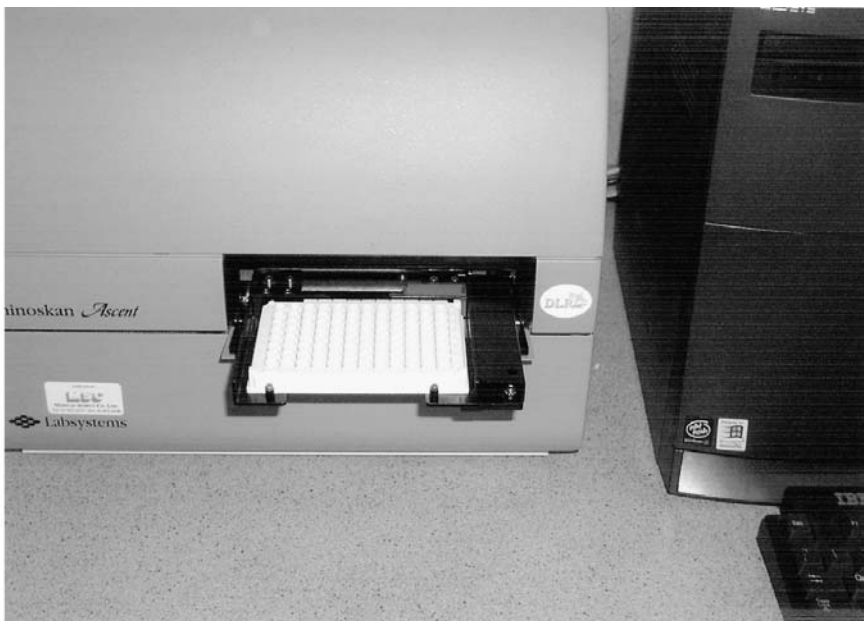


Figure 4 (continued)

began as a pilot scale unit capable of testing 1000 samples over a 24-h period. From this basic unit the Enfer system has expanded to allow multiples of this number to be tested on a nightly basis. The basic Enfer consists of:

Sample homogenizer: Stomacher homogenizers are currently being used to avoid the generation of aerosols and also to prevent cross-contamination. All sample dissection and homogenization disposables are inexpensive and single use (Fig. 4a).

Microplate washer: Ninety-six-head microplate washers are being used at all stages of the Enfer process and are probably the most important component of this ELISA, as becomes apparent when the assay methodology and assay principle are examined. Two washers are ideally required to effectively run the Enfer assay, one washer to be strictly dedicated to the removal of Enfer Buffer 1 from the testing system once it has completed its task. Carryover of Enfer Buffer 1 downstream in the testing procedure will lead to reduced proteinase K activity, resulting in poor duplication of results due to incomplete digestion of the PrP^c present in the samples (Fig. 4b).

Incubator: The incubators used in the Enfer assay are also of critical importance as these items of equipment must have the capability to change temperature and shake speed to reach optimal sensitivity (Fig. 4c).

Chemiluminometer: It is of utmost importance that the chemiluminometer used is validated and standardized using validation-light-emitting microplates. This step is essential in maintaining a standard positive cutoff point (Fig. 4d).

Depending on the volume of samples to be tested, a laboratory will require only some additional equipment and personnel over and above those listed above to scale up to the required level of testing, as seen with the Irish experience (Table 3, Fig. 5a,b). The system also lends itself to complete automation, with a fully automated system currently being developed in conjunction with Abbott Laboratories, which will offer an even higher level of assay reproducibility. The versatility of the Enfer system therefore allows ease of use whether testing 100 samples or 8000 samples on a daily basis.

C. Overview of Test Method

The technology involved in the test can be divided into four main categories.

1. Taking of a Sample of CNS Tissue

The required sample tissue is taken from the carcass immediately after slaughter. It is a matter of end-user choice as to which part of the tissue

Table 3 Equipment Requirements to Run Varying Volumes of Samples per Annum Using the Enfer Test

Equipment	5,000–20,000/yr	21,000–50,000/yr	51,000–75,000/yr	76,000–150,000/yr
Bottle top dispenser	1	1–2	2	3
Stomacher	2	3	4	6
Balance	1	1–2	2	2
Washer	2	2	2	3
Pipettes	1 set	2 sets	2 sets	3 sets
Centrifuge	1	1	1	1–2
Barcoder	1	1	1	1
Incubator/shaker	1	1	2	3
PC	1	1	1	2
Chemiluminometer	1	1	1	1
Staff	2	4	5	8

The Enfer system is highly adaptable and can as easily be used to test 100 as 1000 samples per day.

sample is used for screening of carcasses. In this case, obex or cervical spinal cord has been selected as the target matrix for this assay (Fig. 6). These tissue have been selected as the matrix of choice for two reasons:

These are tissues in which PrP^{sc} occurs consistently at high levels (46).
It is practical to take sample of obex and cervical spinal cord, rapidly, at an abattoir and sample collection may be carried out without the risk of sample cross-contamination.

The Enfer assay has been validated for use on both obex and cervical spinal tissue (47) and has also been tried (though not extensively) on a number of differing CNS tissue types. Evidence of differing sensitivities with different tissue samples is presented in Table 4 where a number of different CNS tissue types were investigated for detectable levels of PrP^{sc}. It is felt that the differing sensitivities are mainly due to the differing distribution of PrP^{sc} through these tissues. All tissues, except cerebral cortex (variable readings expected owing to distribution pattern of PrP^{sc} in this tissue), yielded excellent results. Theoretically, therefore, any of these tissues could be used for PrP^{sc} detection, but difficulties in sample taking and dissection effectively rule out many of them as a matrix for a rapid screening test in the slaughter plant. It must also be stated that the test matrix must be chosen on the basis of basis of early, rapid, and practical detection of PrP^{sc}, rather than on the basis of assay sensitivity with tissue type. The general consensus in the EU at present is that cervical spinal tissue should be used for mass screening, will

Figure 5 (Top) A basic Enfer system capable of testing 1000 samples/day. (Bottom) The highest sample throughput laboratory in the world, capable of testing over 8000 samples/12 h.

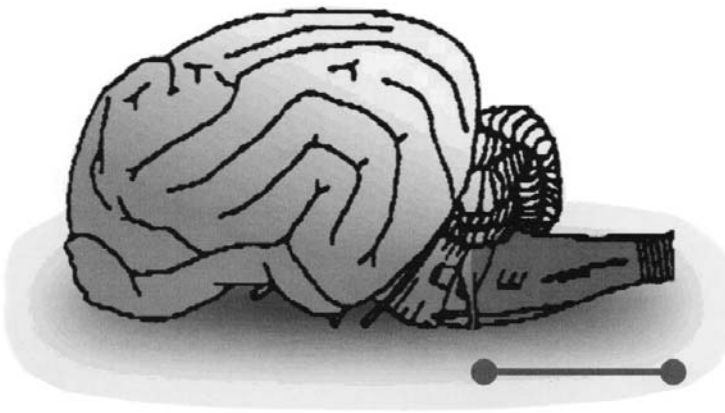


Figure 6 Brain stem: optimal sampling area for use in rapid and confirmatory tests for TSE.

Table 4 Use of Enfer Assay on a Number of Different Bovine CNS Tissues

Sample no.	Case no.	Tissue (CNS)	Replicate 1 (LU)	Replicate 2 (LU)	Replicate 3 (LU)	Mean value (LU)
1	1	Cord	1254	857	1546	1219
2	1	Medulla	2670	2541	2157	2456
3	1	Midbrain	2870	3324	3774	3323
4	1	Cerebellum	851	795	824	823
5	1	Thalamus	3426	3681	4004	3704
6	1	Anterior cortex	320	337	328	328
7	1	Posterior cortex	149		157	153
8	1	Basal ganglia	1363	1293	1132	1262
9	2	Cord	465	345	343	384
10	2	Medulla	1536	1438	1255	1410
11	2	Midbrain	3317	3540	3399	3419
12	2	Cerebellum	551	670	688	636
13	2	Thalamus	1710	1701	1883	1765
14	2	Anterior cortex	152	159	159	157
15	2	Posterior cortex	10	9	9	9
16	2	Basal ganglia	505	412	347	421

Sixteen CNS tissue samples, spinal cord, medulla, midbrain, cerebellum, thalamus, anterior cortex, posterior cortex, basal ganglia, from two confirmed clinical BSE cases were examined using the standard Enfer TSE assay protocol. All tissues yielded positive results (>5.5 LU) with anterior and posterior cortex producing the least optimal results.

leave the obex to be maintained intact for confirmatory testing of suspect positives. Others believe, however, that a sample of the obex should be used as it is felt that this is the region of the hindbrain that will yield most sensitive results. It has been demonstrated, however, that in material that has undergone only minor postmortem delay before being examined, the cervical cord was no less sensitive than other regions of the brain previously shown to be most sensitive for prion detection (48). In the end, the final choice is entirely a decision for the end user and the regulatory authorities of a particular region. It is imperative, however, that the test be validated for use on both regions of the brain and also that the sample is removed from the carcass in such a way that it is also suitable for use in a confirmatory analysis using the recognized confirmatory methods, histopathology, immunohistochemistry, and the OIE western blot. When suitable samples are received by the laboratory, they are dissected, homogenized, and are ready to enter the Enfer immunoassay.

2. Preparation of the Sample

The sample preparation is a unique method devised by Enfer Laboratories and is the essential component contributing to the success of the test. It is the only sample preparation technique in existence that will allow under 4-h testing of a sample for PrP^{sc} (Fig. 7). The sample preparation system

	Assay Step	Time
•	Plate setup (CP) – add controls & samples	10 min/45 samples
•	Centrifugation	5 min/plate
•	Add EB2 to well & transfer samples from CP	5 min/plate
•	Incubate @ 34°C, shake speed 5	60 min
•	Wash 1	
•	Add Enfer buffer 3, incubate @ 34°C, shake speed 5	15 min
•	Wash 2	
•	Insert peptide positive control wells	
•	Primary antibody, incubate @ 34°C, shake speed 5	40 min
•	Wash 2	
•	Add secondary antibody, incubate @ 34°C, shake speed 5	30 min
•	Wash 2	
•	Add substrate, incubate @ 34°C, shake speed 5	10 min
•	Read results, software data reduction, and calculation - print out	2 min

Figure 7 Major steps in the Enfer assay and processing times for each step.

incorporated in the Enfer assay involves sample treatment with a series of three buffers, Enfer Buffer 1, Enfer Buffer 2, and Enfer Buffer 3. Each buffer plays a dual role in solubilization and isolation of PrP^{sc}, a primary role related to the solubilization and isolation of PrP^{sc} and a secondary role in confirming a high degree of specificity on the Enfer assay (Fig. 8). The roles of each of the Enfer buffers is discussed in greater detail in Sect. III.D.

3. Immunoassay (Testing) of the Prepared Sample

The sample is tested by immunoassay, i.e., a test involving the use of antibodies to target the abnormal BSE prion protein (Fig. 9).

4. Detection of Results

The detection of the abnormal protein is carried out using enhanced chemiluminescence techniques.

The test, as it stands, can analyze 45 samples in approximately 3.5 h including system setup, with subsequent runs of 45 sample being available every 20 min after that, making it the quickest means of prion detection available at the present time. A step-by-step assay protocol is included in Fig. 10 with a breakdown of the assay workflow in Fig. 11.

D. Assay Principle

As mentioned previously, the Enfer assay sample preparation technique is based on a triad buffer system: sequential treatment of the sample with Enfer Buffer 1, Enfer Buffer 2, and Enfer Buffer 3. Each of these buffers has a very specific and vital role in the isolatiion of PrP^{sc}.

1. Enfer Buffer 1

This buffer is used to homogenize the tissue sample. It contains a set of reagents with the unique ability to solubilize the high level of lipids contained in CNS tissue and the prion proteins in the tissue sample. This homogenization step therefore liberates the prions present in the tissue sample allowing access to the prions for the next processing step with Enfer Buffer 2. This buffer also plays a secondary role by inactivating any protease inhibitors present in the sample (owing to bacterial contamination of the sample) as is often the case particularly in autolyzed tissue. This is a highly important consideration when the objective of the test system is to produce a 0% occurrence of false positives.

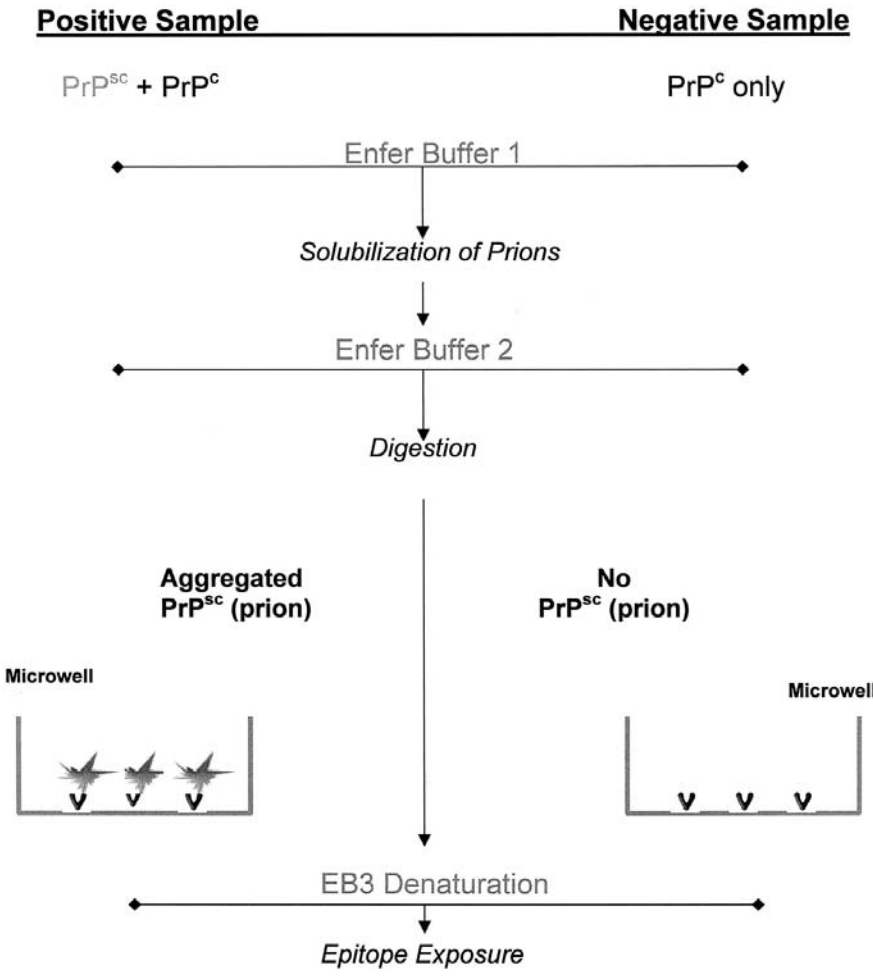


Figure 8 Enfer sample preparation principle that allows specific isolation of PrP^{sc}. Enfer Buffer 1 acts to solubilize the prion protein; Enfer Buffer 2 digests all PrP^{c} present in the tissue sample and results in aggregation and capture of the abnormal PrP^{sc} to the microplate well; Enfer Buffer 3 denatures the captured PrP^{sc} thereby exposing the epitope required for antibody recognition.

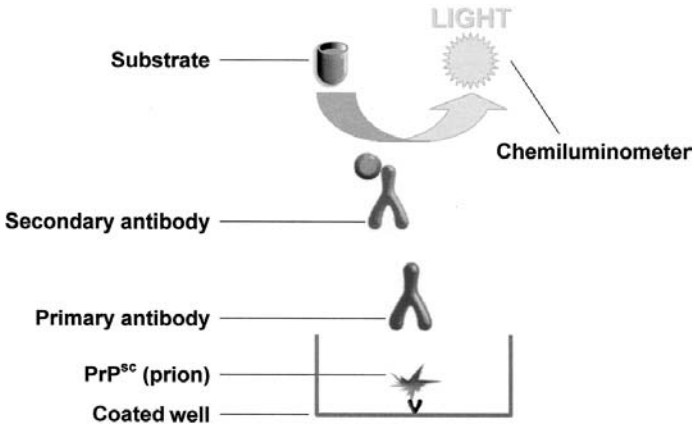


Figure 9 Enfer ELISA technique. The Enfer ELISA is a standard antibody-antigen recognition technique, rabbit anti-PrP being utilized to bind to prion aggregates. Goat anti-rabbit-HRP is used for signal generation in conjunction with an enhanced chemiluminescent reagent.

2. Enfer Buffer 2

Enfer Buffer 2 contains proteinase K. When one returns to the action of proteinase K in relation to prions, it is this step that is most critical in the isolation of PrP^{sc} from the normal prion protein PrP^{c}. An effective proteinase K digestion step will act to completely destroy the PrP^{c} present in the sample thereby isolating PrP^{sc} for detection by the Enfer ELISA. Again this buffer plays a dual role , as it also processes PrP^{c} into a form that will allow aggregation of the abnormal prion protein now existing as the PrP27–30 form. This PrP27–30 aggregation must occur to allow capture of the abnormal prion aggregates onto the Enfer test microplate. Only PrP27–30 has the ability to form these prion aggregates, a factor that contributes greatly to the specificity of the Enfer assay. The assay is essentially isolating the prion using an inherent aggregation characteristic of the prion. No other protein will be present in a form suitable for capture onto the Enfer test plate, hence the high degree of specificity of the Enfer assay.

3. Enfer Buffer 3

The final Enfer buffer in the series, Enfer Buffer 3, is a denaturing solution. It acts to denature PrP27–30, resulting in epitope exposure to allow

Assay Protocol

Sample Preparation

1. Cut a slice of CNS tissue weighing approximately 0.5–1.0 g or 1.5–2.0 g in the case of autolyzed tissue.
2. Place the tissue into a labeled stomacher bag and weigh sample.
3. Add the relevant quantity of Enfer Buffer 1 to the stomacher bag at a ratio of 1:15 w/v.
4. Homogenize the sample for 2 min in a stomacher.
5. Sample Preparation is complete.

Note : Do not store diluted samples, use on the same day.

Immunoassay Procedure

All reagents should reach room temperature and be crystal free prior to commencement of assay. Where crystals are present, incubation of buffer at 37°C for a short period is recommended.

1. Dispense 170 µl of negative control, blank, and each sample in duplicate onto a centrifuge plate except A1, A2. (See Plate Layout).
2. Cover the plate with a plate sealer.
3. Centrifuge the plate at 2500 g for 5 min.
4. Add 20 µl of Enfer Buffer 2 to the Enfer-coated plate except A1, A2.
5. Transfer 100 µl of each centrifuged sample to the corresponding position on the Enfer-coated plate containing Enfer Buffer 2.
6. Cover the plate with a plate sealer.
7. Incubate the plate for 60 min at 34°C. Wash the plate using Enfer Wash 1 solution.
8. Tap the plate after washing to remove any remaining liquid.
9. Add 150 µl of Enfer Buffer 3 to the plate.
10. Cover the plate with a plate sealer.
11. Incubate the plate for 15 min at 34°C.
12. Wash the plate using Enfer Wash 2 solution.
13. Tap the plate after washing to remove any remaining liquid.
14. Place 2 positive control wells into positions A1 and A2 of the Enfer-coated plate.
15. Dispense 150 µl of 1°Ab solution into each well.
16. Cover the plate with a plate sealer.
17. Incubate the plate at 34°C for 40 min.
18. Wash the plate using Enfer Wash 2 solution.
19. Tap the plate to remove any remaining liquid.
20. Dispense 150 µl of 2°Ab onto the plate.
21. Cover the plate with a plate sealer.
22. Incubate the plate at 34°C for 30 min.
23. Wash the plate using Enfer Wash 2 solution.
24. Tap the plate to remove any remaining liquid.
25. Add 150 µl of substrate solution to the plate.
26. Incubate for 10 min.
27. Read the light signal using a chemiluminometer.

Following these recommendations allows 45 samples to be assayed in duplicate per EIA microtiter plate in approximately 3.5 h.

Figure 10 Enfer assay protocol.

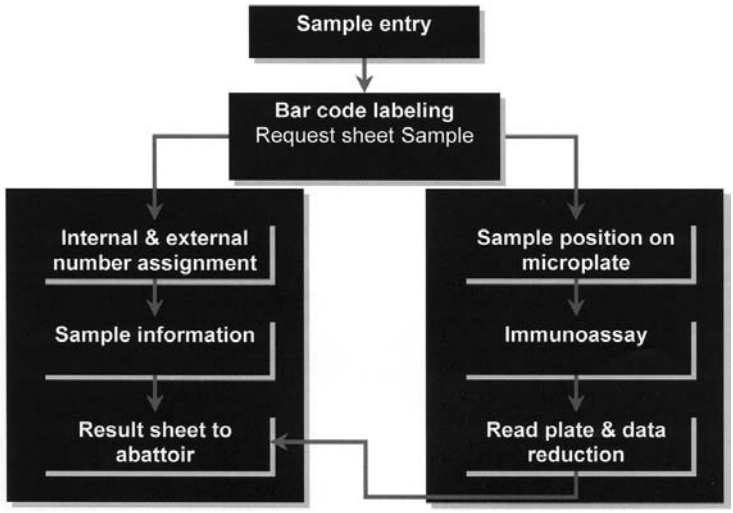


Figure 11 Workflow of a typical Enfer TSE testing laboratory.

binding anti-PrP. This buffer also assists in maintaining test specificity by acting as a protease inhibitor. Should the washing procedure after the proteinase K digestion step not act to remove all of the proteinase K from the microwell, Enfer Buffer 3 will act to destroy any residual protease activity. This is of enormous importance when one takes into account that continued proteinase K activity will eventually begin to destroy the abnormal prion protein. Continued proteinase K activity could therefore have the potential to create a false-negative result by destroying PrP27–30, should the prion be present in very low levels in the sample, for example. Complete removal or inactivation of proteinase K is essential and Enfer Buffer 3 will act to guarantee this.

Sequential treatment with Enfer Buffers 1, 2, and 3 therefore acts to selectively and specifically isolate PrP27–30 and allow downstream ELISA detection using a prion-specific antibody and chemiluminescent reagent (Figs. 8 and 9).

E. Assay Performance and Evaluation Work

1. Enfer Studies

Internal studies have shown the Enfer ELISA to be an excellent performer with examinations of inter- and intra-assay variations yielding

Table 5 Interassay Variation Data Generated on a Single Positive and Negative Tissue Control Sample and a Single Positive Peptide Control Sample, in 20 Assays over 4 Consecutive Days

Assay number	Blank	Negative tissue control	Positive tissue control	Peptide control
1	3.1	0.5	1089	5027
2	3.1	0.5	1282	5526
3	1.7	0.4	1047	5262
4	1.8	0.9	965	5417
5	2.2	0.7	1045	5215
6	1.9	0.5	1009	5032
7	1.7	0.6	893	5061
8	2.1	0.4	1072	5046
9	2.4	0.5	963	5704
10	2.3	0.5	1064	5319
11	2.3	0.6	916	6206
12	1.0	0.4	1374	5537
13	0.7	0.4	982	7306
14	0.9	0.4	1172	6517
15	1.3	0.8	1295	5718
16	1.4	0.5	1240	5524
17	1.2	0.7	1087	5554
18	0.5	0.4	1330	5671
19	0.7	0.5	1090	5046
20	1.1	0.9	1079	5309
	Blank	Negative tissue control	Positive tissue control	Peptide
Mean	1.6	0.6	1099	5550
Standard deviation	0.8	0.2	160	568
Coefficient of variation (%)			14.5	10
n	40	80	80	40

All values are quoted in chemiluminescence light units (LU).

excellent results. Interassay variation data have been generated on a positive tissue control and a negative tissue control in a total of 10 assays (Table 5). These controls have been deemed positive and negative by two unrelated methods, histology (HIS) and immunohistochemistry (ICC), methods that will ultimately be used to confirm results reported using the Enfer test. The interassay variation based on the positive control was

calculated at 14.5%. An additional synthetic peptide control was also included in this study, again showing a very acceptable interassay variation of 10%. Intra-assay variation has also been studied and was determined using the same range of controls as those used for the interassay study (positive, negative, and peptide). A total of 23 replicates of each control were placed on a single plate. Data generated on these controls are provided in Table 6, yielding 9% variation for the positive control and 11% for the peptide control.

A number of stability studies on kit components have also been carried out, but perhaps the most one is the examination of the homogenate stability in Enfer Buffer 1. This study is carried out yearly and the data generated from the most recent study (August 2001) are presented in Table 7. Should an Enfer Buffer 1 homogenate be allowed to stand overnight at an incorrect storage temperature, as can be seen from the data presented in Table 7, the amount of detectable PrP^{sc} will be dramatically reduced and may yield a misleading result. For the purpose of this examination, a positive and negative sample were homogenized in Enfer Buffer 1 and divided into aliquots. Over a 7-day period, the homogenates were tested using aliquots stored at −20°C, 2–8°C, room temperature (16–25°C), and 37°C. The result show that the positive homogenate is not stable overnight at 2–8°C, room temperature (16–25°C), or 37°C. Only slight deterioration of the homogenate is recorded after 1 day at −20°C. It must be noted, however, that this study has been carried out on a strongly positive sample for the purpose of examining the prion deterioration curve over time and yielded a light signal of over 2000 LU on initial testing. Should a weakly positive be subjected to similar treatment, the level of detectable PrP^{sc} would decrease over a shorter time period. Homogenates should therefore be tested within 3 h of production to avoid ambiguous results. As an aside to this, when a positive sample is retested, it is imperative to always recut the sample and test the new homogenate as well as retesting the original homogenate.

In terms of specificity and sensitivity, all 485 suspect positive samples flagged by the Enfer asssay to date, both evaluation (300) and field samples (185), have been confirmed by either hispathology, immunohistochemistry, or Western blot, yielding a sensitivity of 100%. In a study of 5632 negative data points in representing 2816 negative samples, only two samples yielded inconclusive results, with one replicate having a light signal above cutoff (5.5 LU) and the second, a signal below cutoff, yielding an inconclusive rate of 0.037%. On repeat testing of these two samples, both samples replicates had negative results (Fig. 12), yielding a repeat reactive rate of 0.0% and a specificity of 100%.

Table 6 Intra-assay Variation Data Generated on a Single Positive and Negative Tissue Control Sample and a Single Positive Peptide Control Sample, 23 Replicates of Each on a Single Plate

Replicate number	Blank	Negative control	Positive control	Peptide control
1	1.7	1.2	993	4832
2	2.5	0.6	887	4799
3	1.1	0.7	918	5601
4	1.5	0.4	982	6099
5	1.9	0.6	949	5954
6	2.6	0.1	991	5919
7	1.7	0.5	823	5185
8	1.9	0.4	913	5329
9	1.2	0.5	992	5957
10	1.6	0.5	1140	5069
11	1.3	0.2	1150	4427
12	0.9	0.7	1108	4981
13	2.9	0.4	1094	4990
14	1.6	0.6	1086	4635
15	1.3	0.8	1053	5882
16	2.9	0.1	926	4118
17	0.9	0.2	982	4422
18	2.3	0.4	1124	4887
19	0.8	0.6	1080	4614
20	1.0	0.2	1124	5774
21	0.7	0.2	1083	5912
22	1.7	0.4	1006	4879
23	0.9	0.5	1000	4686
	Blank	Negative tissue control	Positive tissue control	Peptide
Mean	1.6	0.5	1018	5172
Standard deviation	0.7	0.3	90	597
Coefficient of variation (%)			9	11
n	23	23	23	23

All values are quoted in chemiluminescence light units (LU).

Table 7 Enfer Buffer 1 Homogenate Stability Study Where an Enfer Buffer 1 Homogenate Has Been Stored at Four Different Temperatures, −20°C, 2–8°C, RT, and 37°C

Positive control					Negative control				
Day	−20°C	2–8°C	RT	37°C	Day	−20°C	2–8°C	RT	37°C
0			2319		0			1.2	
1	2193	1327	1257	428	1	1.3	1.9	1.4	1.3
3	1927	1729	297	96	3	1.7	1.5	1.1	1.8
5	1905	1238	153	14	5	1.1	1.2	2.6	2.5
7	1739	973	42	12	7	1.7	1.3	1.3	2.3
30	902	73	39		30	0.9	0.8	3.1	
90	532	25	28		90	1.7	2.4	2.7	
180	90	32	21		180	2.1	1.8	2.5	

The mean value at each data point is represented by three replicates. Enfer Buffer 1 homogenates are stable for short periods (1–7 days) at −20°C only. This study relates to a single BSE-positive spinal tissue sample. Results may vary with differing sample type and tissue.

2. EU Evaluation of Enfer Assay

Internal assay performance examinations should be carried out continuously but caution should be applied when interpreting results. It is always essential to evaluate a testing system independently. To this end, in 1998, the European Union (EU) (then DGXXIV, now renamed DG SANKO) issued a request for all TSE scientific researchers to submit BSE test methods to the Commission for the purpose of scientific evaluation to assess their potential use in the field of control of BSE. A total of 10 test methods were submitted, and four were eventually chosen to proceed for evaluation. Test methods were chosen on the basis of existing validation work, experience in the field, and suitability of the test procedure for rapid screening. Examinations on the Enfer ELISA have shown it to have acceptable inter- and intra-assay variations (Tables 5 and 6). Enfer had been testing commercial samples in Ireland since January 1998 after successful completion of an evaluation protocol for the Irish Department of Agriculture, Food, and Rural Development, Veterinary Research Laboratories. This evaluation involved testing of some 30 histologically confirmed positive BSE samples from varying CNS tissues (mainly spinal tissue) and over 500 histologically confirmed negative samples. All samples were identified correctly by the Enfer asssay. On the basis of this track record, the Enfer ELISA based assay was chosen to partake in the EU Evaluation along with Prionics, Switzerland, Western blot procedure;

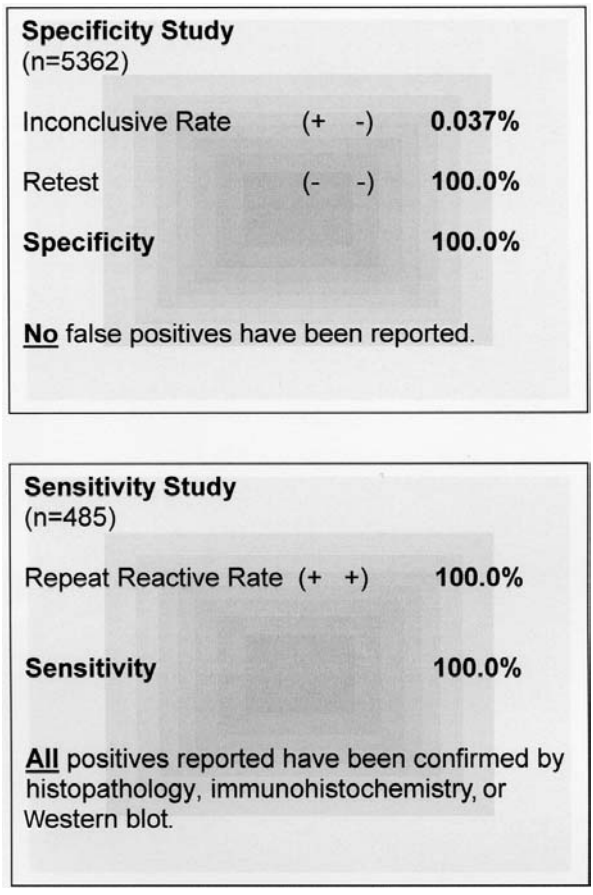


Figure 12 Enfer assay performance; sensitivity and specificty of the Enfer assay. Based on field experience and evaluation work carried out on the Enfer assay, the assay has displayed 100% sensitivity and 100% specificity in the detection of PrP^{sc} in CNS tissue samples.

CEA, France, Sandwich, ELISA-based procedure; and, Wallac, UK, ELISA-based procedure. All four testing sites received 1560 samples, 300 positive tissue samples, 960 negative tissue samples, and 300 homogenized, diluted positive samples. The results of this evaluation for the Enfer test are presented in Table 8 (47,49) and agree well with Enfer's findings in terms of test specificity and sensitivity.

Table 8 Performance of the Enfer Assay in Evaluation of Tests for the Diagnosis of TSE in Bovines[a]

	True positive	True negative	Total	%	95% confidence limit one-sided poisson
Tested positive	300	0	300	100%	99.0%
Tested negative	0	997	997	100%	99.7%
Total	300	997	1297		

[a] Ref. 47.

3. Quantitative Versus Qualitative

No limit of detection (LOD) or limit of quantification (LOQ) can be stated for the Enfer assay, as PrP^{sc} exists as an aggregated protein making it difficult to develop official PrP^{sc} reference standards to determine such limits. This test therefore, similar to all other available assays, is a qualitative procedure, yielding positive/negative results rather than concentrations of PrP^{sc}. Attempts have been made to produce a panel of QA samples that could, for example, be used to compare the specificities and sensitivities of different testing systems. However, a number of difficulties have been encountered in this regard. As there is unequal distribution of PrP^{sc} even in adjacent tissue sections from the same sample, the only means of standardizing a sample is to homogenize it. Prions however, maybe not surprisingly, present a unique situation where it has proved, as yet, impossible to produce a panel of standard samples for such studies. The main reasons for this trace back again to the unusual properties of the prion protein. All testing systems currently approved for use operate by means of three very different prion isolation principles, one using Western blot (Prionics), the second (BioRad) using a Sandwich ELISA, and the third (Enfer) using a prion aggregation technique coupled with an ELISA. The difficulties arise when one tries to produce a homogenate to suit all three assays for the purpose of comparison. Certain homogenizing buffers may not, for example, suit a particular testing system, whereas it has recently been recorded that particular homogenization techniques are not suitable for the Enfer assays (unpublished data, Enfer Scientific). Experiences in a number of European laboratories led to the examination of homogenization effects on the sensitivity of the Enfer ELISA with initial studies suggesting that vigorous homogenization of a tissue sample in the absence of a buffer will destroy the sensitivity of the assay. The reasons for this appear to be strictly physical with the aggregation properties of the prion being affected

by the high temperatures and shear stress generated by vigorous homogenization. Use of a buffer does seem to reduce these effects but sensitivity remains greatly affected. Some initial data are presented in Table 9 and Fig. 13; samples were dissected bilaterally, with one-half retained as a tissue sample (T) and the second homogenized (H) using the procedure outlined by the EU (47). These samples were supplied to Enfer by the EU for the specific purpose of examination of potential "homogenization effects" in relation to different assay procedures. On the basis of the results produced and the clear homogenization effects on the Enfer assay, studies are ongoing to determine the ideal homogenate preparation buffer for use with the Enfer assay. The exact reasons why certain homogenization procedures lead to decreased

Table 9 Investigation of "Homogenization Effects" on Positive Tissue Samples When Using the Enfer Assay

Sample ID	Enfer ID	Reading (LU), Version 1	Reading (LU), Version 2		Result	Sample type
1T	501	118	261	(n = 6)	Pos	Tissue
1H	502	3	4	(n = 6)	Neg	Homog
2T	503	186	624	(n = 6)	Pos	Tissue
2H	504	75	226	(n = 6)	Pos	Homog
3T	505	462	969	(n = 6)	Pos	Tissue
3H	506	52	213	(n = 6)	Pos	Homog
4T	507	903	1785	(n = 6)	Pos	Tissue
4H	508	84	257	(n = 6)	Pos	Homog
5T	509	736	1449	(n = 6)	Pos	Tissue
5H	510	321	658	(n = 6)	Pos	Homog
6T	511	284	639	(n = 6)	Pos	Tissue
6H	512	14	34	(n = 6)	Pos	Homog
7T	513	348	826	(n = 6)	Pos	Tissue
7H	514	307	579	(n = 6)	Pos	Homog
8T	515	898	1697	(n = 6)	Pos	Tissue
8H	516	168	262	(n = 6)	Pos	Homog
9T	517	449	843	(n = 6)	Pos	Tissue
9H	518	9	20	(n = 6)	Pos	Homog
10T	519	120	187	(n = 6)	Pos	Tissue
10H	520	7	15	(n = 6)	Pos	Homog

Ten confirmed positive samples were dissected bilaterally, one-half prehomogenized (H), the other maintained as a tissue sample (T). These 20 samples (501–520) were tested using Version 1.0 and Version 2.0 of Enfer assay. Both protocols show severe "homogenization effects," up to 10-fold reductions in light signal being recorded on both versions of the assay, and a single sample yielding a negative result on prehomogenization. Prehomogenized samples must not be used as a sample matrix for the Enfer assay.

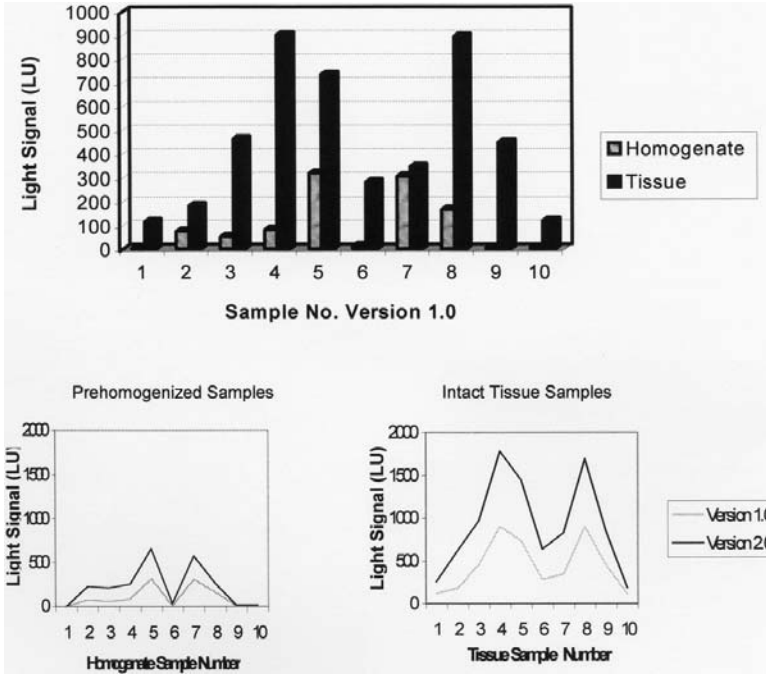


Figure 13 Effect of prehomogenization of tissue before use in the Enfer TSE assay. Plot of paired tissue/homogenate samples showing clear "homogenization effects" in samples prepared using a homogenization procedure identical to that used for the 1999 EU evaluation dilution study. For each of 10 samples, one-half of the sample was prehomogenized (▒) and the second half was maintained as an intact tissue sample (■) before being tested with the Enfer assay. Results are shown as vertical bars proportional to the chemiluminescent light signal generated against sample number, both prehomogenized and intact. Up to a 10-fold drop in signal can be seen in prehomogenized samples. Plots examining the increase in sensitivity in samples using the increased sensitivity Version 2.0 (solid line) of the Enfer TSE assay are also included. Version 1.0 (dotted line) of the assay across all 10 samples, prehomogenized and intact, produces consistently lower light signals than Version 2.0. Homogenization effects are, however, still present even with increased assay sensitivity.

sensitivity using the Enfer assay are as yet unknown and continue to be studied. Note: These homogenization effects are only related to homogenization of a sample before it enters the Enfer system, i.e., before homogenization with Enfer Buffer 1.

The ideal, of course, is to produce some form of artificial control for the assay for the purpose of sensitivity studies, a project in which Enfer is heavily involved at present.

4. Experience in the Field

The Enfer assay has been utilized in the field since 1998, with the majority of testing thus far having been completed in 2001. Significant numbers of samples have been tested in Ireland, Northern Ireland, Lithuania, and Denmark, where the entire testing requirements for slaughtered animals are carried out using the Enfer system. From November 2001, the total testing requirement for fresh slaughtered animals in the United Kingdom will be carried out using the Enfer test. A number of additional smaller-scale reference laboratories are operational in other sites throughout Europe and Asia Pacific. In all, over 1 million commercial tests have been carried out using this technology in just five sites in 2001, these sites representing approximately 20% of the testing capacity of the EU, identifying, in all, 155 positive animals, justifying Enfer's reputation as a highly effective mass screening system.

An important consideration in determining the effectiveness of a test procedure in the field is its ability to test all sample types presented to it, whether they be fresh, frozen, or autolyzed samples. The 1999 EU evaluation was carried out on fresh/frozen tissue samples with all test procedures performing equally well (47). It is highly important, however, again to consider the effectiveness of assays under field conditions. With all EU member states now obliged to test all casualty and fallen animals over 24 months of age (49), it is necessary that the effectiveness of a testing system be investigated with regard to autolyzed samples. To this end, Enfer has monitored the number of autolyzed samples received by the Irish testing laboratory, those recorded as positive and those subsequently confirmed as positive. In all, in excess of 300 autolyzed samples have been received by the Enfer facility in Ireland. Of these, three samples were identified as positive, two of which were confirmed positive by immunohistochemistry and a third that could not be confirmed owing to the extremely poor nature of the sample. Additionally, an autolysis study has been carried out on seven histopathologically confirmed BSE-positive samples. These samples were dissected bilaterally, one-half frozen to be used as a control and the second half left at room temperature (16–30°C) for 10 days to autolyze.

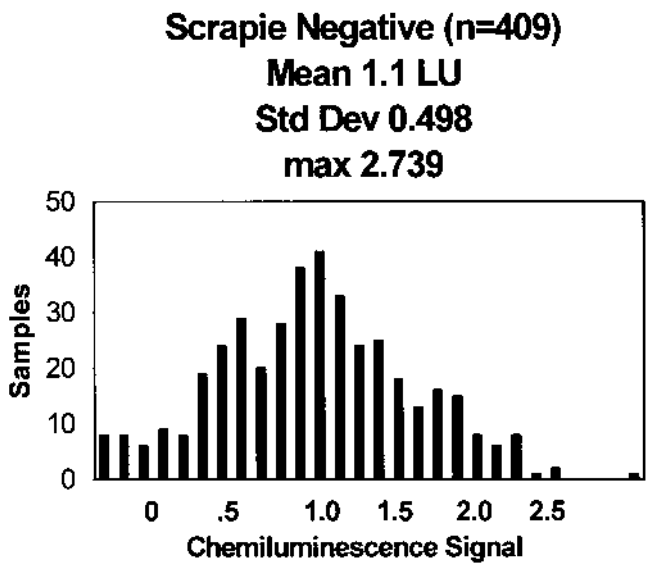
Such treatment should be representative of a worst-case scenario, with the majority of autolyzed samples received into a laboratory being, at most, 5 days old. The results of this experiment are presented in Table 10. From the results, it can be seen that all positive samples remained positive, even after 10 days. A drop in relative light units is seen for all positive samples. However, this is believed to be related to the level of detectable prion remaining in the sample after 10 days of autolysis rather than a decrease in sensitivity of the assay. It can been seen from the data that even a very weak positive, yielding a control mean value of 31 LU, still remains positive after treatment. Equally important, all autolyzed field samples examined to date have not generated a false-positive result using the Enfer test and a random selection of negative samples has been confirmed negative by immunohistochemistry.

An autolyzed sample would be expected to cause problems in terms of increasing the likelihood of false positives. As autolysis proceeds, bacterial contamination of the sample would significantly increase. The level of protease inhibitors present in the sample will therefore also increase, these inhibitors being produced by microbial contaminants as a means of self-protection. With the use of proteinase K being a critical step in the testing system, by acting to digest any PrP^C present in the sample, any external contaminant, such as a microbial inhibitor, can have deleterious effects on an assay. The Enfer assay has proved successful in correctly identifying autolyzed samples both in artificial studies and in the field, as there are two mechanisms present, described earlier, that prevent interference with the assay from autolysis effect, namely, the harshness of Enfer Buffer 1 and the

Table 10 Use of the Enfer TSE Assay on Autolyzed Samples

Sample ID	Frozen sample (LU)	Status	Autolyzed sample (LU)	Status	Positive cut off
BSE 1	1186	Positive	511	Positive	> 5.5
BSE 3	1884	Positive	769	Positive	> 5.5
BSE 4	31	Positive	6.2	Positive	> 5.5
BSE 5	544	Positive	401	Positive	> 5.5
BSE 6	3459	Positive	953	Positive	> 5.5
BSE 8	5236	Positive	4984	Positive	> 5.5

Two tissue sections were dissected from a total of six confirmed-positive BSE cases. One section was stored frozen at −20°C (frozen sample), the second at RT (16–30°C) for 10 days (autolyzed sample). Light readings were generated on both tissue sections, the frozen sample acting as a control. A decrease in light signal was recorded for all autolyzed samples; however, all samples, both frozen and autolyzed, yielded positive results (>5.5 LU).



Scrapie Positives (n=14)

Mean	**958**
max	**2100**
Min	**6.22**

>5.5 LU : Positive

Scrapie Positive Data — Mean (n=2)

Date	Enfer ID	Tissue type		Sample L.U.
14/12/99	661412992481	Spinal Cord		1572
14/12/99	661412992500	Spinal Cord		1438
14/12/99	661412992507	Spinal Cord		1714
02/10/00	660210001435	Spinal Cord		809
09/10/00	660910001859	Spinal Cord		1980
16/10/01	991610000001	Spinal Cord		474
02/11/00	650211004508	Spinal Cord		470
16/11/00	661611004795	Spinal Cord		530
17/11/00	661711005571	Spinal Cord		320
3/5/01	063030501017	Spinal Cord		2030
11/5/01	066110501119	Spinal Cord		1600
11/5/01	066110501120	Spinal Cord		80
15/5/01	375150501013	Spinal Cord		28
19/7/01	375190701278	Spinal Cord		170
19/7/01	375190701215	Spinal Cord		2100

Figure 14 Specificity and sensitivity data relating to testing of ovines for the presence of scrapie using the Enfer test. On the basis of these data, it can be seen that the Enfer assay would prove a highly useful tool in screening ovines for the presence of TSE, yielding a mean negative light signal of 1.1 LU and positive light signals ranging from 28.0 LU to 2100 LU for 15 spinal tissue samples detected during routine screening of animals not showing clinical signs of disease. A positive cutoff value of 5.5 LU has been applied.

specific aggregation of PrP^{sc} onto the Enfer microplate. It must be noted, however, that samples treated with a fixative, e.g., formal saline, cannot be run through the system.

5. Applicable Species

As PrP is a highly conserved protein throughout evolution, it was presumed highly likely that a testing system capable of detecting PrP^{sc} in cattle would prove useful in testing other species for the presence of the diseased prion. Obviously, the testing system has been extensively evaluated both at laboratory level and in the field for detection of clinical and subclinical cases of BSE. However, although BSE is of major concern in Europe, TSE also occurs naturally in a variety of other species such as sheep, mink, and deer, all species of economic importance. While work is continuing on investigations into the usefulness of the test for mink and deer, the majority of work in differing species has been carried out on TSE in sheep. Initial studies centered on evaluating the test using ovine spinal tissue, as this had proved highly successful in bovines. Results are presented in Fig. 14 and highlight a clear use for the assay in detecting scrapie in sheep.

IV. ONGOING AND FUTURE STUDIES

A. Antibody Used in the Enfer Assay

The use of synthetic peptides to immunize animal hosts, thereby generating high-titer-specific antibodies to a compound of interest, is well documented (50,51). Large quantities of high-purity synthetic peptides can be easily and quickly produced by solid-phase synthesis (52) and are often highly useful for immunization rather than using a parent compound, which may be difficult to purify in large quantities or is simply not well characterized. Use of peptide immunogens also allows immunization with only the essential epitopes and eliminates all other unwanted portions of the protein (53).

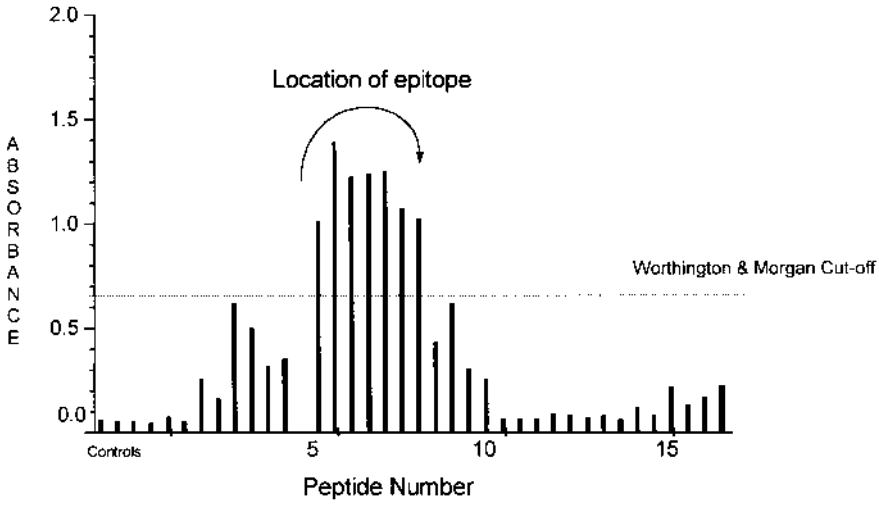
Such a synthetic peptide is used in the Enfer assay for prion detection. This epitope was originally identified using extensive epitope algorithm prediction (54–56) of the prion protein and tested for specificity against PrP using Western blot (unpublished data, Proteus, UK). This antigen, designated Efa, is located at the N-terminus of the prion protein, PrP, and has the following primary sequence: Efa—GQGGSHSQWNKPSKPKTNMKHVAG (24-amino-acid sequence).

PrP protein is unique in the world of science. Its apparent ability to exist in two different conformational states while retaining its original

primary sequence (57), thereby causing disease, is highly unusual. It therefore becomes crucial to examine the interactive potential of prions in as much detail as possible. Further characterization of lengthy synthetic antigens, such as the prion antigen used in the Enfer assay, often identifies a shorter epitope within the original sequence as a single B-cell epitope can encompass as few as three to six amino acids (58). Peptide characterization manipulations would be difficult if working with a lengthy 24-amino-acid epitope, making further resolution and definition of the lengthy epitope essential. Identification of a shorter epitope also raises the possibility of producing an antiserum capable of distinguishing between PrP^{c} and PrP^{sc} without the use of extensive sample preparation techniques, which would be extremely important in furthering the rapid diagnosis of TSE. Also, identification of the antibody-binding site may help to obtain a species-specific prion epitope that would highlight conformational differences in prions from different species (35), now a highly important consideration when one considers the potential for BSE to exist in the ovine population masked as scrapie. Further resolution and redefinition of Efa prion epitope makes detailed study of the protein-protein interactions that occur during antibody-antigen binding possible (58), while a study on the lengthier epitope may prove wasteful and inconclusive. Detailed characterization of the lengthy epitope can also lead to the exclusion of excess amino acid residues, which may result in the introduction of interfering factors (e.g., cross-reacting antibodies) during the antibody production procedure (59). It is also suggested that detailed characterization of a peptide antigen can lead to considerable improvement of the antigenicity of the peptide (60). Therefore, a study allowing isolation of the exact antibody-antigen-binding site within this parent sequence is a useful introductory step (61), eventually leading to full characterization of the antibody-binding site, using amino acid modification studies, and the production of guaranteed monospecific or engineered antibodies.

The desired "short" sequence for identification within this epitope would be the antibody-antigen-binding site within Efa and the aim of this ongoing study therefore was to identify and define the boundaries of the antibody-binding site within Efa.

A modified epitope-mapping technique, known as PEPSCAN technology (62,63), detects sites that can be identified and isolated using such a technique. Octapeptides with an overlap of a single amino acid were synthesized to provide the most comprehensive study of the sequence and allow identification of the minimum sequence necessary for antibody binding within the parent sequence (61). The absorbance results obtained on reacting Efa pepset with anti-Efa antibody at a wavelength of 405 nm are listed in Fig. 15. Duplicate absorbance values recorded were plotted versus



PEPTIDE NUMBER & SEQUENCE	DUPLICATE 1 405nm	DUPLICATE 2 405nm	MEAN VALUE 405nm
1 GQGGSHSQ	**0.075**	**0.054**	**0.065**
2 QGGSHSQW	**0.257**	**0.161**	**0.209**
3 GGSHSQWN	**0.616**	**0.496**	**0.556**
4 GSHSQWNK	**0.317**	**0.351**	**0.334**
*5 SHS**QWNKP***	——	*1.011*	*1.011*
*6 HS**QWNKP**S*	*1.392*	*1.224*	*1.308*
*7 S**QWNKP**SK*	*1.236*	*1.248*	*1.238*
*8 **QWNKP**SKP*	*1.069*	*1.018*	*1.044*
9 WNKPSKPK	**0.433**	**0.615**	**0.524**
10 NKPSKPKT	**0.304**	**0.252**	**0.278**
11 KPSKPKTN	**0.060**	**0.060**	**0.060**
12 PSKPKTNM	**0.064**	**0.085**	**0.075**
13 SKPKTNMK	**0.080**	**0.071**	**0.076**
14 KPKTNMKH	**0.079**	**0.061**	**0.070**
15 PKTNMKHV	**0.119**	**0.077**	**0.098**
16 KTNMKHVG	**0.216**	**0.131**	**0.174**
17 TNMKHVGC	**0.165**	**0.221**	**0.193**

Figure 15 Epitope map of the synthetic peptide used to generate antisera used in the Enfer ELISA with absorbance units (AU) at 405 nm plotted versus peptide number. Overlapping duplicate octapeptides homologous to amino acids 1–8. 2–9, etc. of the Enfer peptide were synthesized. The peptides were reacted with a 1:1000 dilution of homologous antiserum, raised in rabbits, against the Enfer peptide. Results are shown as vertical lines proportional to the absorbance value at 405 nm in the ELISA against the location of the N-terminal amino acid of each duplicate peptide within the sequence. The Worthington and Morgan cutoff point is shown as a dotted horizontal line. Duplicate peptide sequences are also outlined in the accompanying table. (Adapted from Ref. 61.)

peptide number to yield graphic results (Fig. 15). Using the Worthington and Morgan algorithm (64), the mean of 70% of the lowest peptide light signals generated was deemed appropriate to calculate a cutoff point over which signals would be highlighted as significant.

$$\text{Mean 70\% of values} = 0.182 \text{ AU} + 3(0.149) = 0.629 \text{ AU}$$

$$\text{Worthington and Morgan significant light signal value} = 0.629 \text{ AU}$$

The results of the pepset ELISA show duplicate peptides 13,14; 15,16; 17,18; and 19,20 as the most reactive peptides, recording high light signals, values over the Worthington and Morgan significant light signal value.

The sequence QWNKP is common to each of these peptides, identifying this five-amino-acid stretch as the antibody-binding site for anti-Efa antibody. Detailed study of these newly identified antibody-binding sites may yield some interesting conformational information about this section of the prion and may indeed lead to identification of an area of conformational difference between PrP^{c} and PrP^{sc}, a critical step in the road to better understanding of this scientific anomaly. Identification of the critical antibody-binding amino acids within these sites could highlight a loop or turn within the binding site, further characterizing the conformational nature of the prion protein. Such detailed antigen characterization continues to be investigated. This shorter sequence can now be synthesized by conventional solid-phase synthesis techniques (52) in large quantities to allow further characterization of this new peptide and immunization of a host animal for antiserum production.

To further the work carried out for this study, a number of experiments are ongoing:

- Epitope mapping of the complete prion protein to identify other sequences within the prion protein important for antiserum generation.
- Amino acid modification studies of newly identified antibody-binding sites, leading to possible identification of discontinuous epitopes (65). Identification of such epitopes would be more useful in furthering our understanding of the infectious prion protein as these epitopes are conformational in nature and the differences between the normal and abnormal prion are believed to be conformational. It is essential to find conformationally nonconserved regions of the prion protein before a peptide can be used as a BSE vaccine or as a means of readily generating specific anti-PrP^{sc} antisera.
- Examination of potential BSE synthetic peptide vaccine candidates. A combination of epitope mapping and modification techniques should advance progress in this area.

X-ray crystallography of short peptide sequences identified by epitope mapping to elucidate the structure and interaction of prion antigens and their respective antisera.

Antibody engineering, involving informed modification of the antibody-combining site, can be carried out on the basis of known, characterized antigens and antisera. Through determination of antibody/antigen contact residues it may be possible to engineer an antibody (66,67) with high specificity for the infectious prion protein by increasing the potential for hydrogen, hydrophobic, and electrostatic bond formation between antibody and antigen.

B. Increased Sensitivity Study

As the Enfer assay principle allows for 100% specificity in the detection of PrP^{sc}, Enfer has continued to examine and optimize the 1999 EU evaluated test protocol (Version 1.0) to increase sensitivity. On the basis of this work, a revised protocol has been devised and evaluated against Version 1.0 using histologically confirmed positive and negative samples. The new protocol, Version 2.0, involves a reduced incubation temperature from 37°C to 34°C and increased shake speed from 650 rpm to 1400 rpm. A change to the assay protocol for the Enfer TSE Diagnostic Kit (ETSEAB25/ETSEAC01) has therefore been proposed on the basis of the following study, the objective of the change being to increase the signal obtained on positive samples and hence improve sensitivity while maintaining a low signal for negative sample, and maintaining specificity.

In the case of negative tissue samples, 2779 samples were tested in duplicate with Version 1.0 2930 samples in duplicate (5860 data points) with Version 2.0 across three TSE kit lots; frequency distribution table for each protocol are presented in Table 11. Owing to a single invalid plate run and a second run recording an invalid blank control, data from 2689 samples (5378 data points) were analyzed for Version 1.0. All data were analyzed from the 2930 samples tested using Version 2.0. From examination of the negative population tested, the following conclusions have been drawn;

There was no significant change in the mean signal for the negative samples between Version 1.0 and Version 2.0.

There was a reduction in the mean signal for blank wells with the new protocol. This leads to an increase in the mean negative-blank result. This change in mean result does not affect the extremes of the distribution of negatives toward the cutoff (Table 12, Fig. 16).

There were no samples in which both the result (blank subtracted) duplicate wells exceeded the assay cutoff of 5.5 LU.

Table 11 Frequency Distribution of Negative Samples Across Three Kit Lots Using Version 1.0 and Version 2.0 of the Enfer TSE Assay Protocol

Light signal interval (LU)	Data points, version 1	Data points, version 2	
0.0–0.5	5234	5452	
0.5–1.0	97	312	
1.0–1.5	17	45	
1.5–2.0	9	13	
2.0–2.5	5	15	
2.5–3.0	5	3	
3.0–3.5	5	5	
3.5–4.0	3	7	
4.0–4.5	1	2	
4.5–5.0	0	4	
5.0–5.5	1	0	
5.5–6.0	0	0	← Enfer positive cutoff value
6.0–6.5	0	1	
6.5–7.0	0	0	
7.0–7.5	2	0	
7.5–8.0	0	0	
8.0–8.5	0	0	
8.5–9.0	0	0	
9.0–9.5	0	0	
9.5–10.0	0	0	
10.0–10.5	0	0	
10.5–11.0	0	0	
11.5–12.0	0	0	
12.0–12.5	0	0	
12.5–13.0	0	0	
13.0–13.5	0	1	
Total data points	5378	5860	

Two samples yielded a single replicate over the 5.5-LU positive cutoff value on both protocols. This is not deemed to be significant, specificity being comparable between Version 1.0 and Version 2.0 of the Enfer assay.

Table 12 Summary Statistics for Negative Samples Across Three Kit Lots Using Version 1.0 and Version 2.0 of the Enfer TSE Assay Protocol

Batch	Protocol	*n*	Mean LU for blank	Mean LU for samples	Mean sample-blank	SD of sample results	Number of individual wells > 5.5 LU
K13	Version 2.0	990	1.04	1.21	0.17	0.66	2
K27	Version 2.0	970	1.08	1.17	0.09	0.25	0
K28	Version 2.0	970	1.15	1.26	0.12	0.37	0
K13	Version 1.0	794	2.24	1.49	−0.75	0.72	0
K27	Version 1.0	925	1.03	0.91	−0.12	0.35	2
K28	Version 1.0	970	1.02	0.92	−0.09	0.30	0

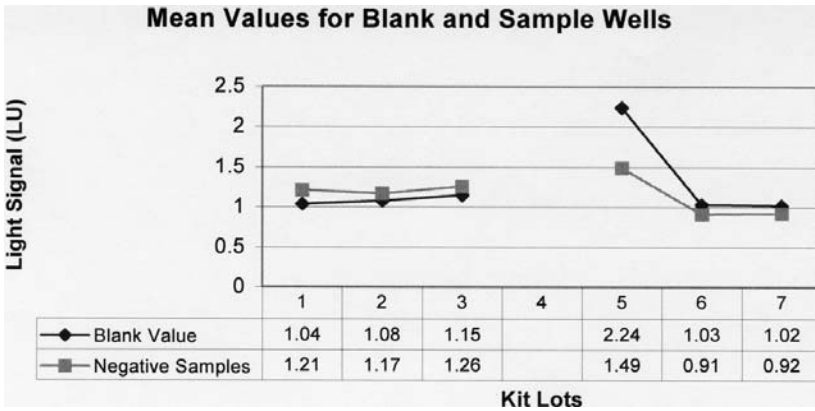


Figure 16 Comparison of mean values for blank and sample wells over three kit lots using Version 1.0 and Version 2.0 of the Enfer TSE Assay Protocol. 1—Version 2.0, Kit K13; 2—Version 2.0, Kit K27; 3—Version 2.0, Kit K 28; 4—N/A; 5—Version 1.0, Kit K13; 6—Version 1.0, Kit K27; 7—Version 1.0, Kit K28. A total of 5378 data points, Version 1.0, and 5860 data points, Version 2.0, were generated using negative field samples to yield negative mean values for three kit lots chosen randomly from existing stocks (K27, K28, K13). This allows examination of the effects on specificity of using Version 2.0 of the Enfer protocol. The blank reading for each of the microplates run was also recorded and the mean value calculated. Mean blank and negative readings are plotted here versus kit lot. There is no significant change in the mean signal for the negative samples between Version 1.0 and Version 2.0. Specificity is therefore unaffected using Version 2.0 of the Enfer protocol.

Table 13 Sensitivity Study of Version 1.0 and Version 2.0 of the Enfer TSE Assay

Sample ID	Version 1.0	Version 2.0	Relative Signal V2.0 vs. V1.0
BSE sample			
BSE 1	1179	3533	3.00
BSE 2	2561	6210	2.42
BSE 3	437.7	1901	4.34
BSE 4	383.7	2192	5.71
BSE 5	631.8	2929	4.64
BSE 6	748	2790	3.73
BSE 7	326.4	2308	7.07
BSE 8	1072	3555	3.32
BSE 9	830.4	3434	4.14
BSE 1 1:2	1084	3558	3.28
BSE 1 1:4	1085	2814	2.59
BSE 1 1:8	1018	2304	2.26
BSE 1 1:16	477	1073	2.25
BSE 1 1:32	120	378	3.16
BSE 2 1:2	2017	5599	2.78
BSE 2 1:4	1819	3788	2.08
BSE 2 1:8	1510	3023	2.00
BSE 2 1:16	733	1395	1.90
BSE 2 1:32	288	557	1.94
BSE 3 1:2	427.7	1531	3.58
BSE 3 1:4	252.4	740	2.93
BSE 3 1:8	68.03	227	3.34
BSE 3 1:16	13.78	49.96	3.63
BSE 3 1:32	3.401	11.89	3.50
BSE 4 1:2	217.9	1092	5.01
BSE 4 1:4	195.6	785.9	4.02
BSE 4 1:8	133.8	451.6	3.38
BSE 4 1:16	43.86	161.5	3.68
BSE 4 1:32	9.588	391.2	
BSE 5 1:2	481.2	1688	3.51
BSE 5 1:4	511.2	1645	3.22
BSE 5 1:8	266.9	768.1	2.88
BSE 5 1:16	91.7	257.3	2.81
BSE 5 1:32	15.66	75.19	4.80
Scrapie sample			
Scrapie 1	419	649	1.55
Scrapie 1:2	175	342	1.95
Scrapie 1:4	29	107	3.72
Scrapie 1:8	9.5	20.3	2.13
Scrapie 1:16	2.4	5.0	2.07
Scrapie 1:32	1.1	1.8	1.61

A total of nine confirmed BSE tissue samples, 24 BSE diluates, one confirmed scrapie tissue sample, and five scrapie diluates were examined using Version 1.0 and Version 2.0 of the Enfer protocol.

Both Version 1.0 and Version 2.0 generated two individual wells (corrected for blank reading) that exceeded 5.5 LU, which is not considered to be significant (Table 11).

Table 12 and Fig. 16 show the summary statistics for the negative samples across the three kit lots examined.

If one examines the data generated on BSE-positive samples (Table 13, Fig. 17) Version 2.0 generated signals an average of 3.42 times higher, ranging from 1.9 to 7.07 times Version 1.0. Some higher sample dilutions did record increases of up to 11.27 times with Version 2.0. However, these are not presented here for reasons of consistency as not all samples were examined at dilutions greater than 1:32. A total of nine BSE-positive samples including dilution series (1:2–1:32 presented) of five of these samples across one kit lot were studied. A scrapie-positive sample and dilutions of this sample were also examined, the average signal using Version 2.0 being 2.83 times the signal generated using Version 1.0 (range 1.55–3.72).

An additional examination of Version 1.0 and Version 2.0 was carried out in conjunction with the EU homogenization study, where six replicates of all samples were tested of both tissue and homogenized samples. Again, Version 2.0 of the assay produce consistently higher results for both tissue samples and homogenates (Table 9, Fig. 13).

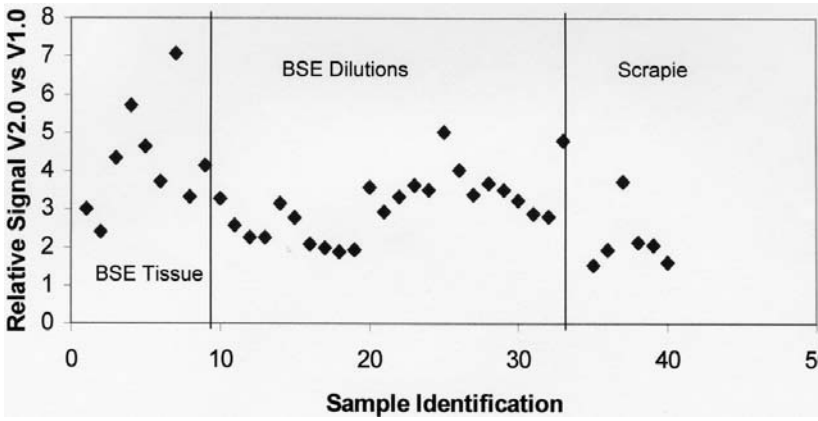


Figure 17 Plot showing the relative light signals, Version 2.0 versus Version 1.0, for a number of BSE tissues, a series of BSE dilutions, a scrapie tissue, and a series of scrapie dilutions. A total of nine confirmed BSE tissue samples, 24 BSE diluates, one confirmed scrapie tissue sample, and five scrapie diluates were examined using Version 1.0 and Version 2.0 of the Enfer protocol. Results are plotted relative to light signal generated by Version 2.0 versus Version 1.0 of the assay against sample number. Sample categories are separated by a vertical line.

C. Future Developments and Prospects

Research continues into further applications of the Enfer TSE assay. Future projects, some of which are already in progress, will include:

Development of uniform quality control materials for test procedure evaluation
TSE detection in body fluids, e.g., blood, milk
Detection of BSE in sheep and differentiation between BSE and scrapie
Extensive epitope characterization of the prion protein
TSE detection in variable species, e.g., deer, felines, mink
TSE vaccine

The Enfer TSE assay is highly adaptable and work is advancing rapidly on these projects.

V. CONCLUSION

When one applies the three criteria of a screening assay (rapid, reliable, reproducible) to the Enfer testing system, its success as a screening system becomes apparent. The Enfer TSE Test Kit may be defined as a complete, rapid, fully traceable screening system for the detection of the TSE infectious agent PrP^{sc}. The assay takes approximately 3.5 h to complete, making it the fastest test for TSE detection currently available. The test has been shown to have a clinically relevant sensitivity and all identified field positives have being confirmed using histopathology, immunohistochemistry, or Western blot. The test is also 100% specific, no false positives having being reported by users of the Enfer testing system. TSEs are a fascinating area of study, and the Enfer test is as useful a research tool as it is a high-throughput test procedure. The coming years will see the Enfer TSE assay at the forefront of TSE research.

REFERENCES

1. Gabriel J-M, Oesch B, Kretzschmar H, Scott M, Prusiner SB. Molecular cloning and structural analysis of a candidate chicken prion protein. In: Prusiner SB, Collinge J, Powell J, Anderton B, eds. Prion Diseases of Humans and Animals. Chichester, England: Ellis Horwood, 1992:407–431.
2. Prusiner SB. Prions. Proc Natl Acad Sci USA 1998; 95:13363–13383.
3. Pan K-M, Baldwin M, Nguyen J, Gasset M, Serban A, Groth D, Mehlhorn I, Huang Z, Fletterick RJ, Cohen FE, Prusiner SB. COnversion of α-helices into

β-sheets features in the formation of the scrapie prion proteins. Proc Natl Acad Sci USA 1993; 90:10962–10966.
4. Anfinsen C. Principles that govern the folding of protein chains. Science 1973; 181:223–230.
5. Gajdusek DC. Unconventional viruses and the origin and disappearance of kuru. Science 1977; 197:943–960.
6. Collinge J, Prusiner SB. Terminology of prion diseases. In: Prusiner SB, Collinge J, Powell J, Anderton B, eds. Prion Diseases of Humans and Animals. Chichester, England: Ellis Horwood, 1992:5–12.
7. Prusiner SB. Novel proteinaceous infectious particles cause scrapie. Science 1982; 216:136–144.
8. Dawson M, Wells GAH, Parker BNJ. Preliminary evidence of the experimental transmissibility of bovine spongiform encephalopathy to cattle. Vet Record 1990; 126:112–113.
9. Diener TO, McKinley MP, Prusiner SB. Viroids and prions. Proc Natl Acad Sci USA 1982; 79:5220–5224.
10. Carp RI, Merz PA, Kascsak RJ, Merz GS, Wisniewski HM. Nature of the scrapie agent: current status of facts and hypotheses. J Gen Virol 1985; 66: 1357–1368.
11. Stahl N, Baldwin MA, Burlingame AL, Prusiner SB. Identification of the glycoinositol phospholipid linked and truncated forms of the scrapie prion protein. Biochemistry 1990; 29:8879–8884.
12. Basler K, Oesch B, Scott M, Westaway D, Wälchli M, Groth DF, McKinley MP, Prusiner SB, Weissman C. Scrapie and cellular PrP isoforms are encoded by the same chromosomal gene. Cell 1986; 46:417–428.
13. Bueler H, Aguzzi A, Saoler A. Mice devoid of PrP are resistant to scrapie. Cell 1993; 73:1339–1347.
14. Prusiner SB, McKinley MP, Groth DF, Bowman KA, Mock NI, Cochran SP, Masiarz FR. Scrapie agent contains a hydrophobic protein. Proc Natl Acad Sci USA 1981; 78:6675–6679.
15. Taylor DM. Bovine spongiform encephalopathy—the beginning of the end. Br Vet J 1996; 152:501–518.
16. Turk E, Teplow DB, Hood LE, Prusiner SB. Purification and properties of the cellular and scrapie hamster prion proteins. Eur J Biochem 1988; 176:21–30.
17. Bolton DC, McKinley MP, Prusiner SB. Molecular characteristics of the major scrapie prion protein. Biochemistry 1984; 23:5898–5906.
18. McKinley MP, Masiarz FR, Prusiner SB. Reversible chemical modification of the scrapie agent. Science 1981; 214:1259–1261.
19. Bolton DC, McKinley MP, Prusiner SB. Identification of a protein that purifies with the scrapie prion. Science 1982; 218:1309–1311.
20. Barry RA, Kent SBH, McKinley MP, Meyer RK, DeArmond SJ, Hood LE, Prusiner SB. Scrapie and cellular prion proteins share polypeptide epitopes. J Infect Dis 1986; 153:848–854.
21. Bolton DC, Meyer RK, Prusiner SB. Scrapie PrP27-30 is a sialoglycoprotein. J Virol 1985; 53:596–606.

22. Prusiner SB, Groth DF, Bolton DC, Kent SB, Hood LE. Purification and structural studies of a major scrapie prion protein. Cell 1984; 38:127–134.
23. McKinley MP, Bolton DC, Prusiner SB. A Protease-resistant protein is a structural component of the scrapie prion. Cell 1983; 35:57–62.
24. Chesebro B, Race R, Wehrly K, Nishio J, Bloom M, Lechner D, Bergstrom S, Robbins K, Mayer L, Keith JM, Garon C, Haase A, A. Identification of scrapie prion protein–specific mRNA in scrapie-infected and uninfected brain. Nature 1985; 315:331–333.
25. Oesch B, Westaway D, Wälchli M, McKinley MP, Kent SB, Aebersold R, Barry RA, Tempst P, Teplow DB, Hood LE, Prusiner SB, Weissman C. A cellular gene encodes scrapie PrP 27–30 protein. Cell 1985; 40:735–746.
26. De Armond SJ, Mobley WC, DeMott DL, Barry RA, Beckstead JH, Prusiner SB. Changes in the localisation of brain prion proteins during scrapie infection. Neurology 1987; 37:1271–1280.
27. Barry RA, Prusiner SB. Monoclonal antibodies in the cellular and scrapie prion proteins. J Infect Dis 1986; 154:518–512.
28. Bolton DC, Bendheim PE, Marmorstein AD, Potempska A. Isolation and structural studies of the intact scrapie agent protein. Arch Biochem Biophys 1987; 258:579–590.
29. Jackson GS, Murray I, Hosszu LLP, Gibbs N, Waltho JP, Clarke AR, Collinge J. Location and properties of metal-binding sites on the human prion protein. Proc Natl Acad Sci Acad Sci USA 2001; 98:8531–8535.
30. Schatzl HM, Da Costa M, Taylor L, Cohen FE, Prusiner SB. Prion protein gene variation among primates. J Mol Biol 1995; 245:362–374.
31. Loftus B, Rogers M. Characterisation of a prion protein (PrP) gene from rabbit; a species with apparent resistance to infection by prions. Gene 1997; 184:215–219.
32. Goldman W, Hunter N, Martin T, Dawson M, Hope J. Different forms of the bovine PrP gene have five or six copies of a G-C rich element with the protein-coding exon. J Gen Virol 1991; 72:201–204.
33. Goldman W, Hunter N, Foster JD, Salbaum JM, Beyreuther K, Hope J. Two alleles of a neural protein gene linked to scrapie in sheep. Proc Natl Acad Sci USA 1990; 87:2476–2480.
34. Gibbs CJ Jr, Gajdusek DC, Amyx H. Strain variation in the viruses of Creutzfeldt-Jakob disease and kuru. In: Prusiner SB, Hadlow WJ, eds. Slow Transmissible Diseases of the Nervous System II. New York: Academic Press, 1979:87–110.
35. Groschup M, Pfaff E. Studies on a species-specific epitope in murine, ovine and bovine prion protein. J Gen Virol 1993; 74:1451–1456.
36. Bockman JM, Kingsbury DT, McKinley MP, Bendheim PE, Prusiner SB. Creutzfeldt-Jakob disease prion protein in human brains. N Engl J Med 1985; 312:73–78.
37. Collinge J, Sidle KCL, Meads J, Ironside J, Hill AF. Molecular analysis of prion strain variation and the aetiology of "new variant" CJD. Nature 1996; 383:685–690.

38. Aguzzi A. Between cows and monkeys. Nature 1996; 381:734–735.
39. Anderson RM, Donelly CA, Ferguson NM, Woolhouse MEJ, Watt CJ, Udy HJ, MaWhinney S, Dunstan SP, Southwood TRE, Wilesmith JW, Ryan JBM, Hoinville LJ, Hillerton JE, Austin AR, Wells GAH. Transmission dynamics and epidemiology and BSE in British cattle. Nature 1996; 382: 779–788.
40. Zerr I, Bodemer M, Otto M, Poser S, Windl O, Kretzschmar HA, Gefeller O, Weber T. Diagnosis of Creutzfeldt-Jakob disease by two-dimensional gel electrophoresis of cerebrospinal fluid. Lancet 1996; 348:846–849.
41. Wells GAH, Handock RD, Cooley WA, Richards MS, Higgins RJ, David GP. Bovine spongiform encephalopathy: diagnostic significance of vacuolar changes in selected nuclei of the medulla oblongata. Vet Rec 1989; 125: 521–524.
42. Mohri S, Farquhar CF, Somerville RA, Jeffrey M, Foster J, Hope J. Immunodetection of a disease specific PrP fraction in scrapie-affected sheep and BSE-affected cattle. Vet Rec 1992; 131:537–539.
43. Wells GAH, Scott AC, Wilesmith JW, Simmons MM, Mathews D. Correlation between the results of a hispathological examination and the detection of abnormal brain fibrils in the diagnosis of bovine spongiform encephalopathy. Res Vet Sci 1994; 56:346–351.
44. Engvall E, Perlmann P. Enzyme-linked immunosorbent assay (ELISA) quantitative assay of immunoglobulin G. Immunochemistry 1971; 8:871–874.
45. Sayers R, Kwasowski P, O'Connor M. A novel screening method for the detection of clenbuterol residues in bovine bile using immunoaffinity and enhanced chemiluminescence immunoassay. In: Haagsma N, Ruiter A, eds. EuroResidue III (Vol 2). Proceedings of the Conference on Residues of Veterinary Drugs in Food, Veldhoven, The Netherlands, pp. 848–852.
46. Wells GAH, Hawkins SAC, Cunningham AA, Blamire IWH, Wilesmith JW, Sayers AR, Harris P. (1993). Comparative pathology of the new transmissible spongiform encephalopathies. Proceedings of a Consultation on BSE with the Scientific Veterinary Committee of the Commission of European Communities, Brussels, 1993, pp 327–345.
47. Moynagh J, Schimmel HT. The evaluation of tests for the diagnosis of transmissible spongiform encephalopathy in bovines. European Commission, DG SANCO, Brussels, 1999.
48. Scott AC, Wells GAH, Chaplin MJ, Dawson M. Bovine spongiform encephalopathy: detection of fibrils in the central nervous system is not affected by autolysis. Res Vet Sci 1992; 52:332–336.
49. Commission Regulation (EC) No 1248/2001 of 22 June 2001 amending Annexes III, X and XI to Regulation (EC) No 999/2001 of the European Parliament and of the Council as regards epidemio-surveillance and testing of transmissible spongiform encephalopathies. Community Legislation in Force. http://europa.eu.int/eur-lex/en/lif/dat/2001/en 301R1248.html. pp. 1–9.
50. Lemer RA. Tapping the immunological repertoire to produce antibodies of predetermined specificity. Nature 1982; 299:592–596.

51. Sutcliff JG, Shinnick TM, Green N, Liu FT, Niman HL, Learner RA. Chemical synthesis of a polypeptide predicted from nucleotide sequence allows detection of a new retroviral gene product. Nature 1980; 287:801–805.
52. Merrifield RB. Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. J Am Chem Soc 1963; 85:2149–2154.
53. Tam JP. Synthetic peptide vaccine design: synthesis and properties of a high-density multiple antigenic peptide system. Proc Natl Acad Sci USA 1988; 85:5409–5413.
54. Chou PY, Fasman GD. Beta-turns in proteins. J Mol Biol 1977; 115:135–175.
55. Hopp TP, Woods KR. Prediction of protein antigenic determinants from amino acid sequences. Proc Natl Acad Sci USA 1981; 78:3824–3828.
56. Welling GW, Weijer WJ, Van der Zee R, Welling-Webster S. Prediction of sequential antigenic regions in proteins. FEBS Lett 1985; 188:215–218.
57. Caughey BW, Dong A, Bhat KS, Ernst D, Hayes SF, Caughey WS. Secondary structure analysis of the scrapie-associated protein PrP 27-30 in water by infrared spectroscopy. Biochemistry 1991; 30:7672–7680.
58. Schoofs PG, Getsen HM, Jackson DC, Brown LE, Tang X-L, White DO. Epitopes of an influenza viral peptide recognised by antibody at single amino acid resolution. J Immunol 1988; 140:611–616.
59. Shukla DD, Tribbick G, Mason TJ, Hewish DR, Geysen HM, Ward CW. Localization of virus-specific and group-specific epitopes of plant polyviruses by systemic immunochemical analysis of overlapping peptide fragments. Proc Natl Acad Sci USA 1989; 86:8192–8196.
60. Meloen RH, Puyk WC, Meijer DJA, Lankhof H, Posthumus WPA, Schaaper WMM. Antigenicity and immunogenicity of synthetic peptides of foot-and-mouth disease virus. J Gen Virol 1987; 68:305–314.
61. Sayers R. The effects of selective modification of the prion protein (PrPsc) on antibody specificity to detect bovine spongiform encephalopathy (BSE) in cattle. Masters dissertation, National University of Ireland, Dublin, 1998.
62. Geysen HM, Meloen RH, Barteling SJ. Use of peptide synthesis to probe viral antigens for epitopes to a resolution of a single amino acid. Proc Natl Acad Sci USA 1984; 81:3998–4002.
63. Geysen HM, Rodda SJ, Mason TJ, Tribbick G, Schoofs PG. Strategies for epitope analysis using peptide synthesis. J Immunol Meth 1987; 102:259–274.
64. Worthington J, Morgan K. Epitope mapping using synthetic peptides. In: Peptide Antigens, a Practical Approach. Wisdom GB, ed. Oxford University Press, Oxford, New York, and Tokyo, 1994:181–217.
65. Geysen HM, Rodda SJ, Mason TJ. A priori delineation of a peptide which mimics a discontinuous antigenic determinant. Mol Immunol 1986; 23:709–715.
66. Churchill MEA, Stura EA, Pinilla C, Appel JR, Houghten RA, Kono DH, Balderas RS, Fieser GG, Schulze-Gahmen U, Wilson IA. Crystal structure of a peptide complex of anti-influenza peptide Fab 26/9: comparison of two different antibodies bound to the same peptide antigen. J Mol Biol 1994; 241:534–556.
67. MacCallum RM, Martin ACR, Thornton JM. Antibody-antigen interactions: contact analysis and binding site topography. J Mol Biol 1996; 262:732–745.

9

Sensitive Detection of Prion Proteins by Immunoassay: CEA/Bio-Rad Test

Emmanuel E. Comoy, Jacques Grassi, and Jean-Philippe Deslys
Atomic Energy Commission, Fontenay-aux-Roses, France

I. INTRODUCTION

Transmissible spongiform encephalopathies (TSEs) have been highlighted during the past years by the bovine spongiform encephalopathy (BSE) epizooty in Great Britain and appearance of BSE cases in many European countries. These diseases, previously known in enzootic or sporadic forms with natural scrapie in sheep or Creutzfeldt-Jakob disease (CJD) in humans, are now considered a major public health problem, since the BSE agent has been shown to be able to contaminate humans with the onset of variant of CJD (vCJD). Since no therapeutic or medical prophylactic measures are currently available against prion diseases, only active or passive prophylactic measures can be performed to avoid new contamination from animal to human or from human to human. In this context, sensitive diagnostic tools are of crucial interest for epidemiological and/or systematic screening to increase food and public health safety.

A. Characteristics of the Problem

Since its first appearance in 1986 (1), BSE, commonly known as mad cow disease, has been registered at a clinical level in more than 180.000 bovine cattle in the United Kingdom, and it is believed that as many as 1 million

undiagnosed infected cattle would have entered the human food chain (2). This transmissible prion disease was transmitted from bovine to bovine through consumption of contaminated meat-and-bone meals (MBM) (3): these MBM were exported nearly worldwide in various proportions, but only a few European countries described autochthon BSE cases in the following years. Epidemiological analysis revealed many years later that almost all European countries were affected, and doubts subsist then for many non-European countries in the absence of large-scale studies (4).

Contrarily to scrapie prion strains, the BSE agent exhibits a high ability to cross the interspecies barrier: TSE cases linked to BSE were first observed in different wild ruminants in zoos (5), and then in domestic cats and wild carnivores (6), all having been fed with contaminated cattle offal. Moreover, transmission experiments showed that BSE is transmissible to sheep by the oral route, leading to a disease very close to scrapie in terms of clinical and lesional signs (7). Since sheep would have been fed with the same contaminated MBM, important attention is pointed against a potent natural contamination of sheep by the BSE agent (8) that would be hidden by enzootic scrapie. Indeed, contrarily to cattle (9), this would constitute a high risk of spreading the BSE agent, since infectivity in BSE-infected sheep would be widely distributed in peripheral organs (10), and even in blood (11).

Finally, in 1996, the British government announced cases of a new form of CJD in young patients (12). This form, called variant Creutzfeldt-Jakob disease (vCJD), is due to contamination of humans by the BSE agent, presumably through the oral route (13–16). Up to now, more than 125 cases of vCJD have been confirmed (with 115 in the United Kingdom). Many parameters remain unknown (e.g., infective dose, susceptibility, duration of incubation period), and mathematical model are still imprecise, even if first models estimating between less than 100 and more than 100,000 are reduced to a few hundred to a few thousand total vCJD cases in the United Kingdom due to direct contamination of humans through consumption of beef products (17). These estimations could be dramatically increased in the case of natural BSE in sheep, because risk exposure would have been maintained through infected peripheral organs (10). Moreover, the unusual risk of secondary transmission from human to human has to be taken into account: indeed, PrPres, the biochemical specific marker of these disease that is supposed to be the infectious agent by itself (18), has been found in peripheral lymphoid organs of vCJD-injected patients (e.g., tonsils, appendix) (19). The theoretical risk of iatrogenic transmission, either by blood transfusion, by organ transplantation, or through contaminated surgical tools, could then be higher than for sporadic CJD (20–26).

To prevent new cases of human contamination, the first major measures has been to remove from human consumption the potent infectious

organs (i.e., central nervous system and some immune organs) (8), corresponding to 99% of the total infectivity. Nevertheless, since clinical signs appeared late in the course of the disease, and since clinical diagnosis of BSE is complicated by the absence of pathognomonic signs (27), an infected animal could still enter the human food chain and then present a risk for human health: indeed, spinal cord can be incompletely removed, and dorsal root ganglia stay included in vertebrae. Moreover, slaughtering practices do not prevent against contamination of healthy materials by infected materials.

In this context, the use of sensitive diagnostic tools was proposed to detect and remove infected animals. First epidemiological studies showed that a large number of previously undiagnosed infected animals could be detected by sensitive tests, even at the slaughterhouse: systematic use of rapid and sensitive tests at the abattoirs were then adopted by the European Community in mid-2001, to enforce consumer protection against new risks of contamination through feeding.

B. Classic Diagnostic Techniques Are Not Efficient

Reference diagnostic tests for prion disease were based on histology (28), by detection of pathognomonic lesions in the central nervous system: neuropil spongiosis, vacuolization, and reactional astrogliosis constitute the specific triad of prion lesions (29). This technique was perfectly adapted for confirmation of clinical cases, but was not able to detect infected animals before development of nervous system lesions. Moreover, it needs well-conserved samples to be correctly performed. Finally, this technique is time-consuming, and therefore not suitable for use at a high-throughput level. Other diagnostic tools were then needed for rapid high-throughput screening.

In the absence of inflammatory or immune reaction during the course of the disease (30), the classic diagnostic approach, commonly used in infectious disease and which consists in looking for antibody response against pathogens, is not adapted in prion disease. Moreover, no modification of biological parameters can be strictly correlated to the onset of these disease, limiting the use of biochemistry as a diagnostic tool (31–33).

Nevertheless, a special protein, the pathological form of the prion protein, constitutes a specific biochemical marker of these diseases (34), and is therefore perfectly dedicated to biochemical analysis of prion disease.

C. PrPres, the Only Specific Biochemical Marker of Prion Diseases

The prion protein, called PrPsens, is a normal protein of unknown biological function found on the membrane of neuronal cells of all mammals

(35,36). The protein, constituted of 269 amino acids in the bovine, can be found unglycosylated, mono-, or diglycosylated. Biophysical experiments have shown that the secondary structure of this protein would be mainly composed of α-helix (37,38). In TSE-affected animals and humans, this protein can be found in a modified form, called PrPres. Even if it shares the same primary structure in amino acids with normal PrPsens, PrPres presents different secondary and tertiary structures than normal PrPsens, with presumably mainly β-sheets structures (37). In fact, PrPres would be generated by a posttranscriptional transconformation of normal PrP (39), following unknown mechanisms.

Different experiments of purification of the TSE agents have shown that PrPres copurifies with infectivity (40), and the amount of PrPres is proportional to infectivity in experimental models of infection (34). Moreover, according to the protein-only hypothesis developed by S. Prusiner, many authors consider PrPres the only component of the prion agent (18). Nevertheless, different experiments have shown, in certain models of interspecies transmission, that disease could occur in the absence of any detectable PrPres, suggesting that PrPres could rather be a factor of virulence (41,42). This protein constitutes a specific marker of these diseases in natural infection, and all currently rapid tests are based on its detection.

Production of anti-PrP antibodies has been impaired for a long time (43), since PrPres shares the same primary structure as PrPsens and therefore is recognized as self-protein. PrP-deficient mice (44) greatly facilitated production of anti-PrP monoclonal antibodies (45). Suprisingly, despite differences of spatial conformation, no anti-PrP antibody produced until now is able to efficiently differentiate between normal and pathological forms of PrP. Therefore, a classic immunometric assay is unable to differentiate correctly a normal sample (PrPsens) from a TSE-infected sample (PrPsens and PrPres). Nevertheless, these two proteins exhibit different physicochemical properties, among which some can be used to selectively detect only PrPres and avoid detection of PrPsens (46).

II. DETECTION OF PURIFIED AND CONCENTRATED PRPRES: THE CEA/BIO-RAD TEST

In diagnostics, sensitivity is supported by maintaining the highest signal-to-noise ratio. In this context, having a very sensitive test for detection of PrPres implies ideally to eliminate all PrPsens without affecting PrPres. Accordingly, the test we have developed is based first on a selective purification of PrPres, prior to its detection (Fig. 1). Indeed, PrPres is partly resistant to enzymatic proteolysis, contrary to normal PrPsens, which is

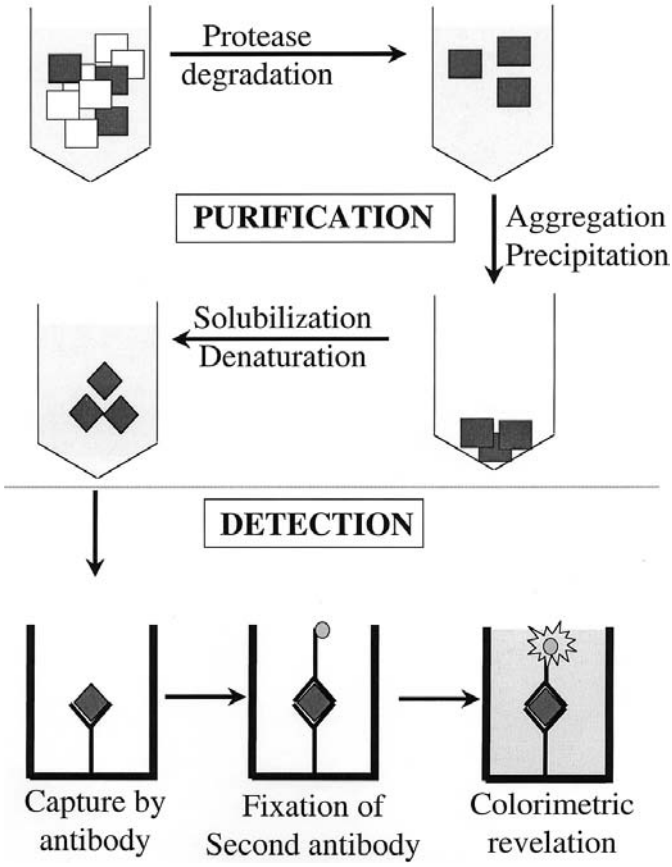


Figure 1 Principles of the CEA/Bio-Rad test. Denaturating buffer with proteinase K (1 vol) is added to the positive sample (1 vol) containing both PrPsens (open square) and PrPres (Filled square) and protease degradation is performed for 10 min at 37°C. Remaining PrPres is then aggregated by the addition of a clarifying buffer (1 vol). After a 5-min centrifugation, the supernatant is discarded and PrPres is denaturated (full losange) by solubilization buffer (1/10 vol). Detection is then performed by incubating the purified sample for 1 h in a microplate well precoated with monoclonal anti-PrP antibody. After extensive washes, peroxidase-linked monoclonal anti-PrP antibody is added for 1 h. After new extensive washes, substrate is added. Colored reaction occurs if PrPres is present in the sample.

sensitive (47). A controlled digestion of sample by proteinase K in defined denaturating conditions completely destroys the PrPsens while poorly affecting PrPres. Moreover, PrPres is able, in certain experimental conditions, to aggregate in fibrils, called scrapie-associated fibrils (SAF) (48), contrary to PrPsens. This second specific property, due to its insolubility in detergents (49), allows concentrating whole PrPres by its selective precipitation through a centrifugation. Design of a special buffer allowed us to shorten this centrifugation from hours to some minutes: the aggregates of PrPres of various sizes are immediately adsorbed in these special conditions on an inert matrix inherent to the sample. Purified PrPres in the resulting pellet is the solubilized with a denaturating treatment, which is known to increase its immunoreactivity (50).

Detection of purified PrPres can be then performed with a classic immunometric assay (Fig. 1). The immunometric technique we chose therefore is based on the enzyme-immunoassay (EIA) sandwich technique (51), which is widely recognized in the diagnostic field as a very specific and sensitive technique, and most important, perfectly dedicated to high-throughput analysis. This technique is based on the recognition of the antigen (here PrPres) by two distinct specific antibodies on two distinct epitopes, thus increasing the specificity of the assay. Among many monoclonal anti-PrP antibodies we produced by immunization of knockout mice, we selected the couple of antibodies allowing the best sensitivity. Practically, a first monoclonal anti-PrP antibody is covalently linked to a solid phase, here the plastic of a microtitration plate. Purified PrPres of the samples, if any, is then bound to these antibodies. After extensive washes to remove nonspecifically fixed molecules of the samples, a second antibody is added in excess, which will link to the fixed PrPres. Previously, this second antibody had been covalently coupled to an enzyme. After new extensive washes to remove any unbound enzyme-linked antibodies, a specific substrate of the enzyme is added. Products of degradation of this substrate by the enzyme develop a colored reaction. This colored reaction is then measured with a spectrophotometer, its intensity being proportional to the amount of degradated substrate. Conditions of the test are determined so that amount of degraded substrate is directly correlated to amount of enzyme, which is itself correlated to amount of fixed PrPres. Then, intensities of coloration can be correlated to amount of PrPres in the samples.

Thus, specificity for PrPres involves three factors, i.e., resistance to protease degradation, insolubility in detergents, and combination of two specific monoclonal antibodies. Moreover, sensitivity of this test is supported by combined use of these two antibodies and concentration of the samples through precipitation of aggregated PrPres.

PrPres is mainly found in the central nervous system in TSEs. In BSE naturally infected cattle, PrPres and infectivity are observed only in the central nervous system, even though infectivity could be observed in the ileum of cattle experimentally infected by the oral route with highly infectious doses (52). The test we have developed has been first optimized for detection of PrPres in the brain of BSE-infected cattle. Nevertheless, the monoclonal antibodies recognize PrP from other species, owing to high homology of PrP genes between species. As we will describe later, this test can then be adapted for use on other animal species for detection of other strains of prions. Moreover, it can be adapted for detection of PrPres on organs-other than brain samples, such as peripheral organs.

The concept of this test was first developed at the experimental level by our research institute (CEA). After the first official evaluation at the European level in 1999, this test was industrialized by Bio-Rad and the commercial version has been used for all further validations and studies.

III. PERFORMANCE OF THE CEA/BIO-RAD TEST FOR SCREENING BSE

A. Validation of the CEA/Bio-Rad Test by the European Community

In 1998, Directorate DGXXIV of the European Community (EC), now called DG Sanco and charged with consumer protection, decided to investigate the performance of the different rapid diagnostic tests for BSE available at this time through a call for interest (53,54). After a first selection of dossiers, four tests were retained for practical evaluation.

This evaluation was performed in May 1999, and consisted in blind analysis of 1600 samples within 2 weeks, under the permanent control of an EC representative, to prevent any contest afterward. Results of the analysis were then officially published after being decoded.

The first part of this evaluation study consisted in 1400 brain samples. Tests of these samples exhibited two very distinct populations, one close to the background level and the other showing saturating absorbances. After decoding, these samples were composed of 1000 samples of BSE-negative cows from New Zealand (New Zealand is, with Australia, the only country presenting neither BSE nor scrapie cases), 300 samples of BSE-positive cows from the United Kingdom (these animals were clinically affected and diagnostics were previously confirmed by histology), and 100 replicates of some of these samples. It appears (Table 1) that our test presented 100% specificity; i.e., no positive result was observed among the 1000 negative samples, and 100% sensitivity at the clinical level, i.e., no negative result was

Table 1 Evaluation of Diagnostic Tests for BSE by DG Sanco, May 1999

	Test A (Wallac)	Test B (Prionics)	Test C (Enfer)	Test D (CEA)
Specificity	89.8%	100%	100%	100%
Sensitivity	69.8%	100%	100%	100%
Dilutions				
Undiluted	6/6	6/6	6/6	6/6[a]
1/10	0/20	15/20	20/20	20/20
1/32		0/20	20/20	20/20
1/100			0/20	20/20
1/317				18/20
1/1,000				1/20
1/3,170				0/20

[a] Positive/tested.

observed among the 300 positive samples (54). Replicates reproduced similar results. In parallel, two other evaluated tests showed similar performances, while the fourth test presented 89.8% specificity and 69.8% sensitivity (Table 1).

The excellent performances of three tests among four in this first part of the evaluation were the minimum required by the authorities. Indeed, specificity was tested on only 1000 samples. Classically, false-positive cases (i.e., healthy samples giving positive results) in diagnostics are mainly due to cross-reaction of antibodies with nonspecific proteins of the samples. In the context of PrPres detection, this risk is strongly decreased by the fact that these tests used protein degradation for PrPres purification prior to detection. This proteolysis degrades other proteins, limiting nonspecific reactions.

Specificity is clearly an important economic issue in the field, to avoid frequent and irrelevant blockades at the abattoir. Nevertheless, in terms of public health, sensitivity is the major point to limit false-negative cases, i.e., undiagnosed infected animals and subsequent lack of confidence in the system. In the first part of the study, all the infected animals analyzed were clinically affected: 100% of sensitivity of these samples corresponded to the level of detection of clinical examination by veterinary practicians. Diagnostic tests needed then a higher sensitivity to present any interest, i.e., detection of infected animals that were not previously diagnosed.

This issue was assessed by the second part of the study. Previous pathogenesis studies in experimental models of prion diseases showed that both infectivity and PrPres slowly accumulate in the central nervous system before the onset of clinical signs (55): therefore, preclinical samples could be

mimicked by serially diluting positive samples in negative samples, as is classically done in experimental models. In accordance with the different competitors, mostly on the nature of the dilution buffer, DG Sanco provided us with these types of samples. The CEA test was able to detect as positive the 300-fold diluted samples, while other evaluated tests were not able to detect lower than 30-fold diluted samples (Table 1): this test turned out to be 10–300-fold more sensitive than the competitor's tests (54).

According to experimental models, the more a test is able to detect high-diluted samples, the earlier in the course of the disease can the detection of positive cases be achieved. After intracerebral inoculation of laboratory animals with stabilized prion strains, it is then possible to detect PrPres all along the second half of the incubation period. However, no data were available on PrPres levels during the natural disease in BSE-infected cattle.

B. Screening of Preclinical Samples with the CEA/Bio-Rad Test

The only model available in this context was a pathogenesis study performed during previous years in the United Kingdom, consisting in feeding calves with a known amount of brain (100 g) of BSE-clinically affected cattle (52). Animals were sacrificed at different times, and the presence of clinical signs and histological lesions was registered, compared to the presence of PrPres by immunohistochemistry, the presence of SAF by electronic microscopy, and the presence of infectivity by bioassay titration in mouse. In this study, while the first clinical signs appeared between 36 and 38 months postinfection, infectivity was detected by the bioassay as soon as 32 months postinfection.

Since the CEA/Bio-Rad test was recognized as the most sensitive of the evaluated tests, DG Sanco proposed to test these precious materials with our test. We were provided with coded samples (brain and cervical, dorsal, and lumbar spinal cord) of some of these animals. Analysis was performed blindly under constant surveillance of a DG Sanco representative with the industrial version of the test, and results were published afterward (56). PrPres was not detected in the brain of control animals, or in the brain of contaminated animals sacrificed earlier than 26 months postcontamination. All samples from animals sacrificed 32 months and above after contamination were found positive, except the sample from one animal that was also found negative at 40 months postexposure with all the other techniques used (histology, immunohistochemistry, presence of SAF, and bioassay).

Comparison of these results with those obtained with the other diagnostic methods used showed that all samples found positive with other methods were found positive with the CEA/Bio-Rad test (56). Moreover, some CEA/Bio-Rad positive samples were not detected by other techniques

(in immunohistochemistry, SAF detection, or histology), while infectivity of these samples was demonstrated through bioassay in the mouse, underlining the higher diagnostic sensitivity of this rapid test compared to classic approaches. For seven animals detected as positive with the CEA/Bio-Rad test in all tested parts of the central nervous system, all techniques were performed (Table 2). If we compare the ability of the different techniques to detect these animals as positive, clinical diagnostic would have detected one animal as positive and three would have been doubtful, while only three animals (plus one doubtful) would have been detected by histology. Fibril detection and immunohistochemistry would have detected six and seven animals, respectively, but only if all samples (brain and cervical, dorsal, and lumber spinal cord samples) had been tested. In fact, if only brain had been tested, as is currently performed in the field, only three and six animals would have been detected, respectively.

This evaluation highlighted the ability of a rapid test as sensitive as the CEA/Bio-Rad test to detect infected animals before the onset of clinical signs. Nevertheless, the onset of clinical signs is not a good reference to evaluate the sensitivity of a test. Indeed, contrary to laboratory models in which animals are syngenic, the course of BSE can be highly variable in cattle, and the oral route increased this variability. In this study, all animals were contaminated with the same amount of infected material, but the presence of clinical signs was highly variable among the animals, and was not correlated with the amount of PrPres (Fig. 2). Moreover, high differences of PrPres level can be observed in this study between animals sacrificed at the same time, suggesting variable kinetics of accumulation. The onset of clinical signs depends on the development of neuronal death in crucial areas of the brain. Moreover, individual sensibility can be so important that one animal seemed to be still uninfected 40 months after exposure in this study. In natural BSE, this high variability is increased by

Table 2 Comparison of Respective Sensitivities of Diagnostic Tools on Preclinical Samples

	Clinical status	Histology	Fibril detection	PrP immuno-histochemistry	CEA/Bio-Rad test
Samples			14/24	20/24	24/24
Animals	1/7 (3 ±)	3/7 (1 ±)	6/7	7/7	7/7
Brain stem			3/7	6/7	6/6
Cervical cord			3/7	5/7 (1 ±)	7/7
Thoracic cord			4/7	6/7	5/5
Lumbar cord			5/7	6/7 (1 ±)	6/6

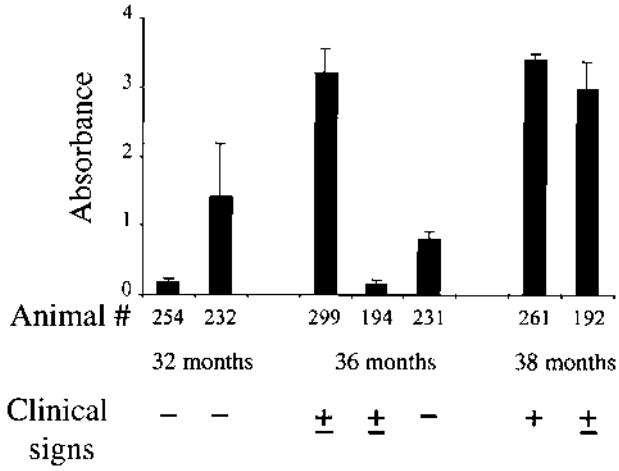


Figure 2 Detection of PrPres in samples from experimentally infected cattle. Cattle experimentally contaminated by the oral route with 100 g of BSE-infected brain were sacrificed at different times postexposure (two, three, and two animals 32, 36, and 38 months postexposure, respectively). The presence of PrPres in corresponding samples was assessed by the CEA/Bio-Rad test. Clinical status is indicated. (Adapted from Ref. 56.)

the variation of infectious dose: clinical signs appear between 22 months and 9 years (1–3,27). Owing to this important variability, the onset of clinical signs cannot be considered a reliable criterion. Conversely, PrPres and presence of infectivity in cattle present a good correlation.

In fact, in terms of public health, the real goal of a sensitive test is to be able to detect infected animals that present an infectious risk: the more sensitive the test is, the higher the level of security this test will bring.

C. Limit of Detection of the CEA/Bio-Rad Test Corresponds to Sensitivity of the Bioassay in Laboratory Models

In the DG Sanco evaluation study of May 1999, the initial positive sample that was used for dilutions was previously titrated by mouse bioassay and showed 1250 lethal doses 50% (LD_{50}) per gram of brain (54). This means that a 1/1250th part of 1 g of this brain, when inoculated into laboratory mice, kills 50% of these mice. Since tests were performed on the equivalent of 25 mg of brain (corresponding to 31 LD_{50}), calculations then tended to show that sensitivity of CEA/Bio-Rad test corresponded to less than 1 LD_{50}, since this sample was still detected when diluted 300-fold.

These estimations were confirmed with the preclinical samples studied as previously described, since the CEA/Bio-Rad test and the bioassay in mice detected the same samples as positive (56). To confirm this fact, DG Sanco provided serial dilutions of a positive BSE sample, which were previously inoculated into laboratory mice (57). The CEA/Bio-Rad test detected all samples until the 1000-fold dilution (Fig. 3): since the bioassay showed that this positive sample titrated 2000 LD_{50} per gram of brain and the test was performed in its industrial version on the equivalent of 33 mg of brain (corresponding to 66 LD_{50}), this new study confirms that sensitivity of this test is close to 0.1 LD_{50} in the mouse model bioassay.

The bioassay is the only test for prion disease that allows measurement of infectivity, and is up to now the reference in term of sensitivity (58). The mouse bioassay has been used for determination of levels of infectivity of different organs in scrapie-infected sheep (59) and BSE-infected cattle (9), and constitutes the basis of all regulations on specified-risk materials (60,61). In the last study comparing sensitivities of bioassay and the CEA/Bio-Rad test (57), it is of note that the inoculation of the 1000-fold-diluted samples induced only one death among 14 mice. The higher-diluted samples, which were detected as negative by the test, induced no mortality by bioassay. This means that contaminated samples not detected by the test presented such low levels of infectivity that they were unable to induce disease in the life span of the mouse when they were inoculated by the intracerebral route. This means that the CEA/Bio-Rad allows protection of the mouse in the case of intracerebral inoculation!

Mice inoculated by the intracebral route are 100-fold more sensitive to the BSE agent than cattle contaminated by the oral route (Fig. 4) [this factor corresponding to experimental data is confirmed by the facts that, on one hand, owing to the species barrier, cattle are approximatively 1000-fold more sensitive than mice to the BSE agent (62) when contaminated by the same route, and, on the other hand, the intracerebral route is more efficient than the oral route, close to 100,000-fold (63)]. The CEA/Bio-Rad test allows then protection of cattle against oral contamination with a 100-fold factor of security. Nothing is known about the sensitivity of humans to the BSE agent by the oral route, but it has been supposed for the risk assessment of BSE for humans that there is a species barrier corresponding to a factor between 1 and 10,000 (64): even in the worst case scenario, the CEA/Bio-Rad test then allows protection of human health with a 100 (to 1,000,000) factor of security. In other words, the infectious material used in the previous study showed 2000 LD_{50} per gram of brain for mouse inoculated by the intracerebral route, i.e., 20 LD_{50} per gram of brain for cattle contaminated by the oral route (1 LD_{50} in 50 mg). Since the test is able to detect samples containing an amount of PrPres 1000-fold lower than this sample, a contaminated brain that would

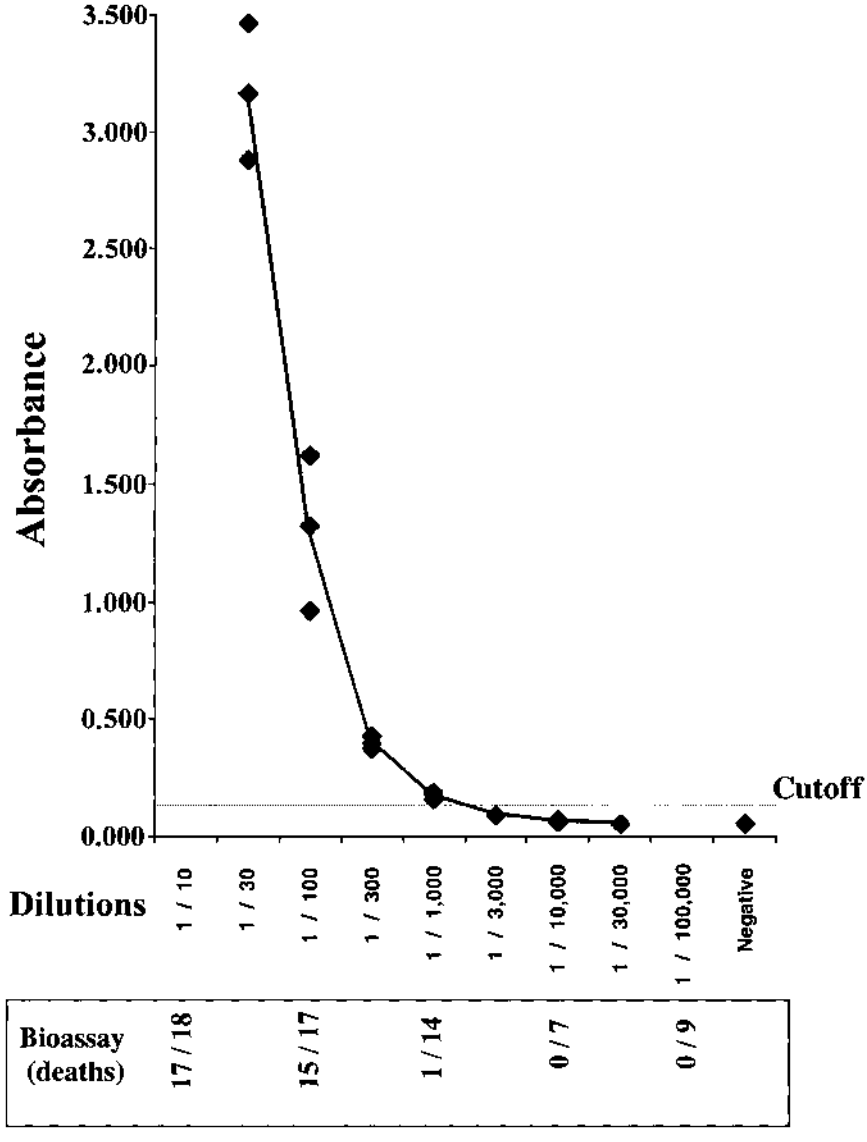


Figure 3 Detection of PrPres in serial dilutions of a titrated BSE-infected sample. Serial dilutions of a brain sample from clinically affected cattle were tested for PrPres by the CEA/Bio-Rad test. These dilutions were previously titrated by intracerebral inoculation to mice. The number of deaths is reported. (Adapted from Ref. 57.)

1 LD_{50}	Cattle oral route	50 mg
1 LD_{50}	Mice intracerebral route	0.5 mg
0.1 LD_{50}	Mice intracerebral route	0.05 mg
100% Detection	CEA/Bio-Rad test	0.03 mg

Figure 4 Comparison of different levels of sensitivity.

not be detected by the test would present a risk for cattle by the oral route at a dose over 50 g. Owing to species barrier, this undetected brain would present a risk for human health if 50 g–500 kg were eaten: even in the worst case (50 g of brain is sufficient), such an amount of banned offal cannot pass through all surveillance measures of the meat industry.

This risk assessment shows that, in addition to the removal of risky materials, such a sensitivity is required to allow security of public health against risk of transmission of BSE by food due to accidental contamination by central nervous system material. Moreover, a lower sensitivity would not be satisfactory in terms of public health, since a sample that would be found negative with a low sensitivity test could be detected as positive (but generally too late) by a second, more sensitive test.

A classic diagnostic policy can then be integrated at the level of the slaughterhouse with such sensitive tests: a first screening prevent positive undiagnosed animals from entering the human food chain, and a second test is then used later to confirm all positive samples detected by screening.

D. Confirmation Test

Indeed, in the context of diagnosis, especially in high-throughput screening, a first reactive sample needs to be confirmed by a second analysis. Then, a reproduced reactive sample needs to be confirmed, preferentially by using another technique. In the case of prion diseases, the currently referenced diagnostic test is histology (28), which highlights pathognomonic lesions.

Nevertheless, these lesions appear late in the course of the disease, and we have previously shown that this technique is not able to confirm all the positive animals detected by our test (56). Detection of PrPres by immunohistology is in regard to its high sensitivity, a diagnostic test that could be used for confirmation of reactive samples (65). But it is important to note that this confirmatory test is laborious and requires an experienced, talented analyst to be correctly performed. Moreover, it takes a few days to be performed, and is generally impracticable on autolyzed or poorly stored samples. Moreover, this technique is not currently standardized.

To have a rapid confirmatory test with equivalent sensitivity to our screening test that could be performed whatever the quality of the sample, we

developed a Western blot test based on detection of PrPres. This technique allows qualitative confirmation of the presence of PrPres in the sample (66), based on the electrophoretic pattern and size of the bands that are detected, and is clearly differentiated from normal PrP, which exhibits bands with higher molecular weights, indicating an incomplete protease digestion.

Based on the same purification procedure as the screening test, and associated to high-affinity specific antibodies, this Western blot has similar sensitivity to the screening test (Fig. 5) and higher sensitivity than other Western blot protocols. Moreover, the rapidity of this protocol allows for

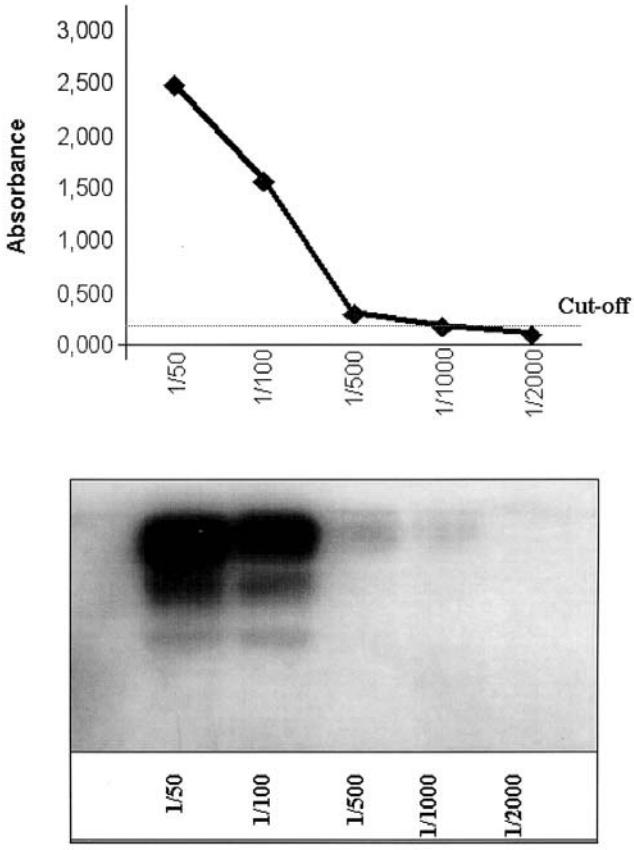


Figure 5 Comparison of sensitivity of the CEA/Bio-Rad immunometric assay and confirmation Western blot. Serial dilutions of a brain sample from clinically affected cattle were tested for PrPres by the CEA/Bio-Rad test. The same samples were confirmed by Western blot.

answers in less than 1 day. This confirmatory test has now been developed under an industrial format by Bio-Rad and is for the moment under homologation by some European Reference Centres in charge with confirmation of samples found positive on field.

IV. PERFORMING CEA/BIO-RAD TEST IN THE FIELD

A. Optimal Sampling for BSE Detection by the CEA/Bio-Rad Test

This test is adapted to be used on every part of the central nervous system. Nevertheless, to get the best benefit of such a sensitivity, it is necessary to perform the test on the part of the brain that contains the highest amount of PrPres. The region of the obex in the brain stem has been widely described as the optimal part for prion diagnosis in histology (67): neuroinvasion occurs along afferent and efferent nerves after peripheral replication, and the obex is the location of nuclei of the vagus nerve, which seems highly involved in neuroinvasion (Fig. 6) (68).

Studies with the immunometric test have confirmed (Fig. 7) that heterogeneous amounts of PrPre can be found in different areas of the same brain, and cerebellum or cortex can be found negative in the brain of some BSE-infected cattle (69). However, brain stem always contains large amounts of PrPres, and studies on different parts of the brain stem showed there is a gradient of PrPres all along the brain stem, with a maximal level in the obex, at the level of the nuclei of the vagus nerve (53).

Practically, sampling of the obex region is performed in two steps. First, brain stem can be easily sampled in the field through the occipital foramen after decapitation: use of a dedicated single-use tool allows a rapid and nonwounding sampling (70) and avoids opening the skull, and then exposing to potent infectious material. The obex is then sent to the laboratory, where it can be sampled.

To avoid any risk of contamination of technicians during sampling of the obex in the laboratory, a dedicated tool is proposed in the industrial version of the test to replace any cutting or sharpening tool. This rapid and nonwounding syringe allows catherizing the brain stem and sampling a carrot centered on the obex (nuclei of vagus nerve).

After sampling of brain stem at the slaughtering place, transportation and storage should be preferentially performed at low temperature, as is used for mostly biological samples: indeed, good conservation of the samples is needed to be able to perform histology or immunohistology as the confirmatory test, if needed. Freeze/thaw cycles do not alter performance of the test, even if samples are stored homogenized, contrary to other tests that were evaluated (71).

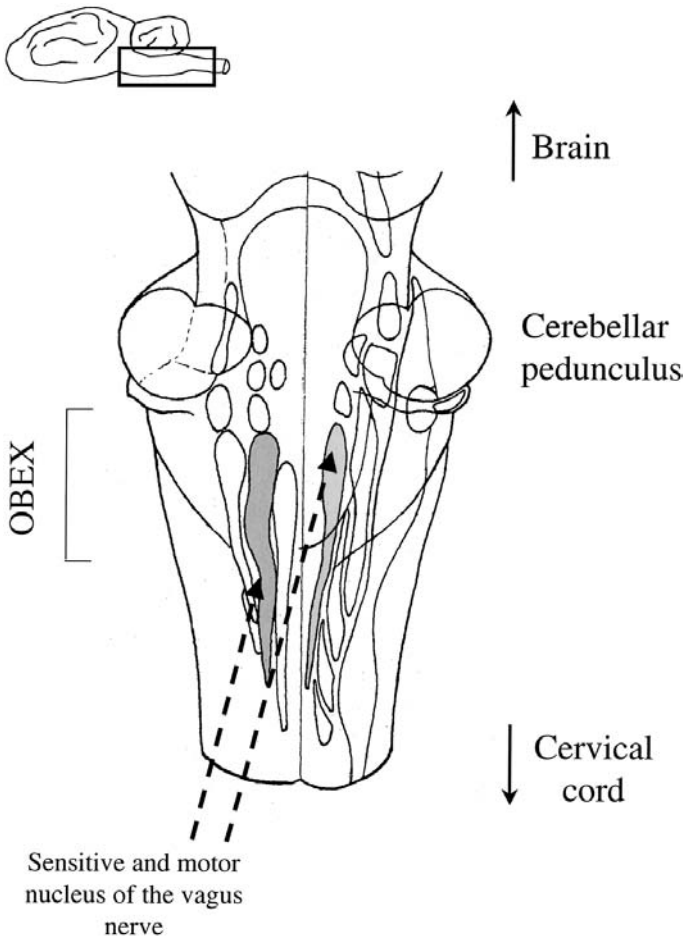


Figure 6 Anatomical projections of sensitive and motor nuclei of the vagus nerve. Sensitive and motor nuclei of the vagus nerve are located at the level of the obex, the part of the brain stem directly behind the cerebellar pedunculi.

Testing can also be correctly performed on autolyzed samples, since PrPres is highly resistant to protease degradation (72). Nevertheless, care should be taken because extensive autolysis can modify the amount of total proteins in the samples by proteolysis, thus disturbing the optimal ratio of proteinase K/proteins in the test. On the other hand, extensive desiccation of the brain stem would increase the relative amount of proteins in the 350 mg of obex sampled, also disturbing this ratio. Moreover, an

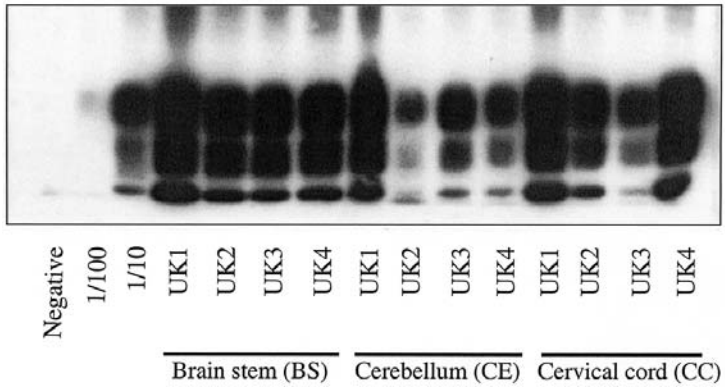


Figure 7 Heterogeneous distribution of PrPres in areas of the brain of clinically BSE-affected cattle. Brain stem, cerebellar, and cervical cord samples of four clinically BSE-affected cattle (UK1–UK4) were assessed for the presence of PrPres by Western blot, exhibiting variable levels of PrPres. Two serial dilutions of a control positive sample and one negative sample were used as references.

extensive autolysis until liquefaction implies a dilution of the obex in the whole brain stem, and therefore diminishes the relative sensitivity of the test.

B. Performing the Test in the Field

The CEA/Bio-Rad test was designed to be used in the field with high throughput, and is currently used in this way in many European countries. The purpose of such a sensitive test was to detect nondiagnosed BSE-affected cattle and to remove them from the human food chain in their entirety. In that way, a systematic screening at the slaughtherhouse was the tightest filter. After slaughtering of animals and cutting, carcasses are stored for some hours for swamping before further preparation: the test must be performed during this period, since it is easier to remove contaminated carcasses rather than searching for several pieces of meat, since they are often grouped in batches with pieces of other slaughtered animals.

Indeed, it was necessary to adapt the test to be easily performed in such a short time. Therefore, the test was developed with short incubation periods: the test can now be performed in less than 3.5 h. Moreover, robustness was particularly assessed to keep efficiency in high throughput. Finally, the standardized format of the microtitration plate is particularly

adapted for high-throughput screening (multichannel pipettes, automatic plate washers and readers), and can be fully automated.

Particular attention was paid to security of users, with regard to the professional risk of contamination with BSE-positive samples. Cutting, stinging, and sharpening materials were then avoided for sampling: a patented nonsharpening tool allows sampling the brain without any risk; calibration of the grinded samples is performed by the passage through a nonstinging needle. Also, grinding is performed in closed grinding units to avoid any aerosols. Finally, the microtitration plate format is perfectly adapted to security of analysis and traceability. Moreover, coloration at different steps ensures good processing.

V. APPLICATIONS OF THE CEA/BIO-RAD TEST TO OTHER PURPOSES

A. Adaptation to Other TSEs in Other Animal Species

Although the CEA/Bio-Rad test has been optimized to detect BSE in cattle, it can be used for detecting other TSEs in other animal species, with some optimization if necessary. Indeed, PrP is highly conserved between species (47), and the antibodies cross-react with different PrP.

Scrapie can be detected in the same way in sheep and goat brains with similar specificity and sensivity (Fig. 8a). Moreover, high amounts of PrPres can be detected in brains of chronic wasting disease (CWD)–affected wild ruminants (deer and elk) (Fig. 8b). It is then technically possible to use this type of test for epidemiological studies and/or systematic analysis of these diseases.

In humans, confirmation of CJD and vCJD suspicion is performed by histology and immunohistology (15,16). Preliminary comparative studies showed that the test gave similar conclusions in its ELISA and Western blot versions with regard to the presence of human PrPres.

Finally, an optimized test is able to detect PrPres in the brains of experimentally contaminated laboratory animals (mouse, hamster, and monkey), which is of special interest for research purposes.

B. Detection of PrPres in Peripheral Organs

It has been widely shown in laboratory models that, after peripheral contamination, neuroinvasion occurs only after a long and silent phase of peripheral replication in some lymphoid organs (63,73): detection of prion disease by testing peripheral organs would then allow an earlier diagnosis. After intraperitoneal inoculation of mouse with either scrapie- or BSE-

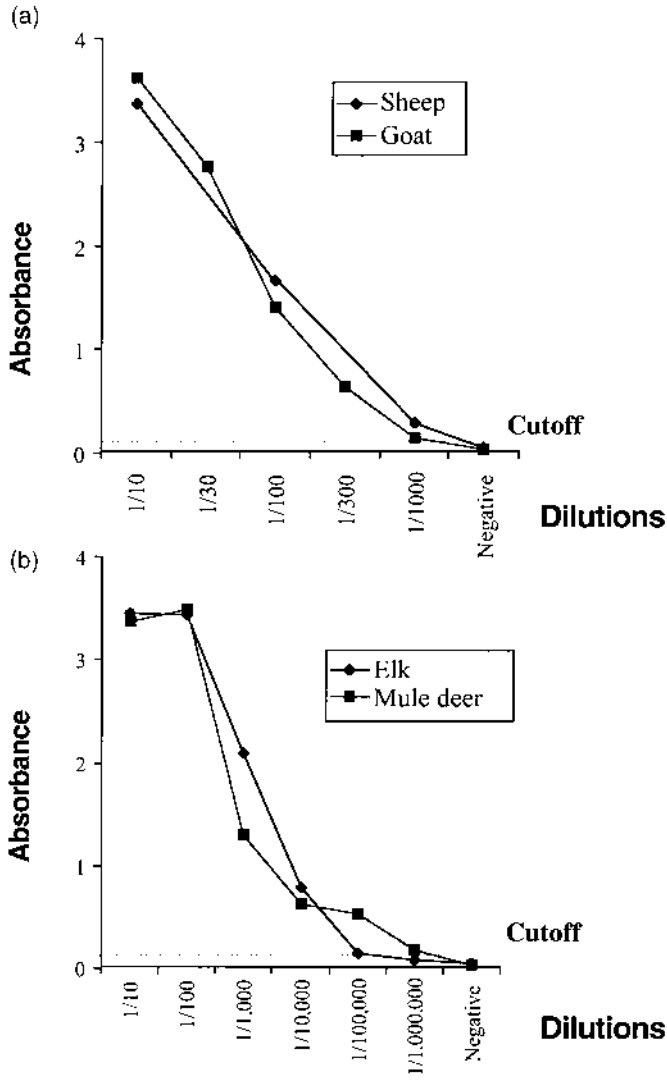


Figure 8 Limit of detection of the CEA/bio-Rad test for detection of scrapie and CWD in ruminants. Serial dilutions of brain samples from scrapie-clinically affected sheep and goat (a), or CWD-affected mule deer and elk (b) were tested for the presence of PrPres by the CEA/Bio-Rad test. Negative control samples were tested in parallel.

adapted strains, PrPres and infectivity rapidly accumulate in the spleen to reach a plateau (73). After oral contamination, Peyer's patches are the first organs of replication, followed by mesenteric lymph nodes and spleen (74,75). While it is currently admitted that PrPres is detected in Peyer's patches 45 days after oral administration to mouse (75), we detected it with our test as soon as 21 days postadministration (E. Comoy, unpublished results).

Infectivity has also been found in some peripheral organs of scrapie-infected sheep by bioassay (59), leading to International Office of Epizooty and World Health Organization classification of specified risk materials (60,61). Also, detection of PrPres in such organs has been performed by immunohistochemistry (76–78). Our first experiments showed that our test can be adapted for detecting PrPres in these peripheral organs of scrapie-infected sheep and goats, even before neuroinvasion and presence of PrPres in the brain (manuscript in preparation). Moreover, detection of PrPres was possible in lymphoid organs of CWD-affected wild ruminants. These different findings are of crucial interest for modeling these diseases, and have important implications in terms of epidemiology and prophylaxis, by explaining the potent routes of contamination and dissemination of infectivity.

In naturally BSE-infected cattle, neither infectivity nor PrPres has been detected in peripheral organs (8,79). Only ileum was found positive in cattle experimentally contaminated with high amounts of infected material by the oral route (52). In this context, detection of PrPres in peripheral organs of cattle will be more difficult than in organs of other ruminants.

These different results showed that tests could be developed for detection of TSEs in ruminants other than cattle, based on detection of PrPres in peripheral organs, in the expectation that these organs always contain PrPres. This would allow for detecting infected animals earlier, before neuroinvasion. To keep similar performance as the current tests, first analysis showed that these tests should be performed on lymphoreticular organs. Since these organs can hardly be sampled during the life of the animal, those tests would certainly be postmortem tests.

C. The Particular Case of Muscle

It has recently been published that prion infectivity and PrPres could be detected in muscle from scrapie-infected mouse (80). With regard to the implication of such a finding in terms of public health, the CEA/Bio-Rad test was used on different muscular samples and corresponding lymph nodes of scrapie-infected sheep and BSE-infected cattle (Fig. 9). No PrPres was detected in muscle samples of these animals. Moreover, no PrPres was

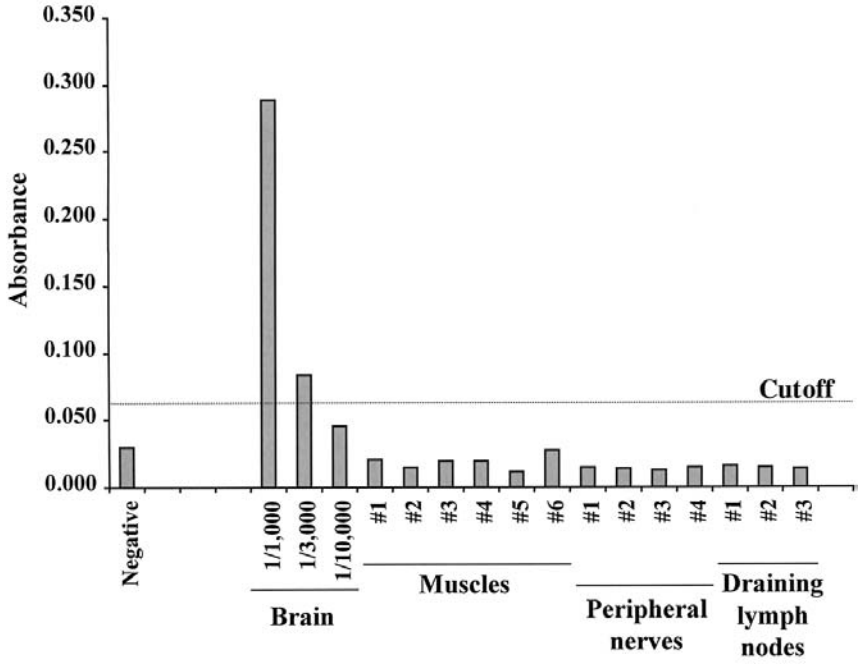


Figure 9 Detection of PrPres in muscle, peripheral nerves, and lymphoid organs of BSE-affected cattle. Different samples of muscle, corresponding nerves, and corresponding draining lymph nodes from clinically BSE-affected cattle were assessed for the presence of PrPres by the CEA/Bio-Rad test. The sample of the corresponding brain was serially diluted in negative muscle homogenate.

detected in lymphoreticular samples from BSE-infected cattle, while brain was still positive when diluted 1000–10,000-fold in cattle muscle. In sheep, no PrPres was detected in muscle while lymphoreticular organs were still positive when diluted 10–100-fold (data not shown) (Fig. 9). Since BSE-infected cattle brain presents infectivity around 10^3 LD_{50} per gram of brain by bioassay in mice by the intracerebral route, then infectivity in muscle, if any, is less than 0.1–1 LD_{50} per gram by bioassay in mice by the intracerebral route. Moreover, no PrPres was found in draining lymphoreticular organs, which are known to concentrate prion and which, in accordance with the sheep model, have at least 10–100-fold more PrPres than muscle (if any): it can then be extrapolated that infectivity in muscle would be less than 0.001–0.1 LD_{50} per gram by bioassay in mice by the intracerebral route. Muscle would then present a potent infectivity 10,000–1,000,000-fold lower than brain does. According to the sheep model,

infectivity in the case of BSE in sheep would be linked to draining lymph nodes and not muscle.

D. Differential Diagnosis of TSE Strains

The BSE agent has been successfully transmitted experimentally to sheep (7), and sheep have been fed with same contaminated meals in previous years (8): important attention has been drawn to potent BSE-contaminated sheep in the field, mostly because it seems that BSE in sheep present a peripheral distribution closer to that of scrapie in sheep than BSE in cattle (10). A major problem is that no difference between BSE and scrapie in sheep can be made regarding clinical signs or histopathological lesions (7).

Typing of TSE strains is possible by bioassay in mice, since they will induce different incubation periods and different lesional profiles, i.e., difference in terms of localization and intensity of lesions (81). Nevertheless, such a diagnosis is expensive and needs more than 1 year of incubation, and therefore is completely useless in the field.

BSE and scrapie PrPres present different resistance to protease digestion. We have therefore developed new controlled purification procedures of PrPres, which allow, in combination with antibodies recognizing different epitopes, differentiation between scrapie and BSE in sheep. Moreover, this differential test is able to selectively diagnose vCJD, thus being an important tool for human diagnosis and public health control.

VI. DEVELOPMENT OF NEW TESTS

Risk assessment of BSE and other ruminant TSEs for human health, on one hand, and risk of secondary transmission of CJD, on the other hand, clearly underlines the crucial necessity of developing new tests exhibiting higher sensitivity. Moreover, with regard to the physiopathology of these diseases, diagnostic tests using peripheral samples would lead to an earlier diagnosis in the course of the disease.

DG Sanco evaluated new diagnostic tests for BSE some months ago (Table 3), with the CEA/Bio-Rad test as the reference test (71). All these tests were based on detection of PrPres in the brain of cattle. In addition to some problems of specificity and sensitivity on clinically affected animals, none of the evaluated tests exhibited higher sensitivity.

Lymphoreticular organs such as the spleen or lymph nodes seem up to now to be the best candidates for peripheral detection of some TSEs. Nevertheless, blood diagnostic tests would be of considerable help in these diseases

Table 3 Evaluation of New Diagnostic Tests for BSE by DG Sanco, 2002

	Test A (ID-Lelystad)	Test B (PerkinElmer)	Test C (Prionics) (LIA)	Test D (UCSF)	Test E (MRC Prion Unit)
Specificity	97.9%	100%	97.9%	100%	100%
Sensitivity	100%	99.3%	100%	100%	100%
Dilution					
Undiluted	1/1	1/1	1/1	1/1	2/2[a]
1/10	4/4	1/4	4/4	4/4	8/8
1/32	0/4		1/4	2/4	8/8
1/100			0/4	1/4	7/8
1/316				0/4	2/8
1/1,000				0/4	0/8
Negative				0/5	

The CEA/Bio-Rad test, as reference test, exhibited similar performances as during the first European validation (1/316).
[a] Positive/tested.

(82,83). Although it has been shown that infectivity could be detected in blood (10,84), no test is available at the moment, since the low blood levels of PrPres, if any, will need higher sensitivity to be detected (85). Therefore, an amplification procedure of PrPres has recently been proposed (86), based on breakdown of PrPres aggregates by ultrasound to allow further conversion of PrPsens in vitro. This technique is currently under optimization. It has also been proposed to develop urinary tests for TSE detection, since PrPres was detected in the urine of several TSE-affected species (87). These findings are also currently under confirmation.

Finally, all the current tests are based on detection of PrPres, which is up to now the only specific biochemical marker of spongiform encephalopathies. Research on new potent, specific markers is currently underway.

In conclusion, the high sensitivity of the CEA test (Bio-Rad for its industrial version), higher than immunohistochemistry and similar to bioassay, ensures a level of security compatible with consumer protection with regard to BSE. Moreover, its short time procedure makes it possible to obtain an answer without disturbing flow in the abattoir, and the test is therefore intensively used for systematic BSE screening (around 70% of slaughtered cattle in Europe in 2002). For other prion diseases, like scrapie and CWD, such a high sensitivity is not indispensable for limited epidemiological purposes. Nevertheless, sensitivity becomes compulsory if consumer protection is involved.

REFERENCES

1. Wilesmith JW, Ryan JBM, Hueston WD, Hoinville LJ. Bovine spongiform encephalopathy: epidemiological features 1985 to 1990. Vet Rec 1992; 130: 90–94.
2. Anderson RM, Donnelly CA, Ferguson NM, Woolhouse ME, Watt CJ, Udy HJ, MaWhinney S, Dunstan SP, Southwood TR, Wilesmith JW, Ryan JB, Hoinville LJ, Hillerton JE, Austin AR, Wells GAH. Transmission dynamics and epidemiology of BSE in British cattle. Nature 1996; 382:779–788.
3. Wilesmith JW, Wells GAH, Cranwell MP, Ryan JBM. Bovine spongiform encephalopathy: epidemiological studies. Vet Rec 1988; 123:638–644.
4. Deslys JP, Picot A. Mad Cow Disease: The Risk for Humans. Dominos, Flammarion, Paris; 2002, pp 71–85.
5. Jeffrey M, Wells GAH. Spongiform encephalopathy in Nyala. Vet Pathol 1988; 25:398–399.
6. Wyatt JM, Pearson GR, Smerdon T, Jones TJG, Wells GAH. Spongiform encephalopathy in a cat. Vet Rec 1990; 126:513–516.
7. Foster JD, Bruce M, McConnell I, Chree A, Fraser H. Detection of BSE infectivity in brain and spleen of experimentally infected sheep. Vet Rec 1996; 138:546–548.
8. Butler DA. Doubts over ability to monitor risks of BSE spread to sheep. Nature 1998; 395:6–7.
9. Bradley R. Experimental transmission of bovine spongiform encephalopathy. In: Court and Dodet, eds. Transmissible Subacute Transmissible Encephalopathies: Prion Diseases. Paris: Elsevier, 1996, pp 51–56.
10. Jeffrey M, Ryder S, Martin S, Hawkins SA, Terry L, Berthelin-Baker C, Bellworthy SJ. Oral inoculation of sheep with the agent of bovine spongiform encephalopathy (BSE). 1. onset and distribution of disease-specific PrP accumulation in brain and viscera. J Comp Pathol 2001; 124: 280–289.
11. Hunter N, Foster J, Chong A, McCutcheon S, Parnham D, Eaton S, MacKenzie C, Houston F. Transmission of prion diseases by blood transfusion. J Gen Virol 2002; 83:2897–2905.
12. Will RG, Ironside JW, Zeidler M. A new variant of Creutzfeldt-Jakob disease in the UK. Lancet 1996; 347:921–924.
13. Lasmézas CI, Deslys JP, Demaimay R, Adjou KT, Lamoury F, Dormont D, Robain O, Ironside J, Hauw JJ. BSE transmission to macaques. Nature 1996; 381: 743–744.
14. Bruce ME, Will RG, Ironside JW, McConnell I, Drummond D, Suttie A, McCardle L, Chree A, Hope J, Birkett C, Cousens S, Fraser H, Bostock CJ. Transmissions to mice indicate that "new variant" CJD is caused by the BSE agent. Nature 1997; 389:498–501.
15. Collinge J, Sidle KCL, Meads J, Ironside J, Hill AF. Molecular analyses of prion strain variation and the aetiology of "new variant" CJD. Nature 1996; 383:685–690.
16. Hill AF, Desbruslais M, Joiner S, Sidle KCL, Gowland I, Collinge J. The same prion strain causes vCJD and BSE. Nature 1997; 389:448–450.

17. d'Aignaux JN, Cousens SN, Smith PG. Predictability of the UK variant Creutzfeldt-Jakob disease epidemic. Science 2001; 294:1663–1664.
18. Prusiner SB. Novel proteinaceous infectious particles cause scrapie. Science 1982; 216:136–144.
19. Hilton DA, Fathers E, Edwards P, Ironside JW, Zajicek J. Prion immunoreactivity in appendix before clinical onset of variant Creutzfeldt-Jakob disease. Lancet 1998; 352:703–704.
20. Will RG, Matthews WB. Evidence for case-to-case transmission of Creutzfeldt-Jakob disease. J Neurol Neurosurg Psychiatry 1982; 45:235–238.
21. Gibbs CJ, Asher DM, Brown PW, Fradkin JE, Gajdusek DC. Creutzfeldt-Jakob disease infectivity of growth hormone derived from human pituitary glands. N Engl J Med 1993; 328:358–359.
22. Thadani V, Penar PL, Partington J, Kalb R, Jansen R, Schonberger LB, Rabkin CS, Prichard JW. CJ disease probably acquired from cadaveric dura mater graft. J Neurosurg 1988; 69:766–769.
23. Manuelidis EE, Angelo JN, Gorgacz EJ, Kim JH, Manuelidis L. Experimental Creutzfeldt-Jakob disease transmitted via the eye with infected cornea. N Engl J Med 1977; 296:1334–1336.
24. Hogan RN, Brown P, Heck E, Cavanagh HD. Risk of prion disease transmission from ocular donor tissue transplantation. Cornea 1999; 18:2–11.
25. Collins S, Law MG, Fletcher A, Boyd A, Kaldor J, Masters CL. Surgical treatment and risk of sporadic Creutzfeldt-Jacob disease: a case-control study. Lancet 1999; 353:693–697.
26. Zobeley E, Flechsig E, Cozzio A, Enari M, Weissmann C. Infectivity of scrapie prions bound to a stainless steel surface. Mol Med 1999; 5:240–243.
27. Brugère-Picoux J, Chatelain J. L'encéphalopathie spongiforme bovine. Bull Acad Vét Prat, 1989; 73:543–567.
28. Wells GAH, Wells M. Neuropile vacuolation in brain: a reproducible histological processing artefact. J Comp Pathol 1989; 101:355–362.
29. Hadlow WJ, Kennedy RC, Race RE, Eklund CM. Virologic and neurohistologic findings in dairy goats affected with natural scrapie. Vet Pathol 1980; 17:187–199.
30. Porter DD, Porter HG, Cox NA. Failure to demostrate a humoral immune response to scrapie infection in mice. J Immunol 1973; 111:1407–1410.
31. Jimi T, Wakayama Y, Shibuya S, Nakata H, Tomaru T, Takahashi Y, Kosaka K, Asano T, Kato K. High levels of nervous system–specific proteins in cerebrospinal fluid in patients with early stage Creutzfeldt-Jakob disease. Clin Chim Acta 1992; 211:37–46.
32. Weber T, Otto M, Bodemer M, Zerr I. Diagnosis of Creutzfeldt-Jakob disease and related human spongiform encephalopathies. Biomed Pharmacother 1997; 51:381–387.
33. Kropp S, Zerr I, WJ Schulz-Schaeffer, Riedemann C, Bodemer M, Laske C, Kretzchmar HA, Poser S. Increase of neuron-specific enolase in patients with Creutzfeldt-Jakob disease. Neurosci Lett 1999; 261:124–126.
34. Bolton DC, McKinley MP, Prusiner SB. Identification of a protein that purifies with the scrapie prion. Science 1982; 218:1309–1311.

35. Bendheim PE, Brown HR, Rudelli RD, Scala LJ, Goller NL, Wen GY, Kascsak RJ, Cashman NR, Bolton DC. Nearly ubiquitous tissue distribution of the scrapie agent precursor protein. Neurology 1992; 42:149–156.
36. Fournier JG, Escaig-Haye F, de Villemeur T Billette, Robain O. Ultrastructural localization of cellular prion protein (PrPsens) in synaptic boutons of normal hamster hippocampus. C R Acad Sci (III) 1995; 318:339–344.
37. Pan KM, Baldwin M, Nguyen J, Gasset M, Seban A, Groth D, Mehlhorn I, Huang Z, Fletterick RJ, Cohen FE, Prusiner SB. Conversion of α-helices into β-sheets features in the formation of the scrapie prion proteins. Proc Natl Acad Sci USA 1993; 90:10962–10966.
38. Riek R, Hornemann S, Wider G, Billeter M, Glockshuber R, Wüthrich K. NMR structure of the mouse prion protein domain (121–131). Nature 1996; 382:180–182.
39. Prusiner SB. Biology of prion disease. J Acq Immun Defic Syndrome 1993; 6:663–665.
40. Prusiner SB, Hadlow WJ, Eklund CM, Race RE. Sedimentation properties of the scrapie agent. Proc Natl Acad Sci USA 1977; 74:4656–4660.
41. Lasmézas CI, Deslys JP, Robain O, Jaegly A, Beringue V, Peyrin JM, Fournier JG, Hauw JJ, Rossier J, Dormont D. Transmission of the BSE agent to mice in the absence of detectable abnormal prion protein. Science 1997; 275:402–405.
42. Race R, Raines A, Raymond GJ, Caughey B, Chesebro B. Long-term subclinical carrier state precedes scrapie replication and adaptation in a resistant species: analogies to bovine spongiform encephalopathy and variant Creutzfeldt-Jakob disease in humans. J Virol 2001; 75:10106–10112.
43. Kascsak RJ, Fersko R, Pulgiano D, Rubenstein R, Carp RI. Immunodiagnosis of prion disease. Immunol Invest 1997; 26:259–268.
44. Büeler H, Fischer M, Lang Y, Bluethmann H, Lipp HP, DeArmond SJ, Prusiner SB, Aguet M, Weissmann C. Normal development and behaviour of mice lacking the neuronal cell-surface PrP protein. Nature 1992; 356: 577–582.
45. Zanusso G, Liu D, Ferrari S, Hegyi I, Yin X, Aguzzi A, Hornemann S, Liemann S, Glockshuber R, Manson JC, Brown P, Petersen RB, Gambetti P, Sy MS. Prion protein expression in different species: analysis with a panel of new mAbs. Proc Natl Acad Sci USA 1998; 95:8812–8816.
46. Lehmann S, Harris DA. Mutant and infectious prion proteins display common biochemical properties in cultured cells. J Biol Chem 1996; 271:1633–1637.
47. Oesch B, Westaway D, Walchli M, McKinley MP, Kent SB, Aebersold R, Barry RA, Tempst P, Teplow DB, Hood LE, Prusiner SB, Weissmann C. A cellular gene encodes scrapie PrP 27–30 protein. Cell 1985; 40:735–746.
48. Merz PA, Somerville RA, Wisniewski HM, Iqbal K. Abnormal fibrils from scrapie-infected brain. Acta Neuropathol 1981; 54:63–74.
49. Meyer RK, McKinley MP, Bowman KA, Braunfeld MB, Barry RA, Prusiner SB. Separation and properties of cellular prion proteins. Proc Natl Acad Sci USA 1986; 83:2310–2314.
50. Serban D, Taraboulos A, De Armond SJ, Prusiner SB. Rapid detection of Creutzfeldt-Jakob disease and scrapie prion proteins. Neurology 1990; 40: 110–117.

51. Grassi J, Créminon C, Frobert Y, Frétier P, Turbica I, Rezaei H, Hunsmann G, Comoy E, Deslys JP. Specific determination of the proteinase K-resistant form of the prion protein using two-site immunometric assays: application to the post-mortem diagnosis of BSE. Arch Virol 2000; 16:197–205.
52. Wells GAH, Hawkins SAC, Green RB, Austin AR, Dexter I, Spencer YI, Chaplin MJ, Stack MJ, Dawson M. Preliminary observations on the pathogenesis of experimental bovine spongiform encephalopathy (BSE): an update. Vet Rec 1998; 142:103–106.
53. Moynagh J, Schimmel H, Kramer GN. Preliminary report: the evaluation of tests for the diagnosis of transmissible spongiform encephalopathy in bovines. European Commission, Directorate-General XXIV, Consumer policy and consumer health protection, 1999.
54. Moynagh J, Schimmel H. Tests for BSE evaluated. Nature 1999; 400:105.
55. Kimberlin RH, Walker CA. Pathogenesis of mouse scrapie: evidence for neural spread of infection the CNS. J Gen Virol 1980; 51:183–187.
56. Grassi J, Comoy E, Simon S, Créminon C, Frobert Y, Trapmann S, Schimmel H, Hawkins SAC, Moynagh J, Deslys JP, Wells GAH. Rapid test for the preclinical postmortem diagnosis of BSE in central nervous system tissue. Vet Rec 2001; 149:577–582.
57. Deslys JP, Comoy E, Hawkins S, Simon S, Schimmel H, Wells GAH, Grassi J, Moynagh J. Screening slaughtered cattle for BSE. Nature 2001; 409: 476–477.
58. Kimberlin RH, Walker CA. Characteristics of a short incubation model of scrapie in the golden hamster. J Gen Virol 1977; 34:295–304.
59. Hadlow WJ, Kennedy RC, Race RE. Natural infection of Suffolk sheep with scrapie virus. J Infect Dis 1982; 146:657–664.
60. Report of a WHO consultation on public health issues related to animal and human spongiform encephalopathies. World Health Organization. 12–14 November 1991.
61. Note for guidance–Guidelines for minimizing the risk of transmissible agents causing spongiform encephalopathies via medicinal products—by the Committee for Proprietary Medicinal Products—Ad Hoc Working Party on Biotechnology/Pharmacy and Working Party on Safety Measures. 11 December 1991.
62. Foster JD, Hope J, McConnell I, Bruce M, Fraser H. Transmission of bovine spongiform encephalopathy to sheep, goats, and mice. Ann NY Acad Sci 1994; 724:300–303.
63. Kimberlin RH, Walker CA. Pathogenesis of mouse scrapie: effect of route of inoculation on infectivity titres and dose-response curves. J Comp Pathol 1978; 88:39–47.
64. Opinion of the Scientific Steering Committee. Oral exposure of humans to the BSE agent: infective dose and species barrier. 13–14 April 2000.
65. Hayward PA, Bell JE, Ironside JW. Prion protein immunochemistry: reliable protocols for the investigation of Creutzfeldt-Jakob disease. Neuropathol Appl Neurobiol 1994; 20:375–383.

66. Doi S, Ito M, Shinagawa M, Sato G, Isomura H, Goto H. Western blot detection of scrapie-associated fibril protein in tissues outside the central nervous system from preclinical scrapie-infected mice. J Gen Virol 1988; 69: 955–960.
67. Fontaine JJ, Parodi AL. Lésions et diagnostic histopathologique de l'encéphalopathie spongiforme bovine. Point Vét 1991; 22:721–732.
68. Beekes M, McBride PA, Baldauf E. Cerebral targeting indicates vagal spread of infection in hamsters fed with scrapie. J Gen Virol 1998; 79:601–607.
69. Schaller O, Fatzer R, Stack M, Clark J, Cooley W, Biffiger K, Egli S, Doherr M, Vandevelde M, Heim D, Oesch B, Moser M. Validation of a Western immunoblotting procedure for bovine PrPsc detection and its use as a rapid surveillance method for the diagnosis of bovine spongiform encephalopathy (BSE). Acta Neuropathol 1999; 98:437–443.
70. Venturini M. Matériel et méthode de prélèvement d'un échantillon de tronc cérébral pour le diagnostic de laboratoire de l'encéphalopathie spongiform bovine (ESB). Bull Acad Vét France 2000; 153:383–390.
71. Schimmel H, Catalani P, Le Guern L, Prokisch J, Philipp W, Trapmann S, Zeleny R, Moynagh J. The evaluation of five rapid tests for the diagnosis of transmissible spongiform encephalopathy in bovines (2nd study). European Commission, Directorate General JRC, Institute for Reference Materials and Measurements, 2002.
72. Race RE, Ernst D, Sutton D. Severe autolysis does not prevent scrapie diagnosis in sheep. J Vet Diagn Invest 1994; 6:486–489.
73. Eklund CM, Kennedy RC, Hadlow WJ. Pathogenesis of scrapie virus infection in the mouse. J Infect Dis 1967; 117:15–22.
74. Kimberlin RH, Walker CA. Pathogenesis of scrapie in mice after intra-gastric infection. Virus Res 1989; 12:213–220.
75. Maignien T, CI Lasmézas, Beringue V, Dormont D, Deslys JP. Pathogenesis of oral route infection of mice with scrapie and BSE. J Gen Virol 1999; 80: 3035–3042.
76. Van Keulen LJ, Schreuder BE, Meloen RH, Mooij-Harkes G, Vromans ME, Langeveld JP. Immunohistochemical detection of prion protein in lymphoid tissues of sheep with natural scrapie. J Clin Microbiol 1996; 34:1228–1231.
77. Schreuder BE, Van Keulen LJ, Vromans ME, Langeveld JP, Smits MA. Tonsillar biopsy and PrPsc detection in the preclinical diagnosis of scrapie. Vet Rec 1998; 142:564–568.
78. Andreoletti O, Berthon P, Marc D, Sarradin P, Grosclaude J, van Keulen L, Schelcher F, Elsen JM, Lantier F. Early accumulation of PrP(Sc) in gut-associated lymphoid and nervous tissues of susceptible sheep from a Romanov flock with natural scrapie. J Gen Virol 2000; 83:3115–3126.
79. Hill AF, Butterworth RJ, Joiner S, Jackson G, Rossor MN, Thomas DJ, Frosh A, Tolley N, Bell JE, Spencer M, King A, Al-Sarraj S, Ironside JW, Lantos PL, Collinge J. Investigation of variant Creutzfeldt-Jakob disease and other human prion diseases with tonsil biopsy samples. Lancet 1999; 353:183–189.
80. Bosque PJ, Ryou C, Telling G, Peretz D, Legname G, DeArmond SJ, Prusiner SB. Prions in skeletal muscle. Proc Natl Acad Sci USA 2002; 99:3812–3817.

81. Fraser H, Bruce ME, Chree A, McConnell I, Wells GAH. Transmission of bovine spongiform encephalopathy and scrapie to mice. J Gen Virol 1992; 73:1891–1897.
82. Schmerr MJ, Goodwin KR, Cutlip RC. Capillary electrophoresis of the scrapie prion protein from sheep brain. J Chromatogr A 1994; 680:447–453.
83. Schmerr MJ, Jenny A, Cutlip RC. Use of capillary sodium dodecyl sulfate gel electrophoresis to detect the prion protein extracted from scrapie-infected sheep. J Chromatogr B Biomed Sci Appl 1997; 697:223–229.
84. Houston F, Foster JD, Chong A, Hunter N, Bostock CJ. Transmission of BSE by blood transfusion in sheep. Lancet 2000; 356:999–1000.
85. Lee DC, Stenland CJ, Miller JLC, Cai K, Ford EK, Gilligan KJ, Hartwell RC, Terry JC, Rubenstein R, Fournel M, Petteway SR Jr. A direct relationship between the partitioning of the pathogenic prion protein and transmissible spongiform encephalopathy infectivity during the purification of plasma proteins. Transfusion 2001; 41:449–455.
86. Saborio GP, Permanne B, Soto C. Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding. Nature 2001; 411:810–813.
87. Shaked GM, Shaked Y, Kariv-Inbal Z, Halimi M, Avraham I, Gabizon R. A protease-resistant prion protein isoform is present in urine of animals and humans affected with prion diseases. J Biol Chem. 2001; 276:31479–31482.

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The Diagnosis of Transmissible Spongiform Encephalopathies Using Differential Extraction and DELFIA

Geoff Barnard
University of Cambridge, Cambridge, England

Man-Sun Sy
Case Western Reserve University School of Medicine, Cleveland, Ohio, U.S.A.

I. INTRODUCTION

The conversion of a normal membrane glycoprotein, the cellular prion protein (PrP^{C}), to an insoluble aggregated isoform (PrP^{Sc}) is thought to be the key process in the pathogenesis of transmissible spongiform encephalopathies (TSEs) (1). Consequently, the specific detection of PrP^{Sc} has formed the basis for the biochemical diagnosis of TSEs, which include bovine spongiform encephalopathy (BSE), scrapie in sheep, and Creutzfeld-Jacob disease (CJD) in humans (2).

Most often, this specificity has been achieved by the differential proteolysis of PrP^{C} using enzymes such as proteinase K (PK) or trypsin, prior to the detection of a protease-resistance core of PrP (designated PrP^{res}) by Western blotting (3). This procedure can currently detect between 10 and 100 pg (10^{8}–10^{9} molecules) of PrP^{Sc}, which is about the same sensitivity as a heterologous bioassay of bovine or human tissue for TSE infectivity in mice (4).

The need for more rapid procedures that would allow for a greater throughput for mass screening purposes has led to the development of immunochemical assays (5–7). In 1996, work began at the Institute for Animal

Health (IAH) in Compton, United Kingdom to develop a sensitivity immunoassay for PrP based upon the principles of time-resolved fluorescence.

II. TIME-RESOLVED FLUORESCENCE

A characteristic of fluorescence is its lifetime, which can be defined as the decay rate of the excited state. The fluorescence from normal components in assay systems (i.e., serum fluors and proteins, plastics, assay buffers, etc.) is usually relatively short-lived, of nanosecond duration. On the other hand, the fluorescent lifetimes of certain beta-diketone chelates of several different lanthanides (e.g., europium or samarium) are up to six orders of magnitude longer (approximately 10^{-3}–10^{-6} seconds). Consequently, the millisecond emission from these metal chelates can be distinguished easily from the background nanosecond fluorescence by using a gated fluorometer with time-resolution capability and employing appropriate delay, counting, and cycle times (8). The measurement principle is illustrated in Fig. 1.

Europium (Eu^{3+}) and samarium (Sm^{3+}) are two of several elements that comprise the lanthanide series. Europium has an atomic weight of 152 and is similar in size to the radioactive isotope iodine-125, which has been commonly used in radioimmunoassay. Europium, however, is a stable nonradioactive isotope and does not denature the material to which it is attached by radiolysis. Like iodine, however, europium requires a carrier molecule to form a conjugate with any immunoreactive component. The

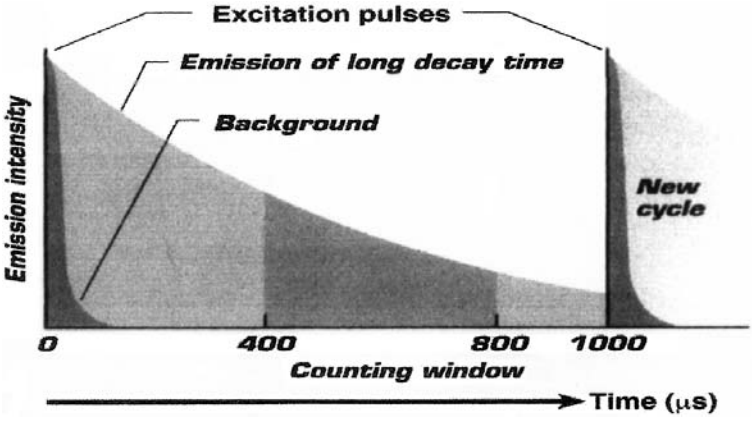

Figure 1 The measurement principle of time-resolved fluorescence.

commercial availability of stable europium or samarium chelates of the EDTA derivative DTTA [N1-(*p*-isothiocyanatobenzyl)-diethylenetriamine N1, N2, N3, N3-tetra-acetic acid] enables the research worker to produce labeled antibodies with high specific activity by very simple and reliable procedures (9).

The decay time and intensity of lanthanide fluorescence are dependent on the structure of the ligands that chelate the metal and on the nature of its physical environment (10). For example, the Eu-DTTA chelate has minimal fluorescence and to measure the fluorescence of europium or samarium with high sensitivity, it is necessary to dissociate the element from the immunoreactive components. This is achieved simply by lowering the pH to 2–3 through the addition of an appropriate buffer (the "enhancement solution") containing a beta-diketone (e.g., 2-naphtoyl trifluoroacetate) that chelates the lanthanide at low pH and possesses the required properties to produce a chelate with high-intensity fluorescence.

In addition, it is necessary to expel water from the complex and this is accomplished by the presence, in the enhancement solution, of a detergent (Triton X-100), which, together with a fatty acid derivative (trioctylphosphine oxide), dissolves the chelate and keeps it inside a micelle. The total immunoassay process may be summed up by the acronym DELFIA (dissociation-enhanced lanthanide fluoroimmunoassay).

III. IMMUNOASSAY FOR PrP

In the initial work at IAH, several monoclonal antibodies were investigated to determine a pair of reagents that might be used to establish a sensitive two-site immunometric assay for PrP. The antibodies included: (1) FH11 with multiple N-terminal epitope specificity (11), (2) KG9/DF7 with similar C-terminal specificity (12), (3) 3F4 from Senetek (Staten Island, NY) binding to an epitope in the hydrophobic region of the molecule (2), and (4) 6H4 from Prionics (Zurich, Switzerland) binding to an epitope in the core region (13). A diagrammatic representation of PrP together with an indication of the epitope specificity of the four antibodies is shown in Fig. 2.

Each of the antibodies was labeled to high specific activity with europium by the established procedures (9) and set aside for use as a detecting reagent. In addition, each of the unlabeled monoclonals was coated onto Nunc Maxisorp microtiter plates to be used as a capture reagent. Briefly, the antibodies were diluted in 0.1M phosphate-buffered saline (pH 7.4) containing 0.1% sodium azide within the range 2–5 μg/ml, and 200 μL of diluted reagent was added to each microtiter well using a multichannel

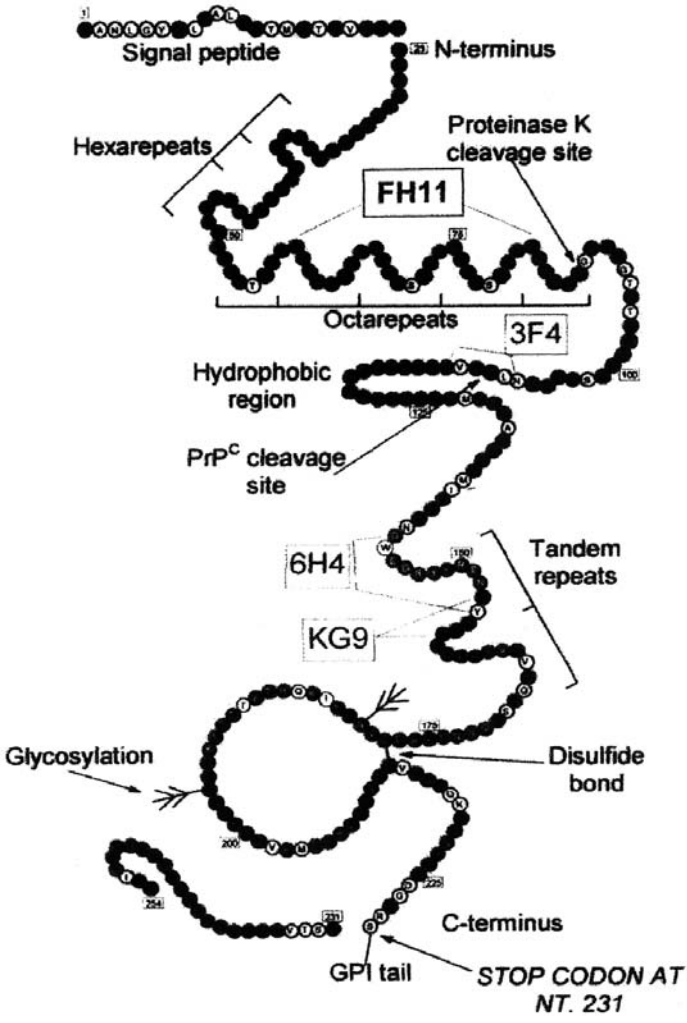

Figure 2 Representation of PrP illustrating the specificity of four different monoclonal antibodies (FH11, 3F4, 6H4, KG9).

pipette. The plates were covered with plastic adhesive sheet and incubated overnight at 4°C. Subsequently, the plates were washed three times and tapped dry on absorbent paper.

Prior to use, the plates were blocked by the addition of 200 μL/well of blocking buffer (2% BSA in 0.1M PBS) using a multichannel pipette and incubated with shaking for at least 1 h at room temperature.

A. Reference Material

Various reference materials have been used as calibrators in the assessment of the alternative immunoassays. These include: (1) recombinant hamster PrP, (2) recombinant bovine PrP, and (3) brain tissue extracts from various species. In addition, in collaboration with Dr. Ian MacGregor of the Scottish National Blood Transfusion Service (Edinburgh, UK), it was discovered that human platelet–enriched plasma was a rich source of PrP that could be used as a readily accessible and appropriate standard (14,15). Calibration curves of human platelet–derived PrP using two different immunoassays are shown in Fig. 3.

Because of availability and cost, it was not possible to use 6H4 as a capture reagent. Consequently, it was decided that FH11, despite its N-terminal specificity, would be a suitable coating antibody. Calibration curves obtained with FH11-coated plates and three different detecting antibodies are shown in Fig. 4.

On the basis of initial studies, antibody availability, and assay specificity, FH11 was adopted as capture reagent with 3F4 as detector. Up to this time, it was not realized that 3F4 would cross-react with bovine or ovine PrP to any appreciable extent. Nevertheless, because of the inherent sensitivity of a two-site immunoassay, a robust detection method was established at IAH by December 1996 and used in subsequent experiments. One of the benefits

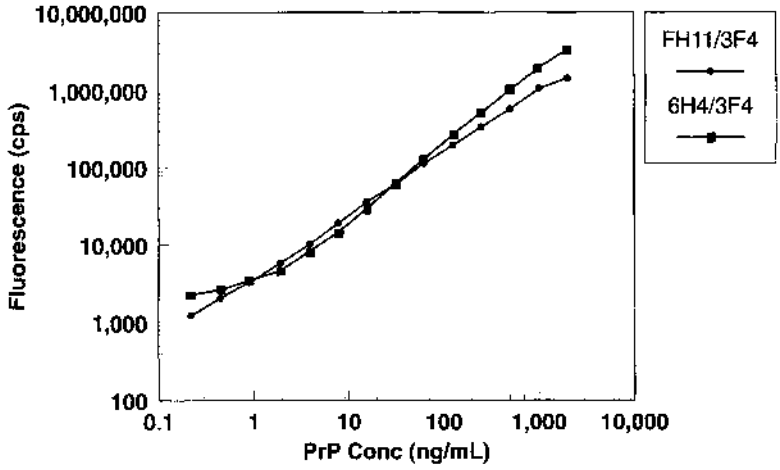

Figure 3 Calibration curves of human platelet–derived PrP using two different immunoassays: (1) capture antibody—FH11; detecting antibody—3F4 and (2) capture antibody—6H4; detecting antibody—3F4.

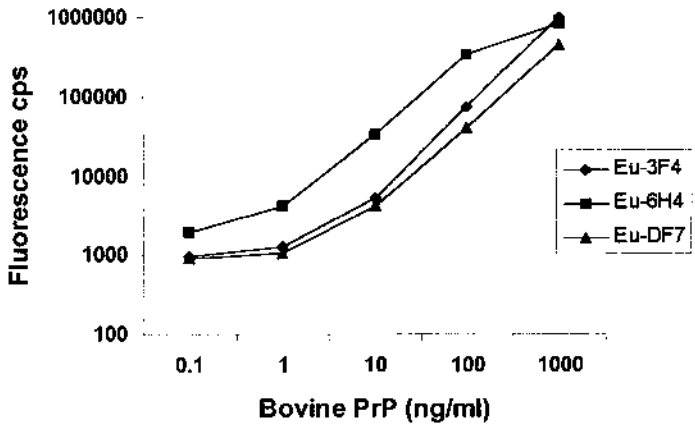

Figure 4 Calibration curves of recombinant bovine PrP on coated FH11 microtiter plates using three different detecting antibodies: (1) europium-labeled 3F4; (2) europium-labeled 6H4; and (3) europium-labeled DF7.

of this procedure was the ability of the immunoassay to detect PrP from several different species, including hamsters, bovine, ovine, and human.

Accordingly, PerkinElmer Life Sciences (formerly E.G. and G. Wallac, Turku, Finland), in contractual association with IAH, took over the preparation of the coated plates (FH11) and europium-labeled antibody (3F4). It is noteworthy that for large-scale commercial production of antibody-coated plates, large amounts of monoclonal antibody are required. For example, 110 μg of FH11 is coated on each plate (1 μg/well). This, however, has resulted in an extremely stable, high-affinity, activated surface. Coated plates have been used for up to a year without any discernible loss in activity.

IV. EARLY STUDIES ON TISSUE PREPARATION USING PROTEINASE K

On separate occasions, brain tissue samples from infected and noninfected hamsters, cattle, and sheep were carefully weighed and transferred to a sterile Dounce Homogeniser. Typically, 1.5 mL of homogenization buffer was addded to each gram of brain tissue. The homogenization buffers that have been used have included: (1) PBS (with or without detergent), and (2) 1 M GdnCL in 50 mM Tris-HC1 pH 7.5 containing 0.5% sulfabetaine (3–14). The brain tissues were homogenized with 10–15 passes using an appropriate "loose" pestle. Subsequently, a further 1.5 mL of homogenization buffer was

added and the brain tissue homogenized for another 10–15 passes using a "tight" pestle. The resulting mixtures were creamy emulsions and corresponded to a 25% brain homogenate based on the assumption that 1 g of brain tissue was equivalent to 1 mL. Aliquots of the emulsions were transferred to polycarbonate ultra centrifuge tubes.

PK (Sigma or Boehringer) was carefully weighed and dissolved in PBS to provide an appropriate stock solution. An aliquot of the PK solution was added to the emulsion in the polycarbonate tubes to give a final concentration of PK within the range 0–100 μg/mL. The tubes were sealed with PVC tape and incubated at 37°C for 30 min with gentle agitation. The digestion was stopped by the addition of 10 μL Pefabloc SC (0.5M solution in PBS) or by some alternative protease inhibitor. The polycarbonate tubes were transferred to a suitable rotor (e.g., Beckman TL100.3) and centrifuged at 100,000 rpm for 8 min at 22°C in an ultracentrifuge (e.g., Beckman TL100). The supernatant was removed and retained. Extraction buffer was added equal to the volume of supernatant removed. Generally, this extraction buffer was 6MGdnHC1 in 50 mM Tris-HC1 pH 7.5 containing 0.5% sulfabetaine (3–14). The pellet was resuspended by trituration with a plastic disposable Pasteur pipette. The samples were incubated for at least 5 min at room temperature.

Prior to the immunoassays, these tissue homogenates were diluted in assay buffer (approximately 1:50 v/v) to minimize the effect of detergents and chaotropic agents on the immunoassays.

A. Immunoassay

In the immunoassay 200 μL of each standard (recombinant hamster or bovine PrP) or diluted sample was added in duplicate to the FH11-coated microtiter plate. The plate was incubated on the shaker at 22°C for 60 min. Subsequently, the plate was washed three times, tapped dry on absorbent paper, and 200 μL of europium-labeled 3F4 (diluted at 1:500 v/v in assay buffer) added to each well. The plate was incubated on the shaker at 22°C for an additional 60 min. The plate was washed six times and tapped dry on absorbent paper, and 200 μL of enhancement solution was added, the plate shaken for 5 min at room temperature, and the fluorescence measured in the time-resolved fluorometer. The concentrations of PrP were determined using the proprietary PerkinElmer data reduction package (Multicalc). The result for hamster, bovine, and ovine tissues are shown in Figs. 5–7.

B. Interpretation

The findings were somewhat unexpected. The release of measurable PrP at low concentrations of PK was a consistent phenomenon in the limited

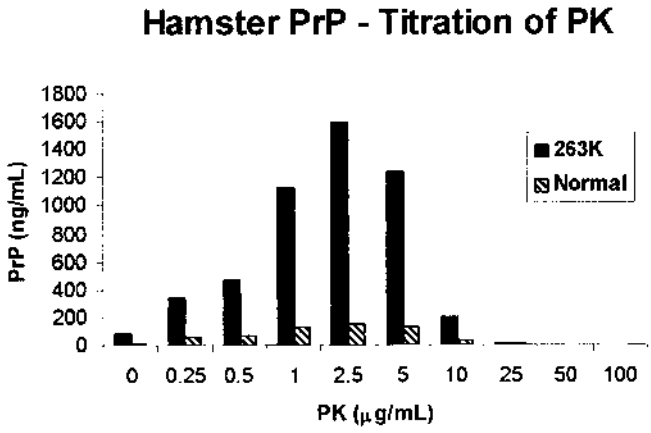

Figure 5 Measurement of disease-associated hamster PrP in brain tissue taken from one experimentally scrapie infected and one normal hamster in the presence of different concentrations of proteinase K.

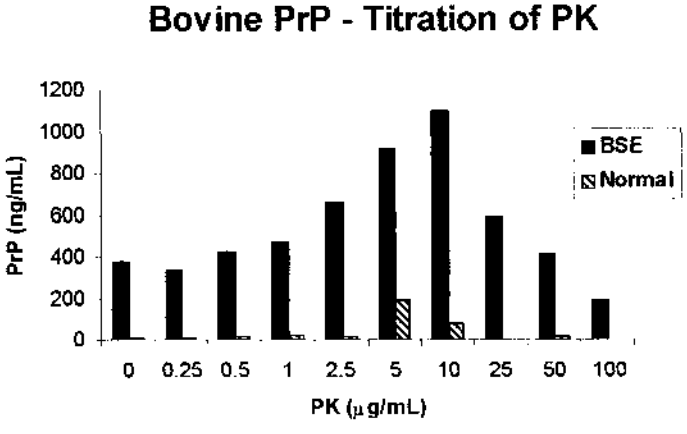

Figure 6 Measurement of disease-associated bovine PrP in the brain tissue taken from one BSE-infected and one normal animal in the presence of different concentrations of proteinase K.

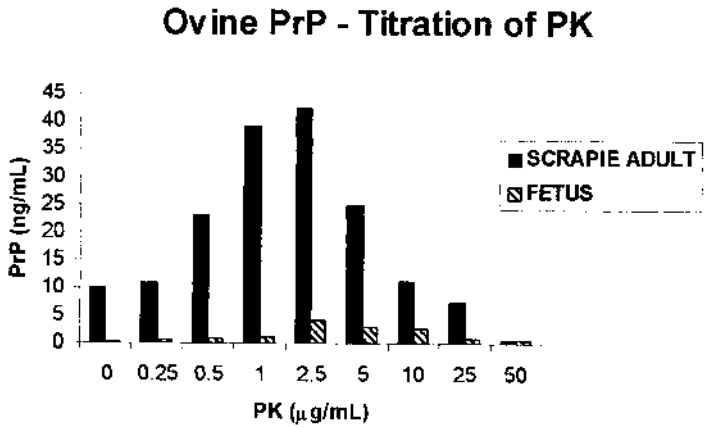

Figure 7 Measurement of disease-associated ovine PrP in brain tissue taken from one scrapie-infected animal and its fetus in the presence of different concentrations of proteinase K.

number of tissues that were investigated between December 1996 and May 1997. A possible explanation is the role that PK might play in the initial disaggregation of the disease-associated PrP^{Sc}. It may be postulated that the one or both of the relevant epitopes of PrP (i.e., for FH11 and 3F4) are masked in aggregated material. Consequently, disaggregation of PrP at very low concentrations of PK might facilitate the binding of the protein to its capture and detecting monoclonal antibodies.

An alternative explanation is supported by studies demonstrating the formation of complexes by disease-associated PrP with other proteins such as plasminogen (16) and putative chaperons such as the enigmatic Protein X (17). It could be postulated that any protein, small molecule [such as heparin (18) or glycosoaminoglycan (19)], or any other polyanion (20) might bind in the hydrophobic region of the PrP molecule. It is noteworthy that this region possesses the 3F4 epitope and the endogenous proteolytic cleavage site (see Fig. 1). Consequently, any molecule interacting with the disease-associated aggregate of PrP might do two things, namely: (1) mask epitopes and prevent the binding of *at least* europium-labeled 3F4 and, possibly, FH11 and (2) cause the aggregate to be resistant to endogenous (and experimental) proteolysis. Accordingly, the addition of PK at very low dose might disrupt aggregate complexes and allow the PrP to be bound and detected.

On the other hand, higher doses of PK begin to hydrolyze the protein with concomitant removal of at least the FH11 epitope. The ability of the immunoassay to detect PrP at higher concentrations of PK is, therefore,

severely compromised. With these limitations in mind, however, the technology was transferred to the Veterinary Laboratories Agency (VLA) (New Haw, Addlestone, Surrey, UK) in March 1998.

Based on these interesting phenomena, a tissue protocol designed to increase sample throughput by utilizing very small sample size (approx. 100 mg tissue), combined with differential proteolysis with PK, was developed by the VLA. This procedure, however, proved to be very difficult to control in the operational conditions of the surveillance laboratory and gave poor performance in the European Union (EU) BSE Tests Evaluation Exercise that was undertaken in May 1999 (21).

V. THE DEVELOPMENT OF A DIFFERENTIAL EXTRACTION PROCEDURE

It would have been easy to abandon the project in the aftermath of the first EU evaluation. Nevertheless, a unique opportunity was presented to rework the tissue preparation. It was decided to do two things. First, it was believed that the small sample size (i.e., 100 mg) was insufficient to represent the nonrandom distribution of aggregated material in diseased brain tissue. Consequently, approximately 1 g of tissue was adopted as standard and conventional homogenization employed. Second, and more significantly, proteolysis was abandoned.

Almost by definition, aggregation and deposited PrP will be more resistant to proteolysis than nonaggregated soluble PrP. Consequently, the extent of protease resistance will be directly related to the concentration, specificity, and potency of the proteolytic enzyme employed in the sample pretreatment. PK has a broad specificity and retains a high activity even in the presence of the the chaotropes and detergents used in the experimental extraction of PrP prior to detection. The concentration of PrP^{res}, therefore, cannot be an absolute. The more enzyme added, the more proteolysis, and vice versa. Consequently, the presence of PrP^{res} is totally dependent on the experimental conditions employed.

The use of differential proteolysis has led to the development of routine Western blots, which are essentially nonquantitative "yes-no" tests (13,22). Careful optimization must be undertaken to discover the exact conditions of proteolysis to ensure that the detectibility of PrP^{res} is a reflection of the presence of disease-associated PrP. Moreover, conditions that have been optimized for one type of tissue (e.g., brain stem) from an animal in the terminal stages of the disease may be inappropriate for the detection of PrP^{res} from other tissues (e.g., spleen and blood) and for the detection of preclinical disease. In these situations, optimum conditions for proteolysis may need to be constantly readjusted.

If the concentration of PK is too high, the enzyme may also remove the small quantities of PrP^{Sc}. On the other hand, if the concentration of PK is too low, the enzyme may not remove all of the relatively high concentration of PrP^{C}. Consequently, the measurement of PrP^{res} following PK digestion will always be technically difficult and, inevitably, limited in sensitivity.

The use of differential extraction with guanidine hydrochloride (GdHCl) has obviated the need for any proteolysis to discriminate between PrP^{C} and PrP^{Sc}. Following homogenization of bovine brain tissue, the more soluble isoform of PrP (equivalent to PrP^{C}) is extracted with 1M GdHCl. Subsequently, the remaining less soluble isoform of PrP (equivalent to PrP^{Sc}) is extracted with 6M GdHCl. For the sake of brevity, these fractions have been designated as "soluble" and "insoluble" PrP, respectively.

The concentration of PrP in the two extracts is determined by the use of the two-site DELFIA using FH11-coated plates and europium-labeled 3F4 as the detector. The diagnostic parameter, however, is not the concentration of insoluble PrP but the percentage of insoluble PrP in the sample. This parameter is independent of protein concentration (which will vary from sample to sample) and provides a reliable index, which clearly differentiates between infected and noninfected animals. The method was published in December 2000 (23).

VI. THE ROLE OF THE CHAOTROPIC AGENT

It has been known for several years that high-molarity guanidine salts may be used to unfold disease-associated aggregates of PrP. For example, Caughey et al. have demonstrated that pretreatment of PrP^{Sc} with concentrations of GdHCl in excess of 3.5M eliminates PK resistance, converting activity and infectivity (24–26). Moreover, Safar et al. have reported the ability to discriminate between eight different prion strains propagated in Syrian hamsters (27). The basis for this discrimination was the monitoring of PrP dissociation and unfolding in the presence of increasing molarity of GdHCl. It is noteworthy that the antibody used to monitor this phenomenon was europium-labeled 3F4 and forms the basis of the conformation-dependent immunoassay.

A. Effect of Chaotrope Concentration on the Solubility of Insoluble PrP

Six samples of bovine brain tissue (3 NZ negative and 3 BSE-confirmed positives) were homogenized and extracted in 1M GdHCl as described previously (23). After removal of the supernatant containing the soluble PrP, the pellets were extracted with differing concentrations of chaotrope

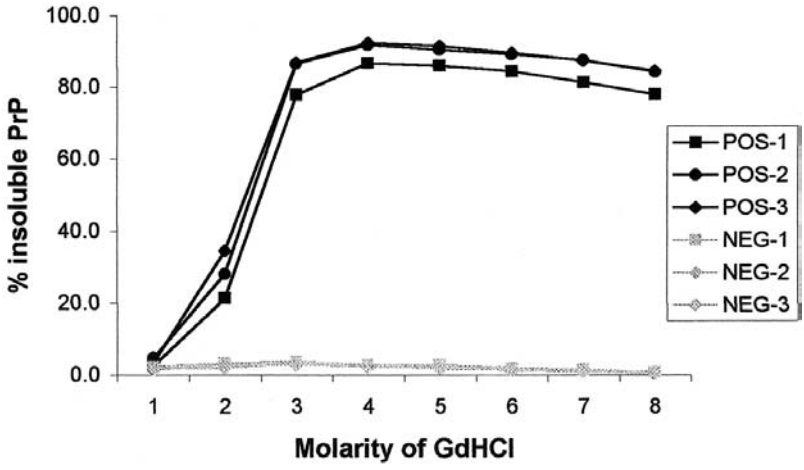

Figure 8 Effect of concentration of GdHCl on the solubility of aggregated PrP.

(GdHCl or urea). The solubilities of the remaining aggregated PrP in aqueous solutions of GdHCl or urea of increasing molarity are shown in Figs. 8 and 9, respectively.

On the basis of these experiments it was decided to use 6M GdHCl to solubilize the disease-associated PrP following the initial extraction of soluble nonaggregated PrP from the brain homogenate with 1MGdHCl.

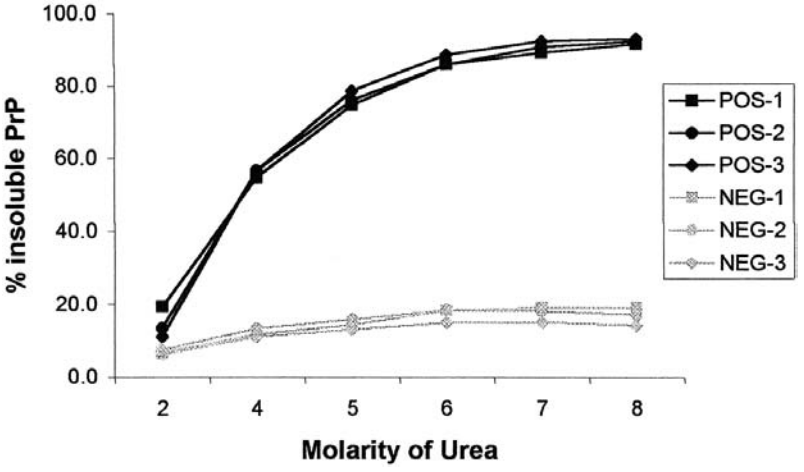

Figure 9 Effect of concentration of urea on the solubility of aggregated PrP.

With hindsight, 6M GdHCl is probably excessive. Even after dilution (typically 1:10 v/v in assay buffer), the continuing presence of GdHCl (approximately 0.6M) is slightly inhibitory in the immunoassay (see Fig. 8). The implications of this are discussed later.

VII. THE VLA BLIND TRIAL UNDERTAKEN IN OCTOBER 1999

To determine the efficacy of the differential extraction procedure, a trial was organized by the VLA in October 1999. Sets of samples were presented blind from the VLA BSE archive. These consisted of brain tissue samples from New Zealand taken from herds that had never been exposed to the disease, histology-confirmed positive and negative samples from BSE cases, and histology-conformed positive and negative samples from the over-30-months scheme (OTMS). All the samples were caudal to the obex, except for the OTMS samples, where caudal and rostral samples from the same animal were analyzed. The method has been described in complete detail and the reader is referred to the publication (23). However, for the sake of completeness, an outline of the method follows.

A. Tissue Preparation Reagents

The basic components of the homogenization buffer (i.e., 0.1M Tris-buffered saline; TBS) and GdHCl (8M; Cat. No. G-9284) were obtained from Sigma-Aldrich Co Ltd. (Poole, Dorset, UK). TBS was prepared by dissolving 1.21 g of Trizma base in 900 mL deionized water and adjusting the pH of this solution to pH 7.4 with 2M HCl. Subsequently, 7.77 g NaCl was added and dissolved with stirring and the solution made up to 1 L. This solution was stored at 4°C until required.

B. DELFIA Reagents

Materials required for the DELFIA were obtained from PerkinElmer Life Sciences (Cambridge, UK) and comprised: (1) anti-PrP microtitration strips (8×12 wells) coated with monoclonal antibody (clone FH11); (2) europium-labeled monoclonal detecting antibody to PrP (Senetek clone 3F4; made available by commercial arrangement with the Institute for Animal Health); (3) assay buffer (250 mL; Cat. No. 1244-111), which is a Tris-HCl buffered (pH 7.8 salt solution with bovine serum albumin, bovine globulin, Tween 40, an inert red dye, and 0.1% sodium azide as preservative; (4) wash concentrate (250 mL; Cat. No. 1244-114), which is a 25-fold concentration of Tris-HCl-buffered (pH 7.8) salt solution with Tween 20 and Germall II as

preservative; and (5) enhancement solution (250 mL; Cat. No. 1244-105) containing Triton X-100, acetic acid, and chelators. The wash solution was prepared by diluting the wash concentrate 25-fold (i.e., 40 mL concentrate diluted to 1 L) with distilled water.

C. DELFIA Equipment

The following equipment obtainable from PerkinElmer Life Sciences is required to perform the DELFIA: (1) time-resolved fluorometer plus printer and computer, e.g., Victor II (Cat. No. 1420-012 or 1420-016); (2) automatic washer, e.g., Platewash (Cat. No. 1296-026/R); (3) automatic shaker, e.g., Plateshake (Cat. No. 1296-003/R); and (4) optional dispenser for the Enhancement Solution (Plate Dispense; Cat. No. 1296-041/R).

D. Homogenization of Sample

Approximately 1 g of brain tissue (rostral or caudal to the obex region of bovine brain) was accurately weighed into a sterile 50-mL Falcon tube. Four volumes of cold 0.1M TBS (pH 7.5) was added and the mixture homogenized with a Camlab Omni homogenizer using a disposable probe. Care was taken to avoid the presence of any lumps of nonhomogenized tissue in the resulting 20% homogenate. The homogenates were stored on ice prior to extraction.

E. Differential Extraction of Sample

The brain homogenate was vortexed and 50 μL carefully pipetted into a 1.5-mL Eppendorf tube. Then 50 μL of freshly prepared 2M GdHCl was added and the tube stoppered and vortexed. Subsequently, 900 μL of assay buffer was added, and the tube stoppered, vortexed, and centrifuged in an Eppendorf Microcentrifuge at 13,000 g for 10 min. The supernatant (designated supernatant 1) containing the soluble PrP was carefully removed and transferred to a separate tube taking care not to dislodge the pellet. Then 100 μL of freshly prepared 6M GdHCl in water was added to the pellet. The tube was stopped and the pellet carefully vortexed into solution. A further 900 μL of assay buffer was added, and the tube stoppered, vortexed, and centrifuged at 13,000 g for 5 min. This supernatant containing the insoluble PrP was designated supernatant 2.

F. Immunoassay

The lyophilized human platelet–enriched plasma standard was reconstituted by the addition of 1 mL of distilled water. This stock solution was arbitrarily

assigned a value of 2000 units/mL (note: 1 unit was approximately equal to 1 ng recombinant PrP). Six standards (including a blank) were prepared by serially diluting the stock material 1 in 5 (v/v) with assay buffer (i.e., 0, 0.64, 3.2, 16, 80, and 400 units/mL); 200 μL of each standards or sample (supernatants 1 and 2) was added in duplicate to the antibody-coated microtiter plate. The plate was sealed and incubated on the shaker at 4°C overnight.

Subsequently, the plate was washed three times with the wash solution, tapped dry on absorbent paper, and 200 μL of filtered europium-labeled anti-PrP-detecting antibody ($3F_4$; diluted at 1:500 v/v in assay buffer) added to each well. The plate was incubated on the shaker at stable room temperature for a further 120 min. The plate was then washed six times, tapped dry, turned around 180°, and rewashed six more times. The plate was again tapped dry on absorbent paper.

Next, 200 μL of enhancement solution was added, the plate shaken for 5 min at room temperature, and the fluorescence measured in the time-resolved fluorometer. The concentrations of soluble and insoluble PrP were determined using the proprietary PerkinElmer data reduction package (Multicalc).

G. Calculation

Percent insoluble PrP was calculated according to the formula:

$$\frac{(\text{Concentration of PrP in supernatant 2}) \times 100}{(\text{Concentration of PrP in supernatant 2} + \text{concentration of PrP in supernatant 1})}$$

The results of the blind trial are given in Table 1. On the basis of these results, the VLA established an initial cutoff of 8% to discriminate between "positive" and "negative" samples. However, a number of points can be made.

Table 1 Results from the VLA Blind Trial

Type of sample	Number of tissues	Mean (%)	SD (%)	Range of values
NZ negatives	100	3.96	1.63	1.1–9.8
CVL BSE histo negative	18	5.78	2.58	3.2–12.2
CVL BSE histo positive	47	51.84	21.71	17.1–89.5
OTMS rostral histo negative	95	6.79	3.63	2.9–19.1
OTMS caudal histo negative	95	6.37	2.13	2.5–14.3
OTMS rostral histo positive	23	41.58	22.83	14.4–81.4
OTMS caudal histo positive	23	42.53	17.51	15.5–82.8

First, the avoidance of PK as the discriminating reagent in the assay allows for the differential extraction method to be potentially much more than a "yes-no" test. While one can understand the desire to have a "simple" result, the disease is not a "yes-no" disease. TSEs are characterized by prolonged incubation periods, during which time the animals are "infected" but do not manifest the characteristic clinical symptoms. This preclinical stage of the disease is likely to be characterized by an increasing accumulation and deposition of aggregated PrP but at low concentrations. It is here that the "sensitivity" of the diagnostic becomes of paramount importance. Histology, which is the accepted reference diagnostic, has limited and operator subjective sensitivity. In addition, it is worth noting that potentially histology is not a "yes-no" test. It becomes so because of the limited sensitivity of the human eye to detect small increases in PrP deposition and the concomitant spongiform changes associated with the diagnosis of TSE.

Any immunodiagnostic, certainly one that aspires to be quantitative or semiquantitative, must inevitably be more sensitive than histology. Furthermore, clinical diagnostic tests are most often not "yes-no" but are characterized by reference ranges and probabilities of diagnosis.

The second point that is worth noting from the results obtained in the blind trial is that as soon as any cutoff is established in a method that is not a "yes-no" test, the potential for false-positive and false-negative results becomes inevitable. Although an initial cutoff of 8% was established, 31 samples in the blind trial gave values between 8% and 10%. In addition, 21 samples gave results that were greater than 10%. The overall mean of these 52 samples was 10.2% with a SD of 2.25%. None of these 52 samples could be confirmed as positive by histology. The possibility that these samples, collected from the UK national herd as part of the OTMS scheme, were taken from animals in the preclinical stage of BSE is worth consideration. The mean age of onset for cattle displaying the clinical signs of BSE is 60 months (28). Nevertheless, prior to onset of clinical symptoms, any animal at any age will be in the preclinical stage of the disease *if* it is infected.

Furthermore, one of the NZ negative tissues in the blind trial gave a result (which was confirmed upon repeat) of 9.8%. The next highest value was 6.9%, well below the cutoff of 8%. In fact, the most statistically realistic cutoff of 8.9% (based on 3 SDs above the NZ negative mean of 3.96%) would not prevent this one sample from being a false positive.

It is highly unlikely that this one animal was in the preclinical stage of BSE. It cannot be ruled out, however, that there might be other clinical conditions, such as viral or bacterial infection (29), that could possibly lead to an increase in PrP aggregation. What is the normal function of PrP? There is some suggestion that it has a physiological role not dissimilar to that of superoxide dismutase in dealing with the local buildup of toxic free

radicals (30). In support of this hypothesis, there are now many reports of the role of copper in defining PrP activity (31–33). Moreover, Requena et al. have investigated copper-catalyzed oxidation of recombinant hamster PrP (34) and have also reported that thioredoxin reduction of recombinant PrP leads to the rapid formation of an insoluble isoform (35).

Accordingly, the role of PrP in dealing with oxidative stress is becoming increasingly likely (36). It is not possible that a reversible oxidation-reduction-mediated aggregation is part of the normal physiological role of PrP? The presence of relatively low concentrations of aggregated material in normal animals might indicate this (see Figs. 5–9 and Table 1).

H. Over-Thirty-Months Scheme (OTMS)

On the basis of the results obtained in the VLA blind trial, the method was extensively used throughout 2001 to analyze over 10,000 brain tissue samples (caudal to the obex) that were collected under the auspices of the OTMS scheme. The data will be published elsewhere.

VIII. EFFECT OF PK ON THE PROTEOLYTIC SUSCEPTIBILITY OF SOLUBLE AND INSOLUBLE PrP

The use of PK as the discriminating reagent to create a "yes-no" test in the TSE diagnostic must inevitably remove a small amount of aggregated PrP. Accordingly; we have investigated the effect of PK on the proteolytic susceptibility of the soluble and insoluble PrP fractions.

Six samples of bovine brain tissue (three NZ negatives and three BSE-confirmed positives) were homogenized using the reported procedure. Subsequently, 50-μL replicate aliquots of the homogenates were incubated with 25 μL of an increasing concentration of proteinase K (range: 0–40 μg/mL deionized water) for 30 min at 37°C. Proteolysis was halted by the addition of 10 μL protease inhibitor cocktail, prepared as described previously (23). Subsequently, 85 μL of 2M GdHCl solution was added (to give a resulting suspension in 1M GdHCl). The tubes were stoppered; vortexed, and 830 μL of assay buffer was added. The enzyme-treated homogenates were differentially extracted with GdHCl using the reported procedure and the concentrations of soluble PrP (supernatants 1) and insoluble PrP (supernatants 2) determined by DELFIA. The results are shown in Figs. 10 and 11.

Consistently, we have demonstrated that the insoluble PrP fraction is more resistant to PK than the soluble fraction. This is not unexpected since, by definition, any aggregated and insoluble protein will be protected against proteolysis. It would not be present as a deposit in an aggregated form if this

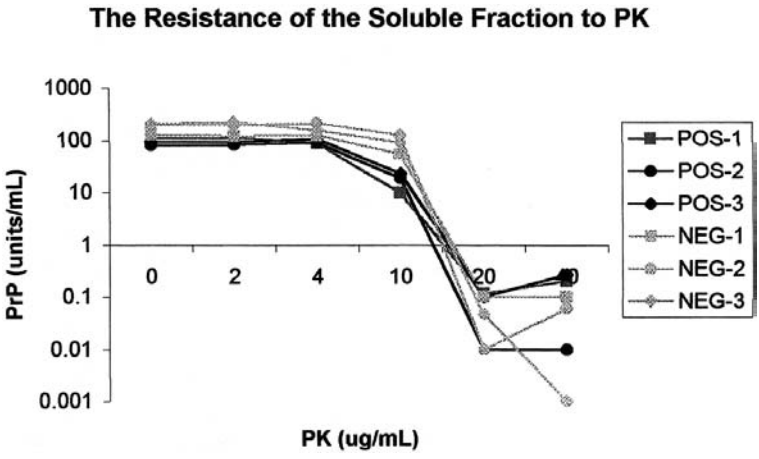

Figure 10 Effect of proteinase K on the proteolytic susceptibility of soluble PrP fractions of BSE-confirmed positive and NZ negative brain tissue.

was not so. Furthermore, we have shown that protease-resistant PrP is not characteristic of the disease per se. It is characteristic of a protein in an aggregated state. Because PrP has the potential to aggregate, one might expect there to be the presence of some aggregated protein even in normal tissues. This is indeed the case. The difference between normal and diseased states, however, is the degree of PrP aggregation. The low levels of insoluble

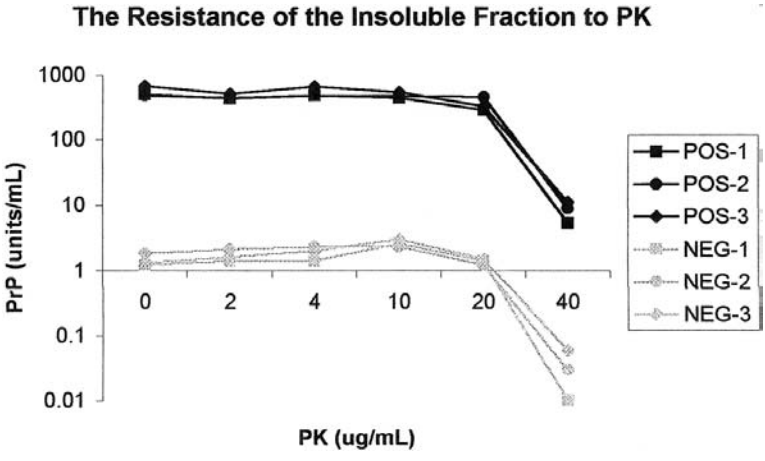

Figure 11 Effect of proteinase K on the proteolytic susceptibility of insoluble PrP fractions of BSE-confirmed positive and NZ negative brain tissue.

PrP may not be seen by methods using experimental proteolysis for two reasons: (1) all diagnostic methods of analysis (including Western blot) will have defined limits of detection; and (2) experimental proteolysis will inevitably remove some aggregated protein.

It is possible that in normal tissue, the soluble and aggregated forms of PrP may be in a state of equilibrium. In disease, however, this equilibrium might be shifted with the concomitant increase in the concentration of aggregated PrP. To investigate the level of aggregation in subclinical disease, therefore, the diagnostic method must be versatile. Moreover, the deposition of PrP, characteristic of TSE, is not uniform. Consequently, it is necessary to take a relatively large amount of tissue to get a homogenate that is representative. This becomes even more critical during the early stages of disease where the degree of aggregation and deposition of PrP is low.

IX. MAPPING THE BRAIN DISTRIBUTION OF AGGREGATED PrP

The percentages of insoluble PrP in paired samples of bovine brain tissue taken from the caudal and rostral regions from nine BSE-confirmed positives are shown in Fig. 12.

The differential extraction method has the potential to map the distribution of aggregated PrP throughout the brain. In Fig. 12, it is readily apparent that the percentages in the caudal samples taken from BSE-

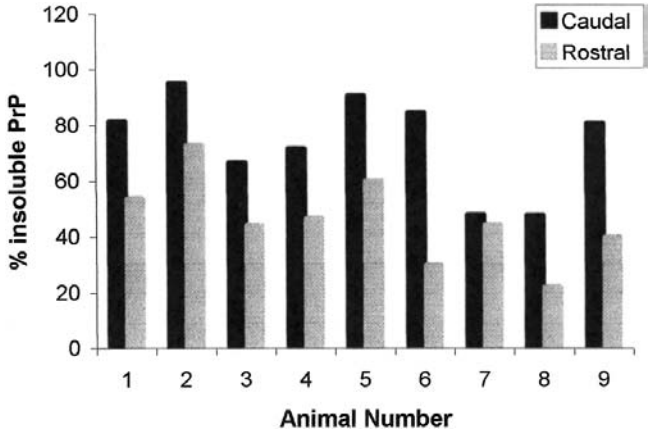

Figure 12 Percentages of insoluble PrP in paired samples ($n = 9$) of caudal and rostral brain tissue from BSE-confirmed positives.

infected animals are higher than the equivalent results obtained from rostral tissue. This preliminary study is an encouragement to go on to investigate the distribution of PrP isoforms throughout the central nervous system at different stages of the disease. In this case, a small sample size (e.g., 100 mg) might be an advantage. Indeed, the smaller the sample sizes, the finer the detail of PrP distribution. We envisage the ability of the method to facilitate an understanding of the progress of the disease from initial infection through to its terminal stage.

A. Application to Scrapie

With the help of Henry Hepburne-Scott (Institute of Animal Health, Compton, UK) tissue samples were obtained from various regions of the brains of six sheep. The animals included: (1) three normal sheep, (2) two scrapie-positive sheep, and (3) one unknown. The regions of the brain were as follows: (1) rostral medulla, (2) caudal medulla, (3) midbrain, (4) cerebellum, (5) thalamus, and (6) cerebrum. The tissues were homogenized by the reported procedure and the concentrations of soluble and insoluble PrP determined by DELFIA as described previously. The results are shown in Table 2.

From the data in Table 2 and from further studies at the Institute of Animal Health, we can conclude that the different extraction method, together with the FH11/3F4 immunoassay, is an appropriate procedure for the diagnosis for scrapie. This is indicated by the relatively high percentages of insoluble PrP in the scrapie animals in contradistinction to the noninfected sheep. The unknown animal is clearly not infected with scrapie. The absent data indicate that the tissue was either not collected or not available for assay.

In addition, the measurement of the soluble fraction of PrP is very interesting in the different regions of the brain. The highest levels of soluble PrP are consistently an found in the cerebrum. On the other hand, the lowest levels of soluble PrP are found in the brain stem. This is likely the reason why *percentages* of insoluble PrP increase significantly in the brain stem in disease whereas *percentages* of insoluble PrP remain low in the cerebum even though there appears to be a significant increase in the actual *concentration* of insoluble PrP in all regions of the brain in the scrapie animals. It is also interesting to note that the region of the brain of normal animals with the highest concentrations of insoluble PrP (as well as soluble) is the cerebrum. This might be an indication of its physiological role. It also is a demonstration of the clinical utility of the differential extraction method that enables the measurement of three parameters, namely, the concentrations of soluble and insoluble PrP together with the percentage of insoluble.

Table 2 Distribution of Aggregated PrP in Regions of the Brains of: (1) Three Normal Sheep, (2) Two Scrapie-Positive Sheep, and (3) One Unknown

Type	Region	Soluble (units/mL)	Insoluble (units/mL)	%	Type		Soluble (units/mL)	Insoluble (units/mL)	%
Normal 1	Rostral	156	2.12	1.3	Scraple 1	Rostral	67.85	157.97	70
	Caudal	56.1	0.24	0.4		Caudal	78.31	169.31	68.4
	Midbrain	270.3	3.5	1.3		Midbrain	138.62	113.03	44.9
	Cerebellum	272.45	3.26	1.2		Cerebellum	663.13	45.23	6.4
	Thalamus	388.21	7.84	2		Thalamus	290.18	370.29	56.1
	Cerebrum	736.59	11.97	1.6		Cerebrum	1063.37	35.78	3.3
Normal 2	Rostral	282.12	4.7	1.6	Scraple 2	Rostral	114.48	157.94	68
	Caudal	187.59	2.05	1.1		Caudal	—	—	—
	Midbrain	—	—	—		Midbrain	362.02	166.61	31.5
	Cerebellum	493.49	6.17	1.2		Cerebellum	389.49	70.22	15.3
	Thalamus	585.5	16.1	2.7		Thalamus	336.14	127.61	27.9
	Cerebrum	1208.31	25.83	2.1		Cerebrum	1937.57	62.51	3.1
Normal 3	Rostral	201.11	3.23	1.6	Unknown	Rostral	209.22	4.85	2.3
	Caudal	306.09	3.93	1.3		Caudal	125.5	1.95	1.5
	Midbrain	—	—	—		Midbrain	525.97	11.28	2.1
	Cerebellum	606.3	7.17	1.2		Cerebellum	365.88	5.27	1.4
	Thalamus	754.83	16.34	2.1		Thalamus	295.9	7.5	2.5
	Cerebrum	1961.67	46.6	2.3		Cerebrum	1309.94	32.4	2.4

B. Application to CJD

Under the auspices of the World Health Organization, the CJD surveillance unit Edinburgh provided sample of gray matter–enriched frontal cortex from four brains. The samples were taken from the following patient types at postmortem: (1) normal non-CJD, (2) one sporadic CJD, nominally type 1, (3) one sporadic CJD, nominally type 2, and (4) one variant CJD brain. All were homozygous for methionine at codon 129, a polymorphism that modulates the disease presentation. Moreover, the disease had been confirmed histologically in all cases. Sample of the infected brains had previously been shown to contain PrP^{Sc} and, at least in the case of the variant CJD, to transmit disease on inocullation of animals while being of acceptably low toxicity (J. Ironside, unpublished).

The specimens were homogenized in 0.25M Tris-buffered sucrose and separated into aliquots in 1.2-mL Nalgene cryovials, flash-frozen in liquid nitrogen, and stored at -86°C for subsequent use. Participants in the study were requested to use established in-house methods to titrate the preparations several times on separate vials, using 10-fold dilutions to establish approximate end-points (37,38).

Accordingly, serial dilutions of the four 10% homogenates were prepared in a negative homogenate pool prepared from calf brain. Each dilution was analyzed by the procedures already described. The results are shown in Table 3.

From the data in Table 3, we can conclude that the concentrations of soluble and insoluble PrP and the percentages of insoluble PrP are significantly elevated in the three cases of CJD. In addition, the three types of CJD, namely sporadic types 1 and 2 and the variant form of the disease, give varying increases. Without doubt, the most significant increases are shown in the variant CJD tissue, and the extremely high concentration of aggregated PrP may be a reflection of the aggression of the disease. Each sample demonstrated linearity of dilution (particularly if the blank is subtracted) with the sporadic type 1 and the variant tissue homogenates being detected even at a further 1000-fold dilution.

One of the objectives of this exercise and, indeed, all dilution experiments is to determine whether an immunoassay can ever approach the sensitivity of a biological infectivity assay. One needs to bear in mind, however, that the amplification available in a biological system, namely incubation period, is not available to any in vitro immunoassay. The sensitivity of the best two-site immunoassays is unlikely to mimic biological assays without a dramatic increase (by several orders of magnitude) in the affinity characteristics of the very best monoclonals that are currently available.

Table 3 Analysis of Soluble and Insoluble PrP in Serial Dilutions Prepared from Four Human Brain Homogenates

		No blank subtract			With blank subtract		
Sample	Code	Soluble (units/mL)	Insoluble (units/mL)	% PRP	Soluble (units/mL)	Insoluble (units/mL)	% PRP
Normal control	CRU97/03	306.34	53.11	14.8	282.49	51.74	15.5
Dilution: 1:10 v/v		52.67	6.61	11.2	28.82	5.24	15.4
Dilution: 1:100 v/v		29.93	1.48	4.7	6.08	0.11	1.6
Dilution: 1:1000 v/v		24.76	1.16	4.5	0.91	—	—
Dilution: 1:10,000 v/v		29.79	1.17	3.8	5.94	—	—
Sporadic CJD type 1	CRU99/009	511.84	481.08	48.6	487.99	479.71	49.6
Dilution: 1:10 v/v		69.29	49.65	41.7	45.44	48.28	61.5
Dilution: 1:100 v/v		35.46	6.29	15.1	11.61	4.92	29.8
Dilution: 1:1000 v/v		30.56	2.28	6.9	6.71	0.91	11.9
Dilution: 1:10,000 v/v		29.79	1.15	3.7	5.94	—	—
Sporadic CJD type 2	CRU97/008	600.79	173.17	22.4	576.94	171.8	22.9
Dilution: 1:10 v/v		79.66	17.94	18.4	55.81	16.57	22.9
Dilution: 1:100 v/v		34.15	2.87	7.8	10.3	1.5	12.7
Dilution: 1:1000 v/v		30.73	1.23	3.8	6.88	—	—
Dilution: 1:10,000 v/v		27.88	1.2	4.1	4.03	—	—
Variant CJD	CRU98/148	671.23	1137.01	62.9	647.39	1135.64	63.7
Dilution: 1:10 v/v		96.13	157.06	62	72.28	155.69	68.3
Dilution: 1:100 v/v		34.44	21.12	38	10.59	19.75	65.1
Dilution: 1:1000 v/v		29.27	2.94	9.1	5.42	1.57	22.5
Dilution: 1:10,000 v/v		29.55	1.36	4.4	5.7	—	—
Negative calf brain (blank)		1.37	23.85	6.4	0	0	0

C. Effects of Autolysis and Sample Quality

Two BSE-confirmed positives and two negative were homogenized by the procedures described previously. The homogenates were divided into 8 × 0.5 mL aliquots in screw-capped Eppendorf tubes and these were stored under various conditions, namely: (1) at −80°C, (2) at −20°C, (3) at 4°C for 24 h, (4) at 4°C for 48 h, (5) at room temperature (22°C) for 24 h, (6) at room temperature (22°C) for 48 h, (7) at 37°C for 24 h, and (8) at 37°C for 48 h. Subsequently, the samples were extracted and the concentrations of PrP were determined in the soluble and insoluble fractions (expressed as units/mL) using two different detection systems, namely: (1) FH11 capture and europium-labeled 3F4 detection, and (2) 6H4 capture and europium-labeled 3F4 detection. The results are shown in Table 4.

The data from the FH11/3F4 study consistently show that, following homogenization, the soluble fractions of PrP are particularly susceptible to proteolysis even at 4°C over 24 h and, more so, at 48 h. If the homogenates are stored at 22°C or 37°C, proteolysis becomes even more pronounced, even affecting the measurement of the insoluble PrP in some cases. The use of 6H4 as the capture antibody, together with europium-labeled 3F4 as detector, may limit the impact of proteolysis on detectibility, but at higher storage temperatures even this method is compromised.

One needs to bear in mind that, in a two-site immunoassay, the integrity of two epitopes needs to be maintained for the detection method to be resistant to the effects of autolysis. This may not be the case in Western blot analysis or in any immunoassay that involves nonspecific capture of PrP onto a chemically activated surface (e.g., glutaraldehyde).

It could be postulated that homogenization might exacerbate proteolysis because of the release of proteolytic enzymes as the tissue is disrupted. To assess the impact of proteolysis prior to homogenization, further experiments were undertaken on brain tissue that was subjected to storage at room temperature and at 4°C. Brain tissue was collected from heads as part of the OTMS2 survey. Rostral material was used for the study because: (1) obex and caudal samples were collected as part of the OTMS2 and were not available for this study, and (2) previous experience had shown that rostral brain stem gives results similar to that of caudal material (see Fig. 12).

Upon removal from the head, the tissue was divided into five pieces weighing between 0.8 and 1.2 g and placed into labeled polypropylene tubes. As a control, one sample was stored immediately at −80°C. In addition, samples were stored in a cold room (at 4°C) or at room temperature (nominally 22°C) for up to 6 days and then frozen prior to assay. The results are shown in Table 5.

Table 4 The Autolytic Stability of Two Positive and Two Negative Homogenates Stored Under Various Conditions

		FH11 plate			6H4 plate		
Sample	Storage conditions	Soluble PrP	Insoluble PrP	%	Soluble PrP	Insoluble PrP	%
Positive 1	Freezer at −80°C	10.84	10.4	49.0	4.48	15.08	77.1
	Freezer at −20°C	8.94	11.13	55.5	2.54	15.37	85.8
	4°C for 24 h	6.81	9.94	59.3	2.25	14.35	86.4
	4°C for 48 h	4.79	10.1	67.8	2.2	14.13	86.5
	22°C for 24 h	2.46	9.93	80.1	2.29	14.45	86.3
	22°C for 48 h	0.44	12.38	96.6	0.001	16.83	100.0
	37°C for 24 h	0.03	11.1	99.7	0.08	17.01	99.5
	37°C for 48 h	0.23	0.86	78.9	0.03	9.38	99.7
Positive 2	Freezer at −80°C	74.52	33.55	31.0	14.82	36.21	71.0
	Freezer at −20°C	73.13	32.8	31.0	16.72	34.89	67.6
	4°C for 24 h	57.18	30.73	35.0	16	34.52	68.3
	4°C for 48 h	43.7	25.6	36.9	15.22	31.75	67.6
	22°C for 24 h	49.57	29.3	37.1	14.02	30.32	68.4
	22°C for 48 h	0.024	25.1	99.9	0.002	27.03	100.0
	37°C for 24 h	33.9	33.7	49.9	1.4	29.47	95.5
	37°C for 48 h	0.023	32.6	99.9	0.11	41.35	99.7
Negative 1	Freezer at −80°C	64.35	1.71	2.6	10.38	0.94	8.3
	Freezer at −20°C	68.53	1.74	2.5	9.67	1.02	9.5
	4°C for 24 h	30.59	1.004	3.2	12.45	1.15	8.5
	4°C for 48 h	7.992	0.626	7.3	11.6	0.85	6.8
	22°C for 24 h	14.57	0.53	3.5	16.99	0.68	3.8
	22°C for 48 h	0.125	0.259	67.4	1.83	0.42	18.7
	37°C for 24 h	0.032	0.45	93.4	0.6	0.36	37.5
	37°C for 48 h	0.011	0.058	84.1	0.38	0.19	33.3
Negative 2	Freezer at −80°C	63.45	0.793	1.2	8.42	0.93	9.9
	Freezer at −20°C	61.34	0.831	1.3	8.45	0.87	9.3
	4°C for 24 h	38.15	0.499	1.3	9.45	0.98	9.4
	4°C for 48 h	7.583	0.265	3.4	8.12	0.78	8.8
	22°C for 24 h	10.74	0.351	3.2	8.62	0.91	9.5
	22°C for 48 h	0.104	0.151	59.2	0.41	0.71	63.4
	37°C for 24 h	7.407	1.073	12.7	0.78	0.53	40.5
	37°C for 48 h	0.104	0.116	52.7	0.56	0.49	46.7

Table 5 The Autolytic Stability of Five Rostral Tissues Stored Under Various Conditions

Sample	Day	Storage conditions	Soluble PrP	Insoluble PrP	% PRP
D1289	0	Baseline	28.13	1.8	6
D1289	1	Cold store	34.92	1.7	4.6
D1289	6	Cold store	111.24	5.29	4.5
D1289	1	Room temp	45.49	3.04	6.3
D1289	6	Room temp	0.05	0.21	80.8
D1290	0	Baseline	39.39	2.44	5.8
D1290	2	Cold store	68.67	3.22	4.5
D1290	6	Cold store	100.43	3.87	3.7
D1290	2	Room temp	0.22	0.39	63.9
D1290	6	Room temp	0.11	0.25	69.4
D1291	0	Baseline	46.09	2.04	4.2
D1291	3	Cold store	51.86	2.76	5.1
D1291	6	Cold store	65.87	3.17	4.6
D1291	3	Room temp	13.02	1.52	10.5
D1291	6	Room temp	0.16	0.45	73.8
D1292	0	Baseline	43.53	2.56	5.6
D1292	4	Cold store	57.69	3.05	5
D1292	6	Cold store	71.17	4.73	6.2
D1292	4	Room temp	OUT	OUT	—
D1292	6	Room temp	0.02	0.13	86.7
D1293	0	Baseline	51.31	3.93	7.1
D1293	5	Cold store	97.58	3.79	3.7
D1293	6	Cold store	70.2	3.51	4.8
D1293	5	Room temp	OUT	OUT	—
D1293	6	Room temp	OUT	0.06	—

These limited data indicate that tissues may be stored at 4°C prior to homogenization without significant deterioration (i.e., autolysis) over the 6-day period (mean on day 0 of 5.7% and day 6 of 5.6%). After day 1, however, there is rapid deterioration of the samples at room temperature, with a decrease in the soluble fraction, giving a false-positive result.

Because of the N-terminal specificity of the capture antibody (FH11), we have to conclude that the method is susceptible to interference from autolytic changes in brain tissue after death. This is unlikely to be a significant problem in abattoir surveys when brain tissue can be collected and rapidly chilled following the slaughter of the animal. Nevertheless, it became a necessity to develop an alternative immunoassay for use in

other surveys involving fallen stock where it was possible that the autolytic integrity of brain tissue received in the laboratory would be of variable quality.

X. THE DEVELOPMENT OF ALTERNATIVE IMMUNOASSAYS

To develop an alternative immunoassay it was necessary to find a significant supply of capture antibody. Fortunately, through the generosity of Professor Chris Bostock, Dr. Chris Birkett, and the TSE Resource Centre at the Institute for Animal Health, two monoclonal antibodies were made available in significant milligram quantities, namely: (1) KG9 and (2) DF7. These antibodies have similar specificity, recognizing an epitope close to one of the glycosylation sites (residue 151; see Fig. 2.). These two monoclonal antibodies were immobilized onto low-fluorescence microtiter plates (provided by PerkinElmer Life Sciences) at 1 μg IgG per microtiter well by the procedures previously described.

In addition, Case Western provided three monoclonal antibodies, namely: (1) 5B2, (2) 8H4, and (3) 7A12. The monoclonal 5B2 was raised in knockout mice and recognizes an epitope in the N-terminal region of the molecule at residues 36–43. On the other hand, 8H4 and 7A12 were raised against murine recombinant PrP and recognize epitopes within the region delineated by residues 158–174 and 122–145, respectively (39).

A. Labeling Antibodies with Europium

All available monoclonal antibodies were labeled with europium using commercially available labeling reagent (Cat. No. 1244-302: PerkinElmer Life Sciences, PerkinElmer House, Cambridge, UK) and a nonchromatographic modification of a method previously described by Barnard (9).

Briefly, approximately 1 mg of each antibody to be labeled was added to a Microcon-30 concentrator (Cat. No. 42409; Millipore Ltd., Watford, Herts, UK). The vial was capped and centrifuged for 15 min in a Microfuge (6500 rpm; MSE Micro-Centaur). The eluate was removed from the vial using a plastic Pasteur pipette and discarded. Then 400 μL of carbonate-labeling buffer (50 mmol/L; pH 8.5) was added to the retentate (approximately 10 μL) in the concentrator. After centrifugation for further 15 min (6500 rpm), the eluate was again discarded and a further 400 μL of carbonate buffer added. This procedure was repeated twice more.

The retentate were carefully diluted with 450 μL of labeling buffer in the concentrator using pipetting action to mix the solutions. This was

transferred to the bottle containing lyophilized labeling reagent (0.2 mg). The bottle was stoppered, and the contents gently mixed and left at room temperature overnight.

The following day, the solution containing the labeled antibody and unreacted labeling reagent was transferred to another Micron-30. The vial was capped and centrifuged for 15 min in the Microfuge. Subsequently, the eluate was removed from the vial using a plastic pasteur pipette and discarded. Then 400 μL of elution buffer (50 mmol/L Tris-HCl containing 0.9% sodium chloride and 0.1% sodium azide; pH 7.8 was added to the retentate. After centrifugation for a further 15 min (6500 rpm), the eluate was again discarded and a further 400 μL of elution buffer added. This procedure was repeated twice more.

The retentate was carefully diluted with 200 μL of elution buffer using pipeting action to mix the solution several times and taking care not to puncture the Microcon membrane. The solution containing purified labeled antibody was transferred to an appropriate glass bottle. The Micocon membrane was washed with 4 × 200 μL aliquots of elution buffer. Each of these aliquots was transferred to the glass bottle containing the labeled antibody.

To remove any aggregated protein, the labeled antibody may be filtered through a 0.22-μ Millex-GV4 filter. Purified BSA stabilizer, contained in the labeling kit, may be added to the labeled antibody solution to give a final concentration of 0.1% BSA. The labeled antibodies were stored at 4°C.

B. Recombinant Bovine Standard

It became immediately apparent that, in some cases, alternative combinations of the antibodies did not cross-react significantly with PrP derived from human platelets. Consequently, it was necessary to obtain recombinant bovine PrP. The TSE Resource Centre (IAH) kindly provided two separate batches. In addition, commercially available bovine recombinant PrP was kindly donated by Dr. Alan Tunnicliffe (Biogenesis, Poole, Dorset, UK). Each batch of recombinant material had a slightly different potency. Calibration curves obtained from the three batches of recombinant PrP prepared in assay buffer using FH11-coated plates and europium-labeled 7A12 are shown in Fig. 13.

Combinations of capture and detection reagents were investigated for their ability to bind human PrP (derived from platelets) and recombinant bovine PrP. Preliminary data were obtained indicating the potential sensitivity, specificity, and potential diagnostic utility of various immunoassays. In particular, two alternative immunoassays, namely: (1) capture antibody

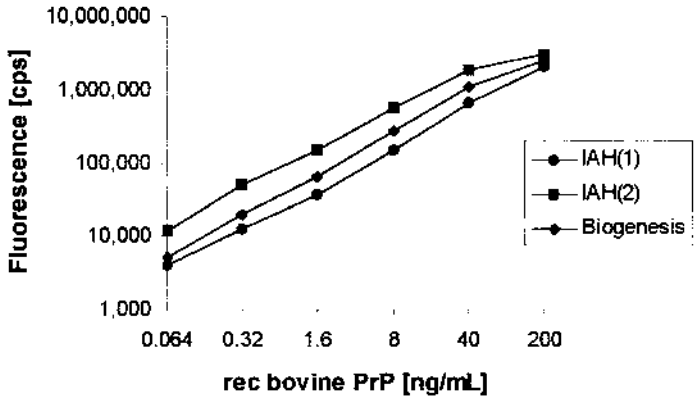

Figure 13 Calibration curves of three batches of recombinant bovine PrP using FH11-coated plates and europium-labeled 7A12.

DF7 using detecting antibody europium-labeled 7A12, and (2) capture antibody KG9 using the same detecting antibody europium-labeled 7A12, were identified as the most promising candidates for further study. It is noteworthy that, unlike FH11/3F4, neither of these two alternative immunoassays had the ability to cross-react with the soluble PrP fraction. It is tempting to speculate that the assays may be specific for the disease-associated PrP.

Accordingly, 39 samples (26 negatives and 13 positives) that had been previously identified by the standard FH11/3F4 assay were reanalyzed using the two new immunoassays (DF7/7A12 and KG9/7A12). The results are shown in Table 6.

To interpret the data in Table 6, it is important to recognize that the results from the new immunoassays are expressed as a concentration (i.e., ng/mL) whereas the results from the original method are expressed as a percentage.

On the basis of the discrimination between the positive and negative samples, it was decided to continue with the KG9/7A12 combination. The data shown in Table 6 were obtained using calibration curves of bovine recombinant PrP prepared in assay buffer alone. It is also important to note, however, that the samples are in a very different matrix. The aggregated PrP is solubilized by the addition of 6M GdHCl. The extraction process dilutes the guanidine 10-fold and the extracted material in assay buffer containing the solubilized PrP and 0.6M GdHCl is added directly to the antibody-coated plate.

Table 6 Initial Data Obtained Using Three Different Immunoassays

Sample	FH11/3F4 Soluble (units/mL)	FH11/3F4 Insoluble (units/mL)	%	DF7/7A12 (insoluble ng/mL)	KG9/7A12 (insoluble ng/mL)
Negatives					
1	152.24	4.36	2.78	0.29	0.30
2	54.5	1.21	2.17	0.12	0.09
3	66.46	1.69	2.48	0.13	0.17
4	31.66	1.62	4.87	0.20	0.55
5	115.15	2.72	2.31	0.47	0.63
6	89.62	1.59	1.75	0.10	0.14
7	145.92	3.6	2.41	0.12	0.10
8	163.51	3.15	1.89	0.23	0.33
9	138.47	3.1	2.19	1.00	0.16
10	110.11	2.84	2.61	0.12	0.15
11	153.69	3.74	2.38	0.20	0.28
12	156.2	3.723	2.33	0.11	0.27
13	165.53	3.62	2.08	0.19	0.16
14	120.19	2.77	2.25	0.19	0.15
15	65.68	1.59	2.36	0.09	0.09
16	81.53	1.39	1.68	0.13	0.01
17	90.35	2.77	2.97	0.28	0.14
18	99.25	2.07	2.04	0.20	0.20
19	109.34	2.37	2.12	0.16	0.16
20	82.52	1.83	2.17	0.12	0.07
21	98.27	3.08	3.04	0.30	0.19
22	93.21	2.04	2.14	0.43	0.28
23	123.26	3.88	3.05	0.41	0.16
24	105.17	2.05	1.91	0.23	0.63
25	125.91	2.88	2.24	0.20	0.18
26	131.85	10.09	7.11	0.18	0.40
Mean			2.69	0.24	0.24
Positives					
1	36.4	30.96	45.96	2.39	10.84
2	73.43	71.96	49.49	4.78	25.24
3	44.96	61.06	57.69	4.75	28.59
4	94.25	84.76	47.36	5.27	36.59
5	41.86	10.81	20.53	0.61	1.42
6	58.32	52.17	47.22	5.24	36.76
7	41.75	56.53	67.52	6.07	35.20
8	28.02	4.57	14.02	0.60	3.32
9	31.96	51.48	61.70	6.48	53.98
10	30.17	35.65	54.16	4.52	31.94
11	16.12	13.02	44.68	2.54	14.34
12	19.15	15.42	44.61	2.71	15.21
13	26.31	41.18	61.02	4.81	26.64
Mean			46.60	3.90	24.61

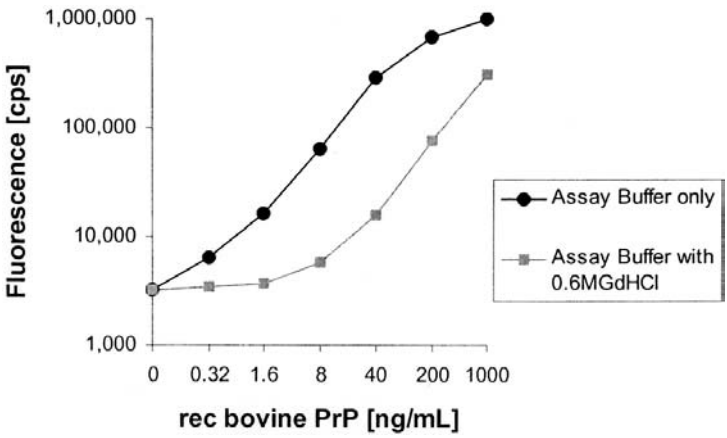

Figure 14 Calibration curves of recombination bovine PrP in the presence and absence of 0.6M GdHCl using KG9-coated plates and europium-labeled 7A12.

The impact of guanidine on the shape of the calibration curve was investigated. Results are shown in Fig. 14.

The presence of guanidine in the diluted standards significantly alters the sensitivity and shape of the calibration curve. This is not totally unexpected. Consequently, it was decided to perform the KG9/7A12 assay with two calibration curves, one with guanidine and one without.

XI. THE SECOND EUROPEAN EVALUATION IN JUNE 2001

The specificity of the new method was assessed when it was run in parallel with the existing DELFIA (FH11/3F4) during another European evaluation exercise that took place in June 2001. Tissues were obtained from 199 animals (48 positives and 151 negatives). These tissues had been stored by the Commission at only $-20°C$ for over 2 years since the last European trial in May 1999. The results are shown in Table 7.

During the course of this evaluation, the quality of the tissues was called into question. Under normal circumstances, tissues are stored at $-80°C$ prior to testing. No data have ever been obtained with tissues stored for prolonged periods at $-20°C$. Consequently, using a threshold of 8% for the standard assay, three false negatives were obtained. Arbitrary thresholds were established for the new assay, namely: (1) 1.5 ng/mL with the standards prepared in buffer, and (2) 45 ng/mL with the standards prepared in buffer containing 0.6M GdHCl. On the basis of these thresh-

Table 7 Results on 199 Tissue Samples Obtained During the EU Evaluation Exercise in June 2001

	Antibodies	Standards	No.	Mean	Range of values
Negatives					
Standard assay	FH11/3F4	Human	154	3.81%	1.8–7.7
New assay	KG9/7A12	Bovine in buffer	153	0.45 ng/mL	0.1–1.4
New assay	KG9/7A12	Bovine in 0.6M GdHCl	153	13.24 ng/mL	5.1–36.5
Positives					
Standard assay	FH11/3F4	Human	45	37.88%	9.1–76.2
New assay	KG9/7A12	Bovine in buffer	46	16.18 ng/mL	2.3–164.1
New assay	KG9/7A12	Bovine in 0.6M GdHCl	46	325.6 ng/mL	54.7–923.1

olds, however, two false negatives were obtained with either version of the new assay.

Up to this time, the new assay was performed on the insoluble fraction obtained by the normal extraction protocol used for the standard procedure (FH11/3F4). As a consequence it was decided to repeat the new assay with a revised tissue protocol.

A. New Tissue Extraction Protocol

The 20% brain homogenate was prepared as previously described. Prior to extraction, the homogenate was vortexed and 200 μL carefully pipetted into a 1.5-mL Eppendorf tube; 200 μL of freshly prepared 2M GdHCl was added and the tube stoppered and vortexed. Subsequently, 800 μL of assay buffer was added, and the tube stoppered, vortexed, and centrifuged in an Eppendorf Microcentrifuge at 13,000 g for 10 min. The supernatant containing soluble PrP was discarded. Then 100 μL of freshly prepared 6M GdHCl in water was added to the pellet. The tube was stoppered and the pellet carefully vortexed into solution. A further 900 μL of assay buffer were added, and the tube stoppered, vortexed, and centrifuged at 13,000 g for 5 min. This supernatant containing the insoluble PrP was taken for assay.

B. Immunoassay

Two sets of calibrators were initially prepared. First, six standards (including a blank) were prepared by serially diluting the stock recombinant bovine PrP (Biogenesis) with assay buffer (calibration range: 0, 0.32, 1.6, 8, 40, and

200 ng/mL). A second set of standards was prepared in assay buffer containing 0.6M GdHCl (calibration range: 0, 1.6, 8, 40, 200 and 1000 ng/mL). Then 200 μL of each standard or sample was added in duplicate to the KG9-coated microtiter plate. The plate was sealed and incubated on the shaker at 4°C overnight.

Subsequently, the plate was washed three times with the wash solution, tapped dry on absorbent paper, and 200 μL of filtered europium-labeled anti-PrP detecting antibody (7A12; diluted at 1:2000 v/v in assay buffer) added to each well. The plate was incubated on the shaker at stable room temperature for further 120 min. The plate was then washed six times, tapped dry, turned around 180°, and rewashed six more times. The plate was again tapped dry on absorbent paper.

Then 200 μL of enhancement solution was added, the plate shaken for 5 min at room temperature, and the fluorescence measured in the time-resolved fluorometer. The concentrations of the insoluble PrP (expressed in ng/mL) were determined using the proprietary PerkinElmer data reduction package (Multicalc). The results obtained are shown in Table 8.

Using thresholds of 0.8 ng/mL (standards prepared in assay buffer alone) or 55 ng/mL (standard prepared in buffer containing 0.6M GdHCl) 100% classification was obtained using the new assay (i.e., KG9/7A12) with the revised tissue protocol taking 200 μL of 20% brain tissue homogenate. Clearly, the increase in sample size improved the overall assay sensitivity and the revised tissue extraction protocol was adopted for subsequent experiments.

Table 8 Results on 199 Tissue Samples Obtained with the KG9/7A12 Assay Using Different Aliquots of Homogenate

Aliquot	Standards	No.	Mean	Range of values
Negatives				
50	Bovine in buffer	153	0.46 ng/mL	0.1–1.4
50	Bovine in 0.6M GdHCl	153	13.24 ng/mL	5.1–36.5
200	Bovine in buffer	151	0.26 ng/mL	0.1–0.7
200	Bovine in 0.6M GdHCl	151	21.49 ng/mL	4.3–65.9
Positives				
50	Bovine in buffer	46	16.18 ng/mL	2.3–164.1
50	Bovine in 0.6M GdHCl	46	325.6 ng/mL	54.7–923.1
200	Bovine in buffer	48	52.3 ng/mL	1.4–454.7
200	Bovine in 0.6M GdHCl	48	1875.8 ng/mL	90.1–6273

C. The Effects of Autolysis

A further study was conducted to determine if the new assay (KG9/7A12) was more resistant to the effects of autolysis. Brain tissue was obtained from four BSE-confirmed positives and negatives. The tissue was divided into two equal pieces and transferred to polypropylene tubes. One set of samples was stored at −20°C and the other set was stored at room temperature for 7 days. Subsequently, the samples were processed using the standard method (FH11/3F4) and the new assay (KG9/7A12) using the revised tissue protocol. The results are shown in Table 9.

We can conclude from the data in Table 9 that the new immunoassay (KG9/7A12) is able to detect aggregated PrP in tissue samples that have been stored at room temperature for 7 days. It is to be noted, however, that there is a significant reduction in the concentrations of insoluble PrP as measured by both methods. Nevertheless, because the standard assay (FH11/3F4) uses the soluble PrP as an internal reference, the impact of autolysis inevitably leads to the creation of *false positives*. On the other

Table 9 Data Indicating the Effect of Tissue Autolysis on the Performance of Two Immunoassays

	Standard assay			New assay	
Sample	Soluble (units/mL)	Insoluble (units/mL)	%	Buffer (insoluble ng/mL)	0.6M GdHCl (insoluble ng/mL)
A. Tissue stored at –20°C for >7 days					
Positive 1	52	33.5	39.2	21.3	2337
Positive 2	34.1	10.8	24.1	12.4	1324
Positive 3	37.3	33.3	46.8	21.3	2326
Positive 4	57.2	36.3	38.8	20.5	2248
Negative 1	25.3	0.68	2.6	0.69	85.3
Negative 2	23	0.44	1.9	0.58	73.2
Negative 3	54.6	1.1	2.0	0.94	112.9
Negative 4	72.3	2.1	2.8	1.46	165.7
B. Tissue stored at room temperature for >7 days					
Positive 1	nil	11	n.a.	3.2	340.3
Positive 2	nil	5.1	n.a.	2	218.1
Positive 3	nil	12.9	n.a.	2.3	253.6
Positive 4	nil	34.8	n.a.	7.7	801
Negative 1	nil	nil	n.a.	0.02	0.59
Negative 2	nil	nil	n.a.	0.03	1.1
Negative 3	nil	nil	n.a.	nil	nil
Negative 4	nil	nil	n.a.	nil	nil

hand, the new assay, which does not require the use of the soluble fraction as an internal reference, the impact of extreme autolysis might lead to the possibility of *false negatives*.

It is probable that all immunoassays will be affected by autolytic changes in brain tissue to a greater or lesser extent. It is interesting to note that an assay that could lead to the possibility of a false-negative result (because of autolysis) is to be preferred to one that would lead to the possibility of a false positive.

At the end of August 2001, the existing procedure (FH11/3F4) and the alternative immunoassay (KG9/7A12) using the increased sample size were evaluated in a second trial organized by the European Commission. The 200 tissue samples (48 positives and 152 negatives) had been stored at −70°C subsequent to collection although many of the positives would have been frozen and thawed several times. The results of this trial are shown in Table 10.

It was immediately apparent that the quality of these samples, particularly the negatives, was considerably better than that of the samples received in June 2001. This was reflected in the fact that the concentrations of PrP in negative tissue as determined by the new assay (KG9/A12) where significantly higher (mean 56.7 ng/mL, SD 18.4 ng/mL) than the values obtained previously in the NZ negative material received in June 2001 (mean 3.24 ng/mL, SD 5.1 ng/mL). Consequently, it was necessary to reestablish the cutoff for the new method. If this was set at three times the standard deviation above the mean of the negative results, namely, 112 ng/mL, one sample was classified as a false negative.

Initially a threshold was set at 8% for the standard method. Accordingly, one sample (8.1%) was above this threshold and was classified as a false positive. A more statistically relevant threshold, however, was 8.7% (i.e., three times the standard deviation above the negative mean of 4.8%).

Table 10 Results on 200 Tissue Samples Obtained During the EU Evaluation Exercise in August 2001

	Antibodies	Standards	No.	Mean	Range of values
Negatives					
Standard assay	FH11/3F4	Human	152	4.80%	2.1–8.1
New assay	KG9/7A12	Bovine in 0.6M GdHCl	153	56.7 ng/mL	15.0–94.9
Positives					
Standard assay	FH11/3F4	Human	48	36.6	9.5–76.1
New assay	KG9/7A12	Bovine in 0.6M GdHCl	47	794.3 ng/mL	123.4–2680

At this threshold, 100% classification was obtained. It is interesting to note that this is almost exactly the same statistical threshold that was determined for the 100 NZ negative tissues by the standard method (FH11/3F4) in the VLA blind trial that was conducted in October 1999 (mean 3.96%, SD 1.63, threshold 8.9%).

Nevertheless, if there has to be a threshold, there may be some epidemiological rationale in setting a lower threshold to obtain at least a small number of false positives. The reason for this is that any positive result will be repeated whereas a false negative remains totally hidden and ignored.

XII. FUTURE DEVELOPMENTS

Work is proceeding to develop alternative immunoassays for the measurement of disease-associated PrP in BSE, scrapie, and CJD. As more monoclonals become available it will be possible to epitope-map the surface of PrP and to recognize confirmational changes, epitope masking, and exposure that may be associated with the earliest stages of infection. The ultimate goal is to develop a high-throughput-screening assay that can be performed on more accessible peripheral tissue or blood. The potential sensitivity, versatility, and robustness of DELFIA and other time-resolved fluorescence technologies lend themselves to this objective.

In particular, it is possible to use up to four different lanthanides (europium, samarium, terbium, and dysprosium) (9). Moreover, the Victor fluorometer has all the relevant filters to allow the use of several detectors simultaneously. Multiarray detection may be the key to the development of the best diagnostics for TSEs.

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REFERENCES

1. Prusiner SB. The prion diseases. Brain Pathol 1998; 8:499–513.
2. Kascsak RJ, Fersko R, Pulgiano D, Rubenstein R, Carp RI. Immunodiagnosis of prion disease. Immunol Invest 1997; 26:259–268.
3. Schaller O, Fatzer R, Stack M, Clark J, Cooley W, Biffiger K, Egli S, Doherr M, Vandevelde M, Heim D, Oesch B, Moser M. Validation of a Western immunoblotting procedure for bovine PrP(Sc) detection and its use as a rapid surveillance method for the diagnosis of bovine spongiform encephalopathy (BSE). Acta Neuropathol (Berl) 1999; 98:437–443.
4. Hope J. Transmissible spongiform encephalopathies in man and animals. In: Krause R, ed. Emerging Infections. New York: Academic Press, 1998:447–461.
5. Grathwohl K-UD, Horiuchi M, Ishiguro N, Shinagawa M. Sensitive enzyme-linked immunosorbent assay for the detection of PrP^{Sc} in crude tissue extracts of scrapie-affected mice. J Virol Meth 1997; 64:205–216.
6. Meyer RK, Oesch B, Fatzer R, Zurbriggen A, Vandevelde M. Detection of bovine spongiform encephalopathy-specific PrP (Sc) by treatment with heat and guanidine thiocyanate. J Virol 1999; 73:9386–9392.
7. Deslys JP, Lasmezas CI, Comoy E, Dormont D. Diagnosis of bovine spongiform encephalopathy. Vet J 2001; 161:1–3
8. Wood P, Barnard G. Fluoroimmunoassay. In: Price CP, Newman DJ, eds. Principles and Practice of Immunoassays: 2nd ed. New York: Stockton Press, 1997:389–424.
9. Barnard G. Time-resolved fluorescence immunoassay. In: Edwards R, ed. Immunodiagnostics: A Practical Approach. Oxford: Oxford University Press, 1999:137–158.
10. Hemmila IA. Applications of Fluorescence in Immunoassays. New York: John Wiley & Sons, 1991.
11. Foster JD, Wilson M, Hunter N. Immunolocalisation of the prion protein (PrP) in the brains of sheep with scrapie. Vet Rec 1996; 139:512–515.

12. Laffling AJ, Baird A, Birkett CR, John HA. A monoclonal antibody that enables specific immunohistological detection of prion protein in bovine spongiform encephalopathy cases. Neurosci Lett 2001; 300:99–102.
13. Oesch B, Doherr M, Heim D, Fischer K, Egli S, Bolliger S, Biffiger K, Schaller O, Vandevelde M, Moser M. Application of Prionics Western blotting procedure to screen for BSE in cattle regularly slaughtered at Swiss abattoirs. Arch Virol 2000; 16:189–195.
14. MacGregor I, Hope J, Barnard G, Kirby L, Drummond O, Pepper D, Hornsey V, Barclay R, Bessos H, Turner M, Prowse C. Application of a time-resolved fluoroimmunoassay for the analysis of normal prion protein in human blood and its components. Vox Sanguin 1999; 77:88–96.
15. Bessos H, Drummond O, Prowse CV, Turner M, MacGregor IR. The release of prion protein from platelets during storage of apheresis platelets. Transfusion 2001; 41:61–66.
16. Fischer MB, Roecki C, Parizek P, Schwarz HP, Aguzzi A. Binding of disease-associated prion protein to plasminogen. Nature 2000; 408:479–483.
17. Pruisner SB, Scott MR, DeArmond SJ, Cohen F. Prion protein biology. Cell 1998; 93:337–348.
18. Gabizon R, Meiner Z, Halimi M, Ben-Sasson SA. Heparin-like molecules combine differentially to prion-proteins and change their intracellular metabolic fate. J Cell Physiol 1993; 157:319–325.
19. Caughey B, Brown K, Raymond G. Binding of the protease sensitive form of prion protein PrP to sulfated glycosoaminoglycans and congo red. J Virol 1994; 68:2135–2141.
20. Brimacombe DB, Bennett AD, Wustemann FS, Gill AC, Dann JC. Characterization and polyanion-binding properties of purified recombinant prion protein. Biochem J 1999; 342:605–613.
21. Moynagh J, Schimmel H. Tests for BSE evaluated. Bovine spongiform encephalopathy [letter]. Nature 1999; 400:105.
22. Madec JY, Belli P, Calavas D, Baron T. Efficiency of Western blotting for the specific immunodetection of proteinase K–resistant prion protein in BSE diagnosis in France. Vet Rec 2000; 146:74–76.
23. Barnard G, Helmick BJ, Madden S, Gilbourne C, Patel RK. The measurement of prion protein in bovine brain tissue using differential extraction and DELFIA as a diagnostic test for BSE. Luminescence 2000; 15:357–362.
24. Kocisko DA, Lansbury PT, Caughey B. Partial unfolding and refolding of scrapie-associated prion protein: evidence for critical 16-kDa C-terminal domain. Biochemistry 1996; 35:13434–13442.
25. Caughey B, Raymond GJ, Kocisko DA, Lansbury PT. Scrapie infectivity correlates with converting activity, protease resistance, and aggregation of scrapie-associated prion protein in guanidine denaturation studies. J Virol 1997; 71:4107–4110.
26. Callahan MA, Xiong L-W, Caughey B. Reversibility of scrapie associated prion protein aggregation. J Biol Chem 2001; 276:28022–28028.
27. Safar J, Wille H, Itri V, Groth D, Serban H, Torchia M, Cohen FE, Prusiner

SB. Eight prion strains have PrP (Sc) molecules with different conformations Nature Med 1998; 4:1157–1165.
28. Wilesmith JW. Manual on Bovine Spongiform Encephalopathy. FAO Animal Health Manual No.2. Food and Agriculture Organization of the United Nations, Rome. 1998; 13–14.
29. Ebringer A, Pirt J, Wilson C, Cunningham P, Thorpe C, Ettelaie C. Bovine spongiform encephalopathy: is it an autoimmune disease due to bacteria showing molecular mimicry with brain antigens? Environ Health Perspect 1997; 105:1172–1174.
30. Brown DR. Prion and prejudice: normal protein and the synapse. Trends Neurosci 2001; 24:85–90.
31. Stockel J, Safar J, Wallace AC, Cohen FE, Pruisiner SB. Prion protein selectively binds copper (II) ions. Biochemistry 1998; 37:7185–7193.
32. Aronoff-Spencer E, Burns CS, Avdievich NI, Gerfen GJ, Peisach J, Antholine WE, Ball HL, Cohen FE, Pruisiner SB, Millhauser GL. Identification of the Cu^{2+} binding sites in the N-terminal domain of the prion protein by EPR and CD spectroscopy. Biochemistry 2000; 39:13760–13771.
33. Shaked Y, Rosenmann H, Hijari N, Halimi M, Gabizon R. Copper binding to the PrP isoforms: a putative marker of their conformation and function. J Virol 2001; 75:7872–7874.
34. Requena JR, Groth D, Legname G, Stadtman ER, Prusiner SB, Levine RL. Copper-catalyzed oxidation of the recombinant sha(29-231) prion protein. Proc Natl Acad Sci USA 2001; 98:7170–7175.
35. Requena JR, Levine RL. Thioredoxin converts the syrian hamster (29-231) recombinant prion protein to an insoluble form. Free Rad Biol Med 2001; 30:141–147.
36. Wong B-S, Liu, T, Li R, Pan T, Petersen RB, Smith MA, Gambetti P, Perry G, Manson JC, Brown DR, Sy M-S. Increased levels of oxidative stress markers in the brains of mice devoid of prion protein. J Neurochem 2001; 76:565–572.
37. Asher DM, Padilla AM, Pocchiari M. WHO Consultation on Diagnostic Procedures for Transmissible Spongiform Encephalopathies: Need for Reference Reagents and Reference Panels. Geneva, Switzerland, 22–23 March 1999. Biologicals 1999; 27:265–272.
38. Minor P, et al, and the WHO Working Group on International Reference Materials for the Diagnosis and Study of Transmissible Spongiform Encephalopathies (TSEs). Standards for the assay of CJD specimens. 2003, in preparation.
39. Zanusso G, Liu D, Ferrari S, Hegyi I, Yin X, Aguzzi A, Hornemann S, Liemann S, Glockshuber R, Manson JC, Brown P, Petersen RB, Gambetti P, Sy M-S. Prion protein expression in different species: analysis with a panel of new Mabs. Proc Natl Acad Sci USA 1998; 95:8812–8816.

11
In Vivo Diagnosis of Prion Diseases

Ruth Gabizon and Gideon M. Shaked
Hadassah University Hospital, Jerusalem, Israel

I. INTRODUCTION

Prion diseases are a group of fatal, neurodegenerative diseases of humans and animals believed to be caused by the accumulation in the brain of the victims of an aberrantly folded protein, denominated PrP^{Sc}. Although the pathology of the disease is unclear, there is a consensus in the field that the presence of the protease-resistant form of PrP, denominated PrP^{Sc}, is the only reliable marker for the disease. PrP^{Sc} is present mostly in the brains of animals and humans affected with prion diseases. We have recently shown (1) that a protease-resistant PrP isoform can also be detected in the urine of hamsters, cattle, and humans suffering from transmissible spongiform encephalopathies (TSEs). Most important, this PrP isoform ($UPrP^{Sc}$) was also found in the urine of hamsters inoculated with prions long before the appearance of clinical signs. Interestingly, intracerebral inoculation of hamsters with $UPrP^{Sc}$ did not cause clinical signs of prion disease even after 400 days, suggesting it differs in its pathogenic properties from brain PrP^{Sc}. We propose that the detection of $UPrP^{Sc}$ in urine can be used to diagnose humans and animals incubating prion diseases, as well as to increase our understanding of the metabolism of PrP^{Sc} in vivo.

PrP^{Sc} is the abnormal isoform of PrP^{C}, a GPI-anchored glycoprotein of unknown function (2,3). Since the appearance of bovine spongiform en-

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cephalopathy (BSE) in 1985 (4,5), a cattle prion disease produced by humans by the common modern practice of feeding animals with bonemeal mixtures signaled the appearance of a new worldwide health problem. For about 10 years it was debated whether the cattle epidemic would transmit to humans. Since 1996, it has become clear that such transmission had occurred and resulted in humans in a disease denominated variant Creutzfeldt-Jakob disease (vCJD), a variant form of the rare human prion disease CJD. The incubation time of vCJD is unknown, but believed to be very long, even in the range of decades. It is therefore possible that today, a very large number of individuals in the world are incubating the disease. Owing to the late discovery of BSE all over Europe, it is clear now that both BSE and vCJD are not confined to the United Kingdom but are a worldwide problem, which may last for many years. Therefore, the need for an in vivo diagnostic test for prion diseases has become acute. In the absence of such a method, an extensive slaughtering of cattle was required once an affected animal was identified within a herd. The need for such an in vivo test was reinforced since the first cases of vCJD were reported in 1996 (6–8). vCJD is a fatal neurodegenerative disease believed to be caused by the consumption of BSE-contaminated meat, and the incubation time between infection to clinical symptoms may be as long as decades (9). As opposed to cattle, the incubating individuals (which at this point can be any of us) will be present for many years, donating blood and in some cases other organs to the nonaffected population.

At this moment, there is no cure for prion diseases. It is our belief that it is unlikely that a treatment for a neurodegenerative disease in an advanced state will be developed, since by the time the disease is diagnosed, the process is mostly irreversible. Therefore, only early and prophylactic treatments are a reasonable approach. Such a therapeutic approach requires an in vivo, early diagnostic test.

The only identified component of the prion, the agent causing prion diseases (also referred to as TSEs), is PrP^{Sc}, a protease-resistant abnormal isoform of PrP^{C}. It has been postulated that prion diseases propagate by the conversion of PrP^{C} molecules into protease-resistant and insoluble PrP^{Sc} molecules by a mechanism in which PrP^{Sc} serves as a template (10). The pathway for PrP^{Sc} synthesis may feature the formation of PrP^{C}-PrP^{Sc} heterodimers (11). Alternatively, the nucleation-dependent protein polymerization model argues that the formation of new PrP^{Sc} molecules depends on the presence of a seed composed of aggregated PrP^{Sc} molecules and that new PrP^{Sc} molecules join previously assembled prion polymers (12). Although many lines of evidence suggest that PrP^{Sc} is the crucial and even the only prion component, until recently infectivity could not be associated with PrP^{Sc}-like PrP molecules produced by an array of in vitro conversion protocols (12a–14).

Although some other markers for prion diseases have been suggested (15–17). PrP^{Sc} remains not only an obligatory prion component, but also the

only reliable and universally accepted marker for this family of diseases (18–20). We have recently shown that a protease-resistant isoform of PrP, hereby denominated $UPrP^{Sc}$, can be detected, after a specific enrichment procedure, in the urine of scrapie-infected hamsters, BSE-infected cattle, and humans suffering from CJD. These results may pave the way for the development of a simple in vivo test for prion diseases.

We are also investigating the properties of urine PrP in normal and prion-affected individuals, to understand the metabolic pathways that may lead the prion protein molecules from the brain or other infected organs to urine.

II. EXPERIMENTAL PROCEDURES

A. Analysis of Urine Samples

Urine samples (2 mL for hamster; 10 mL for human; 15 mL for bovine) were sedimented for 5 min at 3000 rpm to discard occasional cell debris, and then dialyzed overnight in a cellulose tubular membrane (pore range 6000–8000 Da, FPI, Texas, USA) against 5 L of saline at 4°C (saline was changed twice during dialysis). For experimental purposes (Fig. 1c), the dialysis step was omitted in some cases. Subsequently, the urine samples were centrifuged at high speed (100,000 gav for 1 hr at 4∞c). Pellets were resuspended in 100 mL 2% sarkosy/ISTE buffer (10 mM Tris HCl pH 7.5, 10 mM NaCl, 1 mM EDTA). Samples were divided and digested in the presence or absence of proteinase K (PK). Digestion conditions were optimized for each species: for hamster urine; 40 mg/mL PK for 60 min at 37°C; for human urine, 40 mg/mL PK for 30 min at 37°C; for bovine urine, 20 mg/mL for 30 min at 37°C.

After protease digestion, the urine samples were boiled in SDS sample buffer, applied to a 12% SDS PAGE, and subsequently transferred to a nitrocellulose membrane. Membranes were blocked with 3% fat milk except for the bovine samples, which were blocked with 5% HAS (Human Serum Albumin, Sigma). A second blocking step was performed with a mixture of 1:3000 antimouse lgG and 1:3000 antirabbit lgG in TBST buffer (for 30 min) to avoid nonspecific binding of the secondary Ab to lgG light chain present in some urine samples. Membranes were then rinsed in TBST for 15 min and immunoblotted with either a PrP mAb 3F4 or 6H4 (hamster, human) at 1:5000 or 6H4 (bovine) at 1:5000.

B. In Vivo Experiments

Syrian hamsters were inoculated with samples containing urine PrP from normal or scrapie-infected hamsters. For inoculation, urine samples were

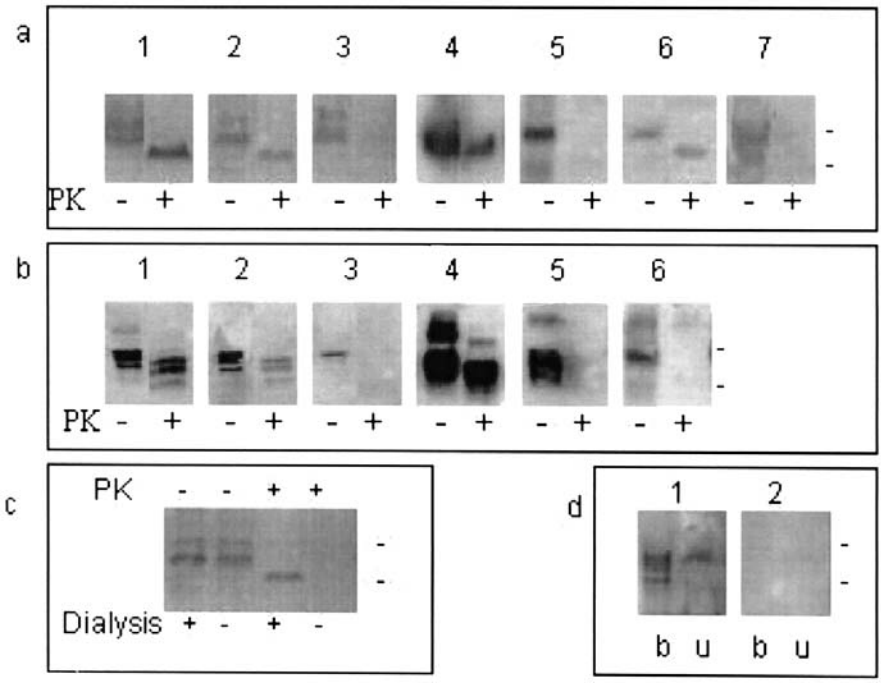

Figure 1 Protease-resistant PrP in urine of TSE-affected humans and animals. (a) Freshly frozen urine samples from hamsters, humans, and cattle were enriched for protease-resistant PrP as described. All samples were digested in the presence or absence of proteinase K, as described, and immunoblotted with either PrP mAb 3F4 (hamster and human samples) or 6H4 (bovine samples): (1) homozygous E200K CJD patient, (2) heterozygous E200K CJD patient (3) human control, (4) scrapie-infected hamster, (5) normal hamster, (6) BSE-infected cattle, (7) normal cattle. The volumes of urine used are described in the text. (b) Five milliliters of 10% brain samples were analyzed for PrP: (1) homozygous CJD patient, (2) heterozygous CJD patient, (3) human control, (4) scrapie-infected hamster, (5) normal hamster, (6) kidney sample from scrapie-infected hamster. (c) Scrapie hamster urine samples were enriched for $UPrP^{Sc}$ with and without the dialysis step. Samples were digested in the presence or absence of PK as described. (d) Human brain sample (b) and human urine sample (u) were immunoblotted with mAb 3F4 in the absence (1) or the presence (2) of 10 mg/mL of the peptide comprising the 3F4 epitope. Molecular weight markers (top to bottom): 36 kDa, 30 kDa.

prepared as described above (including PK digestion but not SDS boiling) and diluted as required in 1% BSA/PBS. Brain samples from scrapie-infected hamsters were diluted to contain similar concentrations of PrP^{Sc} (Fig. 3) and inoculated into additional groups of hamsters. To achieve similar concentrations of protease-resistant PrP in brain and urine inoculi, each animal was inoculated, depending on the appropriate experimental group, with a 50-mL sample containing PrP originating from either 0.5 mL urine or 1.25 mL of 10% scrapie hamster brain homogenate.

C. Collection of Urine from Hamsters

After inoculation, animals were examined daily for scrapie-associated symptoms. For time course experiments, groups of three hamsters in an equivalent stage of disease incubation were housed each week in a metabolic cage for urine collection from 3:00 P.M. to 8:00 A.M. the next day. Urine was collected in the morning and immediately frozen at (−80°C). Food and water were supplied ad libitum. A similar procedure was applied to scrapie-infected hamsters.

D. Tissue Homogenates

Whole brain or kidney was homogenized in 10 vol homogenization buffer (10 mM Tris HCl, pH 7.5, 300 mM sucrose). After centrifugation (2000 rpm, 15 min, °C), the supernatant was frozen (−80°C).

E. Human Urine Samples

Whenever possible, the human samples from CJD patients and controls were the first-morning urine. Some CJD and poststroke patients were using catheters, and in these cases urine was collected for a period of up to 8 h in a urine-collecting bag. All samples were frozen until further use.

F. Bovine Urine Samples

All BSE and most control bovine urine samples were obtained from the Veterinary Laboratory Agency (VLA) in London. The VLA samples constituted 51 samples of 24 cows, all coded for blind testing. Additional freshly frozen control samples were obtained from the Hebrew University Veterinary School. According to VLA records, most samples were frozen following collection, while some were kept chilled. No information was provided regarding time of day for sample collection.

III. RESULTS

Human prion diseases are manifested as sporadic, infectious, or inherited forms. Inherited prion diseases, including familial Creutzfeldt-Jakob disease (fCJD), Gerstmann-Straussler-Scheinker disease, and fatal familial insomnia, are transmitted as autosomal-dominant disorders, and are caused by mutations in the human prion protein (PrP) gene, designated PRNP (21). To date, more than 20 pathogenic mutations in PRNP have been discovered in families with inherited prion diseases, and for five of them genetic linkage has been demonstrated (22). The largest focus of fCJD in the world was identified among Jews of Libyan origin, carrying a missense mutation in codon 200 of PRNP (substitution of lysine for glutamate, E200K) (23).

The research on the focus of fCJD in Libyan Jews is important not only because of its size but also because of the clinical and pathological resemblance to sporadic CJD (24). The mean age of the disease onset (58 years), the mean disease duration (4.3 months), and clinical and electroencephalographic findings are similar. The brain pathology in both forms shows spongiform changes with profound gliosis and absence of PrP plaques. A unique finding in fCJD in Libyan Jews is the identification of CJD patients who were homozygous for the E200K mutation owing to the genetic isolation of this community and the high rate of consanguinity (24). The disease in these patients occurred significantly earlier, at the age of 50 years as opposed to 58 years, suggesting a dose effect contribution of the mutation to the onset of the disease (25). The identification of these homozygous patients allowed us to investigate the properties of the mutant protein E200KPrP in the absence of wtPrP (wild-type).

Most of the CJD patients tested in this work were genetic patients carrying the E200K mutation (23, 26–28). One of the patients was a 52-year-old individual homozygous for this mutation (25). Among the other genetic patients, four were MM at codon 129 and one was MV. The E200K mutation is located at a methionine 129 allele (27). The human controls ($n = 15$), were either healthy individuals ($n = 7$) or patients suffering from diverse neurological disorders, such as Alzheimer's disease ($n = 3$), multiple sclerosis ($n = 2$), or stroke ($n = 3$). Urine from BSE-infected cattle, as well as most of the bovine controls used in this work, were obtained from the VLA laboratory in England. For the bovine samples, the urine test was performed as a blind study (Tables 1 and 2); samples were sent to us in a coded form and codes were open only after results were delivered back to the VLA investigators. Urine from Syrian hamsters, either inoculated with the 263K prion strain (29) or normal controls, were collected with the use of a metabolic cage (as explained earlier), as pools from three animals.

Table 1 Blind Urine Test for VLA Cattle

Number	Clinical	Histopathology	Urine test
1	BSE negative	Negative	Negative
2	BSE negative	Negative	Negative
3	BSE positive	Positive	Positive
4	BSE negative	Negative	Negative
5	BSE negative	Negative	Negative
6	BSE negative	Negative	Negative
7	BSE positive	Positive	Positive
8	BSE suspect	Negative	Positive (low signal)
9	BSE suspect	Positive	Positive (low signal)
10	BSE suspect	Positive	Nonspecific background
11	BSE negative	Negative	Negative
12	BSE suspect	Negative	Positive (low signal)
13	BSE positive	Positive	Positive
14	BSE negative	Negative	Negative
15	BSE positive	Positive	Positive
16	BSE suspect	Positive	Positive (low signal)
17	BSE positive	Positive	Positive
18	BSE suspect	Negative	Positive (low signal)
19	BSE suspect	Negative	Positive (low signal)
21	BSE positive	Positive	Positive
22	BSE positive	Positive	Positive
23	BSE negative	Negative	Negative
24	BSE positive	Positive	Positive
25	BSE suspect	Negative	Negative
26	BSE suspect	Positive	Nonspecific background
27	BSE negative	Negative	Negative
28	BSE negative	Negative	Negative
29	BSE negative	Negative	Negative
30	BSE positive	Positive	Positive
31	BSE negative	Negative	Negative
32	BSE negative	Negative	Negative
33	BSE positive	Positive	Positive
34	BSE suspect	Negative	Negative
35	BSE negative	Negative	Negative
36	BSE positive	Positive	Positive
37	BSE suspect	Negative	Positive
38	BSE positive	Positive	Positive
39	BSE negative	Negative	Negative
40	BSE suspect	Negative	Negative
41	BSE negative	Negative	Negative
42	BSE suspect	Positive	Positive (low signal)
43	BSE positive	Positive	Positive
44	BSE suspect	Negative	Positive
45	BSE positive	Positive	Positive
46	BSE suspect	Positive	Positive (low signal)
47	BSE positive	Positive	Positive
48	BSE negative	Negative	Negative
49	BSE positive	Positive	Positive
50	BSE positive	Positive	Positive
51	BSE negative	Negative	Negative

Table 2 Blind Urine Test for VLA Cattle

Clinical	Histopathology	VLA	Urine test
BSE positive 2 nonspecific background	20	20 positive	18 positive
BSE suspect	13		
	4 positive	4 positive (low signal)	
	9 negative	2 positive	
	3 negative		
	4 positive (low signal)		
BSE negative	18	18 Negative	18 negative
Total	51	51	

Urine samples from scrapie-infected hamsters, CJD patients, and BSE-infected cattle, as well as from their appropriate controls, were processed for enrichment of $UPrP^{Sc}$ as described earlier, and subsequently immunoblotted for PrP. Human and hamster urine samples were immunoblotted with either mAb 3F4 or 6H4 (not shown), while bovine samples were blotted only with mAb 6H4. Parallel samples were blotted only with a secondary mouse antisera to rule out the possibility of interfering signals.

Figure 1 demonstrates the results of such an experiment. While a precipitable and protease-resistant form of PrP could be detected in the dialyzed urine of prion disease–affected humans and animals, this was not the case for the urine of the appropriate controls. The PrP urine assay was negative for all control humans ($n = 15$), hamsters ($n = 10$) when each sample represents a pool from three hamsters), and cattle ($n = 15$), positive for all CJD patients tested ($n = 8$), for a large number of hamster groups ($n = 20$), and for 10 of 12 BSE-infected cattle, while the other two BSE-positive cows showed a positive but poor signal. As can be seen in Fig. 1c, dialysis was essential for detection of the protease-resistant $UPrP^{Sc}$ in urine from scrapie-infected hamsters. The fact that the PrP signal in urine could be blocked by the 3F4 peptide and did not react with the secondary antibody provides strong evidence that this signal belongs to a PrP peptide (Fig. 1d). Nevertheless, it is important to prove more directly that the reacting protein is indeed a PrP isoform, and thereby we are going to sequence the urine protein.

A surprising result depicted above is that a protease-sensitive PrP isoform is present in the precipitable fraction of the normal urine samples, as opposed to what is expected for PrP^{C}. It should be noted, however, that no

detergent was added to the urine before ultracentrifugation as performed in membrane extractions that result in a soluble PrP^{C} (30, 31). It is also possible that all PrP molecules are present in urine in a partly denatured state owing to the presence of urea, which may encourage protein aggregation. Also, dialysis of normal urine may induce aggregation of the PrP^{C} isoform, which, as opposed to $UPrP^{Sc}$, is protease sensitive. Although the exact chemical nature of $UPrP^{Sc}$ is yet to be determined, its molecular weight seems to be slightly higher than full-length and fully glycosylated PrP^{C} or PrP^{Sc}. In addition, the pattern of $UPrP^{Sc}$ in the immunoblots suggests it may be composed mostly of the higher molecular band of PrP, and not of the less glycosylated species. This may indicate that partly or nonglycosylated PrP is less resistant to the conditions encountered by PrP^{Sc} until it is excreted in urine as $UPrP^{Sc}$. That normal PrP is excreted in urine is not entirely surprising since this is also the case of other GPI-anchored proteins (32–34). As stated in the Introduction, we are in the process of testing the biochemical properties of PrP^{Sc} and PrP^{C} in urine, so that we can elucidate the metabolic pathways that result in finding PrP isoforms in urine.

One metabolic pathway we have ruled out so far is that $UPrP^{Sc}$ originated directly from the kidneys. This was possible if PrP^{Sc} was found at considerable concentrations in kidney tissue. However, this was not the case, since no PrP^{Sc} could be identified in the kidney tissue of the scrapie-infected hamsters (Fig. 1b). This result therefore suggests $UPrP^{Sc}$ originates from other organs and arrives to the urine from blood through the filtration process. Interestingly, this result will mean that although PrP^{Sc} was never identified directly in blood, it must be present there even if at very low quantities, so as to arrive to the urine.

The detection of PrP^{Sc} at the end stages of prion disease may result from some degree of blood-brain-barrier disruption by brain degeneration (35). Contrarily, the presence of PrP^{Sc} in prion-infected urine early in the incubation time would suggest a clearance pathway for the aberrant PrP protein either from brain or from a peripheral organ, through its excretion into urine. To address this question, we inoculated Syrian hamsters either intracerebrally (i.c.), or intraperitoneally (i.p.) with hamster prions and collected urine samples, as described earlier, every week during the incubation period. Each sample was frozen immediately after collection. At the end of the experiment, similar volumes of these urine samples were thawed, enriched for PK-resistant $UPrP^{Sc}$ as described above, and subsequently immunoblotted with PrP mAb 3F4.

The results of such an experiment can be seen in Fig. 2. A light signal of prion-specific PrP was detected in the urine samples of the i.c.-inoculated hamsters after only 17 days (Fig. 2a), followed by the disappearance of the PrP signal until day 35. Subsequently, the signal for protease-resistant PrP

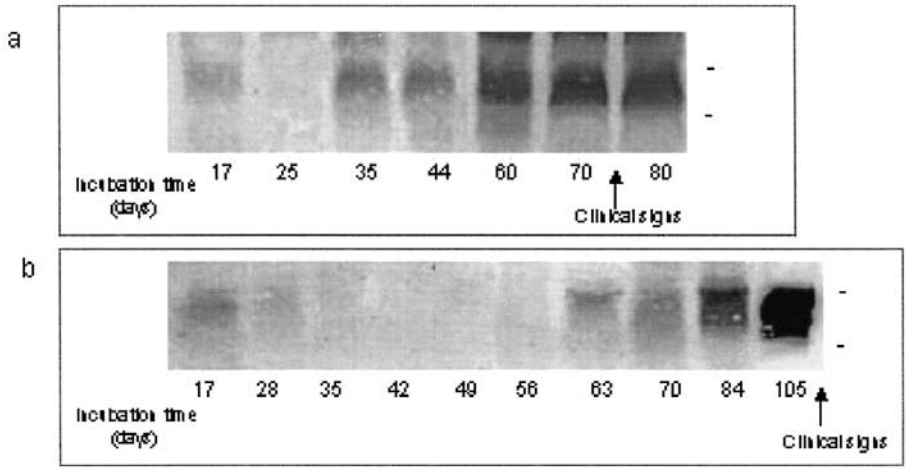

Figure 2 Prion-specific PrP can be detected during scrapie incubation time. Urine samples were collected weekly from Syrian hamsters inoculated either i.c. (a), or i.p (b) with hamster 263K prions, and enriched for $UPrP^{Sc}$ as described. Samples were immunoblotted with aPrP mAb 3F4. Arrows represent the onset of clinical signs. Molecular weight marker: 30 kDa.

increased until the appearance of clinical signs. Similar results were obtained for the i.p.-inoculated hamsters. A PrP signal was detected in the first weeks following the inoculation, disappeared at later dates, and reappeared at about 60 days. These results may imply that some of the prion inoculum is immediately secreted after inoculation. Thereafter, no PrP signal appeared in urine until the first stages of prion protein accumulation in brain. Indeed, while scrapie incubation time for i.c.- or i.p-inoculated hamsters with the 263 strain is about 75 and 120, days respectively, PrP^{Sc} can be identified in enriched brain samples of these hamsters at about 40 (i.c.) or 70 (i.p.) days (36–38). Our experiments therefore suggest that $UPrP^{Sc}$ is excreted in urine in parallel to its accumulation in brain.

The results of the experiments described in both Fig. 1 and 2 indicate therefore that urine testing for protease-resistant PrP can be used not only to diagnose prion diseases in animals and humans at terminal stages of the disease, but also to diagnose these diseases in the subclinical stages of infection. If indeed the PrP signal detected at the first weeks postinfection is due to clearance of the inoculum, the PrP urine test may serve to diagnose a potential new occurrence of infection. This will allow, in the future, provided an effective antiprion therapy becomes available, treating individuals at risk of

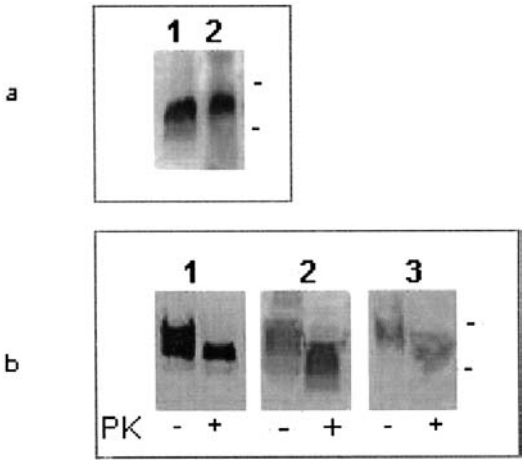

Figure 3 Intracerebral inoculation of Syrian hamsters with $UPrP^{Sc}$. Syrian hamsters were inoculated with equivalent amounts of PK-resistant PrP from brain or urine of scrapie-infected hamsters. All samples were immunoblotted with 1:5000 mAb 3F4. (a) PK-resistant PrP^{Sc} equivalents originating from 5 mL of 10% hamster brain homogenate (1) as compared to 2-mL scrapie hamster urine (2). (b) (1) Brain sample from a scrapie-infected hamster. (2) Urine samples collected (at 60 dpi) from hamsters inoculated with $UPrP^{Sc}$. (3) Brain sample of one of the animals inoculated with $UPrP^{Sc}$. All samples were digested in the presence or absence of PK. Molecular weight markers (top to bottom): 36 kDa, 30 kDa.

a new prion exposure, as well as treating prophylactically carriers of pathogenic PrP mutations.

The detection of $UPrP^{Sc}$ raises the alarming possibility that urine from prion-infected individuals, either ill or as yet incubating the disease, can somehow transmit prion diseases. This prospect is especially disturbing in the case of BSE-infected cattle as well as in natural scrapie in sheep. Since the mechanism by which these diseases are transmitted among animals within the herd was never well elucidated (39,40), it is conceivable that urine can contaminate the living areas of these animals.

To investigate whether urine from TSE-infected animals can be infectious, we inoculated 20 hamsters with $UPrP^{Sc}$ pooled and enriched from urine of 10 hamsters terminally ill with scrapie. Twenty hamsters were inoculated with similarly prepared samples from 10 normal hamsters. Brain samples from scrapie-infected hamsters, which were diluted to contain similar concentrations of PrP^{Sc} (1.25 mL of 10% homogenate) as the enriched $UPrP^{Sc}$ (from 0.5 mL urine), were inoculated in additional groups of hamsters (Fig. 3a).

Hamsters were observed daily for symptoms of scrapie infection. Urine was collected periodically from animals inoculated with $UPrP^{Sc}$. At different time points during the experiment, some of the hamsters inoculated with $UPrP^{Sc}$ were sacrificed and tested for the presence of PrP^{Sc} in their brains.

As expected, the animals inoculated with scrapie-infected brain samples suffered from fatal disease symptoms at about 80 days postinoculation. Contrarily, none of the animals inoculated with urine samples (normal or scrapie infected) have developed clinical symptoms of prion disease to date (270 dpi). Twelve hamsters (four groups of three) were tested for the presence of $UPrP^{Sc}$ and all were found positive from about 60 days postinoculation (Fig. 3b, group 2). In addition, low concentrations of PrP^{Sc} could be identified in the brain of one out of three hamsters sacrificed at about 120 days (Fig. 3b, group 3). All other hamsters in this experiment are still under observation to determine whether they will develop a fatal prion disease at a later date. These results suggest that $UPrP^{Sc}$ inoculation can result in a subclinical or carrier state prion infection. The clinical and epidemiological implications of this finding are yet to be determined. More experiments are being performed to test whether TSE urine can be infectious in other experimental setups.

IV. DISCUSSION

Although much is known about the biochemistry and genetics of prions, this is not the case for the diagnosis or treatment for the disease. Diagnosis can be done easily by looking immunologically for PrP^{Sc} in the brains of affected animals and humans, and this is indeed the basis for the present kits for screening cattle for BSE (Prionics, Bio-Rad, etc). However, no methods for monitoring BSE in alive cattle are in the market. There is also no method for discovering BSE during the disease incubation time, although it has been shown, for all prion disease, that incubating animals that are not yet sick can transmit the disease. There is also no method for screening humans before blood donation to assess whether they are incubating the disease and can transmit it to others. Today, individuals living in the United Kingdom cannot donate blood in many countries, but with the spread of the disease the situation becomes more complicated.

For these reasons, our finding that PrP is present in urine long before clinical signs may have great clinical importance. Why is $UPrP^{Sc}$ excreted into urine? Since most urine proteins originate from blood, we speculate that some PrP^{Sc} molecules, either from brain or from a peripheral organ, is released during the disease incubation into the blood serum in a nonaggregated form, although at a low and undetectable concentration. Owing to its protease resistance, this PrP^{Sc} is not digested by blood proteases. However, and since

the MW of PrP is below the cutoff size for filtering through kidney cells (about 40 kDA) (41), PrP may subsequently be secreted into the urine and thereby be concentrated, like other proteins, at about 120 times more than its concentration in blood (41). The concentration by the kidney, makes it possible to detect $UPrP^{Sc}$ in urine much more easily than in blood. Since dialysis of the urine seems to be a necessary step in our detection procedure, we propose that $UPrP^{Sc}$ is present in a semidenatured form, probably owing to the relatively high concentrations of urine-denaturing agents, and is subsequently renatured during the dialysis step. This denaturation/renaturation effect may also occur in the field owing to absorption of the urea by the soil.

$UPrP^{Sc}$ may differ in its conformation from brain PrP^{Sc}, thereby explaining the fact that inoculation of comparable amounts of both protease-resistant PrP isoforms produced such different results It must be remembered, however, that not all protease-resistant PrP molecules carry prion infectivity. Indeed, in vitro conversion experiments of PrP^{C} to PrP^{Sc}, in which protease resistance was achieved by a denaturation/renaturation procedure, resulted in a protease-resistant but not infectious PrP isoform (13,14). Contrarily, $UPrP^{Sc}$ may resemble the new protease-resistant soluble isoform we have identified lately, which is associated with very low levels of infectivity, if any (1,42). The latest possibility is consistent with the fact that $UPrP^{Sc}$ is found in urine since it would be difficult for an aggregated molecule to be filtered through the kidney.

It can also be speculated that $UPrP^{Sc}$ is a component of a new prion strain, less virulent than the original 263K strain, which may produce not a fatal but a subclinical or a carrier-state prion disease. Recent publications indicate the presence of low levels of PrP^{Sc} in the brains of animals that did not succumb to prion disease (43,44), suggesting a subclinical state of prion disease may exist. In some cases, the brains of these animals transmitted a fatal prion disease to other rodents, suggesting that apparently healthy carriers of prion disease can transmit disease.

To summarize, we have identified a prion-specific, protease-resistant PrP isoform in the urine of prion-infected animals and humans ($UPrP^{Sc}$), which may be used for the in vivo early diagnosis of ill as well as seemingly healthy but prion-infected individuals. Our findings, in addition to their practical aspects, may also open new avenues to investigate the metabolism and clearance mechanism of PrP^{Sc} during prion infection and disease.

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REFERENCES

1. Shaked GM, Shaked Y, Kariv Z, Halimi M, Avraham I, Gabizon R. J Biol Chem 2001; 21:21.
2. Oesch B, Westaway D, Walchli M, McKinley MP, Kent SB, Aebersold R, Barry RA, Tempst P, Teplow DB, Hood LE, et al. Cell 1985; 40(4):735–746.
3. McKinley MP, Bolton DC, Prusiner SB. Cell 1983; 35(1):57–62.
4. Hope J, Reekie LJ, Hunter N, Multhaup G, Beyreuther K, White H, Scott AC, Stack MJ, Dawson M, Wells GA. Nature 1988; 336(6197):390–392.
5. Fisher E, Telling G, Collinge J. Mamm Genome 1998; 9(7):497–502.
6. Zeidler M, Johnstone EC, Bamber RW, Dickens CM, Fisher CJ, Francis AF, Goldbeck R, Higgo R, Johnson Sabine EC, Lodge GJ, McGarry P, Mitchell S, Tarlo L, Turner M, Ryley P, Will RG. Lancet 1997; 350(9082):908–910.
7. Bruce ME, Will RG, Ironside JW, McConnell I, Drummond D, Suttie A, McCardle L, Chree A, Hope J, Birkett C, Cousens S, Fraser H, Bostock CJ. Nature 1997; 389(6650):498–501.
8. Ironside JW, Head MW, Bell JE, McCardle L, Will RG. Histopathology 2000; 37(1):1–9.
9. Bruce ME, Will RG, Ironside JW, McConnell I, Drummond D, Suttie A, McCardle L, Chree A, Hope J, Birkett C, Cousens S, Fraser H, Bostock CJ. Nature 1997; 389(6650):498–501.
10. Pan KM, Stahl N, Prusiner SB. Purification and properties of the cellular prion protein from Syrian hamster brain. Protein Sci 1992; Oct; 1(10):1343–1352.
11. Prusiner SB, Scott MR, DeArmond SJ, Cohen FE. Prion protein biology. Cell 1998; May 1; 93(3):337–348.
12. Come JH, Fraser PE, Lansbury PT Jr. A kinetic model for amyloid formation in the prion diseases: importance of seeding. Proc Natl Acad Sci USA 1993; Jul 1; 90(13):5959–5963.
13. Kocisko DA, Come JH, Priola SA, Chesebro B, Raymond GJ, Lansbury PT, Caughey B. Nature 1994; 370(6489):471–474.
14. Hill AF, Antoniou M, Collinge J. J Gen Virol 1999; 80(Pt 1):11–14.
15. Miele G, Manson J, Clinton M. Nature Med 2001; 7(3):361–364.
16. Rosenmann H, Meiner Z, Kahana E, Halimi M, Lenetsky E, Abramsky O, Gabizon R. Neurology 1997; 49(2):593–595.
17. Hsich G, Kenney K, Gibbs CJ, Lee KH, Harrington MG. N Engl J Med 1996; 335(13):924–930.

18. Safar J, Wille H, Itri V, Groth D, Serban H, Torchia M, Cohen FE, Prusiner SB. Nature Med 1998; 4(10):1157–1165.
19. Serban D, Taraboulos A, DeArmond SJ, Prusiner SB. Neurology 1990; 40(1):110–117.
20. Prusiner SB. Proc Natl Acad Sci USA 1998; 95(23):13363–13383.
21. Prusiner SB. Inherited prion diseases. Proc Natl Acad Sci USA 1994; 24(91): 4611–4614.
22. Goldfarb LG, Brown P, Cervenakova L, Gajdusek DC. Molecular genetic studies of Creutzfeldt-Jakob disease. Mol Neurobiol 1994; Apr–Jun; 8(2–3): 89–97.
23. Hsiao K, Meiner Z, Kahana E, Cass C, Kahana I, Avrahami D, Scarlato G, Abramsky O, Prusiner SB, Gabizon R. N Engl J Med 1991; 324(16):1091–1097.
24. Meiner Z, Gabizon R, Prusiner SB. Familial Creutzfeldt-Jakob disease. Codon 200 prion disease in Libyan Jews. Medicine (Baltimore) 1997; Jul; 76(4):227–237.
25. Simon ES, Kahana E, Chapman J, Treves TA, Gabizon R, Rosenmann H, Zilber N, Korczyn AD. Ann Neurol 2000; 47(2):257–260.
26. Gabizon R, Telling G, Meiner Z, Halimi M, Kahana I, Prusiner SB. Nature Med 1996; 2(1):59–64.
27. Gabizon R, Rosenmann H, Meiner Z, Kahana I, Kahana E, Shugart Y, Ott J, Prusiner SB. Am J Hum Genet 1993; 53(4):828–835.
28. Goldfarb LG, Korczyn AD, Brown P, Chapman J, Gajdusek DC. Lancet 1990; 336(8715):637–638.
29. Kimberlin RH, Walker CA. J Gen Virol 1986; 67(Pt 2):255–263.
30. Meyer RK, McKinley MP, Bowman KA, Braunfeld MB, Barry RA, Prusiner SB. Proc Natl Acad Sci USA 1986; 83(8):2310–2314.
31. Gabizon R, McKinley MP, Prusiner SB. Proc Natl Acad Sci USA 1987; 84(12): 4017–4021.
32. Nakano Y, Noda K, Endo T, Kobata A, Tomita M. Arch Biochem Biophys 1994; 311(1):117–126.
33. Rindler MJ, Naik SS, Li N, Hoops TC, Peraldi MN. J Biol Chem 1990; 265 (34):20784–20789.
34. Meri S, Lehto T, Sutton CW, Tyynela J, Baumann M. Biochem J 1996; 316(Pt 3):923–935.
35. De Armond SJ, Gonzales M, Mobley WC, Kon AA, Stern A, Prusiner H, Prusiner SB. Prog Clin Biol Res 1989; 317:601–618.
36. Czub M, Braig HR, Diringer H. J Gen Virol 1988; 69(Pt 7):1753–1756.
37. Czub M, Braig HR, Diringer H. J Gen Virol 1986; 67(Pt 9):2005–2009.
38. Taraboulos A, Jendroska K, Serban D, Yang SL, DeArmond SJ, Prusiner SB. Proc Natl Acad Sci USA 1992; 89:7620–7624.
39. Bradley R, Matthews D. Rev Sci Tech 1992; 11(2):605–634.
40. Chatelain J, Dautheville Guibal C. Eur J Epidemiol 1989; 5(1):113–116.
41. Berne RM, Levy MN. 4th ed. Physiology, 1998.
42. Shaked GM, Fridlander G, Meiner Z, Taraboulos A, Gabizon R. Protease-

resistant and detergent-insoluble prion protein is not necessarily associated with prion infectivity. J Biol Chem 1999; Jun 18; 274(25):17981–17986.
43. Hill AF, Joiner S, Linehan J, Desbruslais M, Lantos PL, Collinge J. Proc Natl Acad Sci USA 2000; 97(18):10248–10253.
44. Tremblay P, Meiner Z, Galou M, Heinrich C, Petromilli C, Lisse T, Cayetano J, Torchia M, Mobley W, Bujard H, DeArmond SJ, Prusiner SB. Proc Natl Acad Sci USA 1998; 95(21):12580–12585.

12

Diagnosis of Prion Diseases by Multispectral Techniques

Richard Rubenstein and Jae-Il Kim
New York State Institute for Basic Research in Developmental Disabilities, Staten Island, New York, U.S.A.

Perry C. Gray
Los Alamos National Laboratory, Los Alamos, New Mexico, U.S.A.

Gary C. Tisone[†]
TW Research Associates, Albuquerque, New Mexico, U.S.A.

I. INTRODUCTION

Prion diseases, which include Creutzfeldt-Jakob disease (CJD) in humans, sheep scrapie, and bovine spongiform encephalopathy of cattle, are transmissible neurodegenerative diseases characterized by the accumulation in the central nervous system of the protease-resistant isoform (PrP^{Sc}) of a host-coded membrane glycoprotein (PrP^{C}) (1–4). The transmissibility and fatal nature of these diseases necessitate their rapid and accurate diagnosis. Polyclonal and monoclonal antibodies to PrP^{Sc} have been very useful for the immunodiagnosis of prion diseases (5,6). However, the accessibility of antigenic sites for antibody binding is restricted by the conformation of PrP. Conformational differences in PrP^{Sc} are associated with the different scrapie strains and influence the properties of the protein. In addition, the available antibodies to PrP do not distinguish between PrP^{Sc} and PrP^{C}.

[†] Deceased.

We are investigating the use of optical sensing technology not only for the sensitive, specific, and direct detection of PrP^{Sc}, but also for the ability to distinguish between different forms of the prion protein.

One of these techniques, which uses more than 1 wavelength of ultraviolet light to excite the samples, is known as multispectral ultraviolet (UV) fluorescence spectroscopy (MUFS) (7). With MUFS, a sample is sequentially illuminated with monochromatic light at chosen wavelengths, λ_{exc}, in the ultraviolet spectrum, and then the fluorescence is measured over a finite bandwidth, λ_{flr}, thus building up a two-dimensional spectral signature. Using this technique, spectra from unknown samples can be analyzed by multivariate techniques to determine the presence and concentration of chemicals and proteins for which known database spectra have been acquired (8). For proteins, the UV fluorescence signal is predominantly influenced by the presence of aromatic amino acid residues (tryptophan, phenylalanine, tyrosine), with the fine structure of the signature due to the relative amounts of these residues and their local environment (9) (i.e., nearest-neighbor effects of the residues, and exposure of the residues to water through folding of the protein).

Two criteria must be met to detect PrP^{Sc} by MUFS. First, the fluorescence cross-section for the protein must be sufficiently large. This quantity, which indicates how large the returned fluorescence signal (in number of photons) will be for a given flux of excitation light, of a given wavelength, is a fundamental property of the protein. Second, differences in the shape of the spectral signatures must be sufficiently large to allow them to be distinguishable. Since the spectra from proteins tend to be broad and diffuse, signatures from different molecules tend to have features that are shared and overlap. As a result, only a fraction of the fluorescence signal contains information that can be exploited to identify a molecule in a mixture or against its natural background. Our approach in dealing with this issue is to compare, on a component-by-component basis, the relative orthogonal differences between spectra (10). The orthogonal difference is the minimum residual from a multidimensional least-squares fitting of the spectrum of one sample to another. This results in the localization of differences in the shape of the spectra, and the relative amount of signal available to distinguish one spectrum from the other. The measure of spectral signature differences determines the sensitivity limits of the technique.

Consequently, our initial goals in measuring the fluorescence spectrum of the prion protein was to calculate fluorescence cross-sections and determine whether there were qualitative differences in signatures between the untreated and the proteinase K (PK)-treated protein, as well as differences between PK-treated PrP^{Sc} from various species. Spectroscopic measurements of fluorescence from untreated and PK-treated PrP^{Sc} have been performed

(7). Significant amounts of fluorescence could be detected from the proteins allowing qualitative distinctions to be observed. Furthermore, the orthogonal differences were ~10% of the overall signal strength, which is more than enough to allow one to distinguish between signals by standard multivariate methods. The spectral analysis was performed using only 1 μL of the ~3 μg/mL PrP^{Sc} preparations. Based on the relatively high fluorescence cross-section measurements using ~3 ng of partially purified PrP^{Sc}, the ability to detect the protein at, or below, the picogram level is likely. The analysis of these spectral signatures is being used to initiate the database for PrP^{Sc}.

The fluorescence cross-section for the protein establishes the limits of detectability for a given instrument. We have performed calculations to estimate the fluorescence cross-section for PrP^{Sc} using their spectral signatures (7). The fluorescence cross-section values are an order of magnitude larger than what could be accounted for from the aromatic residues alone. Since there are relatively small differences in the PrP sequence between species, the results suggest that conformational differences between these proteins, along with spatial interactions between specific aromatic and nonaromatic amino acids, play an influential role in contributing to both the significant amount of fluorescence detected and the distinctive spectral signatures.

Raman spectroscopy comprises the family of spectral measurements made on molecular media based on inelastic scattering of monochromatic radiation. During this process, energy is exchanged between the photon and the molecule such that the scattered photon is of higher or lower energy than the incident photon. The difference in energy is made up by a change in the rotational and vibrational energy of the molecule and gives information on its energy levels. Recent advances in optical filter technology have led to a renewed interest in this detection technology. Raman spectroscopy continues to be used widely as a probe of structure and function in biochemical systems and as a quantitative technique for biomarkers. Greater interest in its use is due to the increased availability of ultraviolet and near-infrared excitation sources (where fluorescence interference is minimized), and by the realization that unique, fingerprint-like spectra can be obtained for species as small as low-molecular-weight metabolites and as large as living organisms.

Several different approaches have been used to probe protein secondary, tertiary, and quaternary structure. A common feature in these experiments is the presence of conformationally and/or environmentally sensitive Raman bands (11,12). UV-resonance Raman has been used to probe the solution conformation of the extracellular domain of human tumor necrosis factor (13) and to follow isomerization dynamics of amides and peptides (14). In addition, techniques such as vibrational Raman optical activity

(ROA) (15,16) and polarized Raman also furnish valuable information about protein structure. ROA studies of poly L-lysine in H_2O and D_2O have provided insight into conformational elements present (e.g., rigid loop, α-helix, β-strand, and left-hand helix) (17). Accordingly, ROA can be used to follow protein folding and/or unfolding (18,19).

Two approaches are widely used to generate information about catalytically active sites within proteins: Raman difference spectroscopy (20) and, when a chromophore is present, resonance Raman spectroscopy (21). In the former, careful subtraction of spectra obtained under different conditions yields information about protein residues undergoing structural or environmental changes.

There appears to have been an increase in medically related (i.e., diagnostic) applications of Raman. The rapidity with which data can be acquired and then analyzed with software, coupled with the ability to manufacture extremely small probes suitable for in vivo work, appears to be a powerful combination for disease detection and identification. The development of a fiberoptic pulsed UV-resonance Raman probe for real-time monitoring of neurotransmitters in the brain has been reported (22). To avoid interference from fluorescence, Dou et al. (23,24) have used anti-Stokes Raman to determine the concentration of glucose, acetone, and urea in urine samples. Longer-excitation wavelengths are important for characterization of analytes in biological matrices such as skin or blood, which absorb much of the visible spectrum but are transparent in the near-infrared (IR). Accordingly, Berger et al. (25,26) have determined concentrations of glucose, lactic acid, and creatinine in aqueous solutions using near-IR Raman and measured glucose and other blood constituents in human whole blood.

II. INSTRUMENTATION

To examine both direct fluorescence detection (MUFS) and Raman and fluorescence-tagged detection for PrP^{Sc}, two instruments have been developed. The first of these is a multispectral fluorometer shown in Fig. 1. The excitation source is a xenon arc lamp, which is first passed through a spectrometer and then imaged into the sample (in this case a cuvette) yielding spectral dispersion in the vertical direction. This light is then imaged through a second spectrometer at 90 degrees (to minimize scattered light) onto a back-thinned, thermoelectrically cooled, CCD detector (Hamamatsu, Inc., San Jose, CA). This optical arrangement allows for a fully two-dimensional measurement (intensity of fluorescence as a function of both excitation wavelength and fluorescence wavelength) of the sample, which can be acquired as fast as the CCD can be read. This design allows the

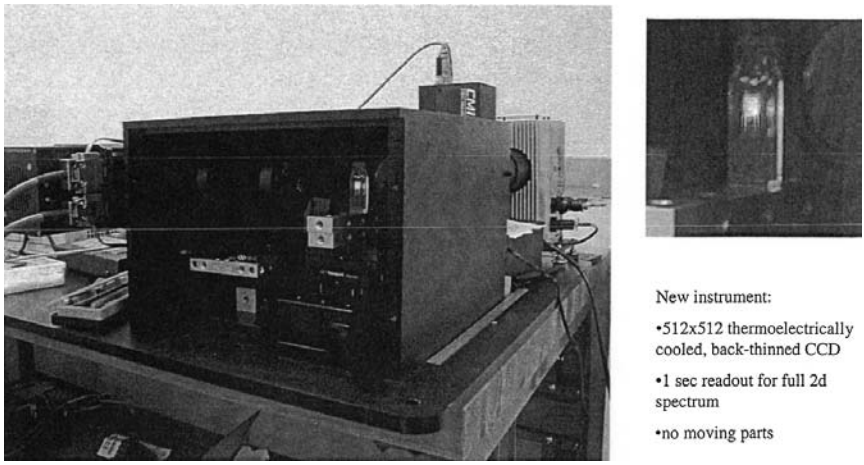
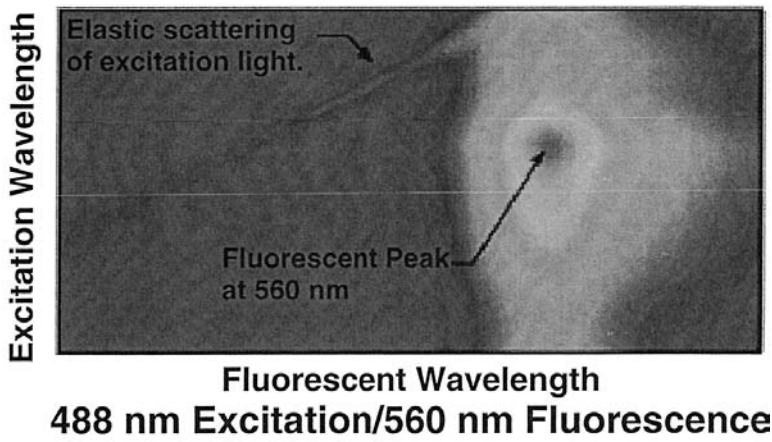


Figure 1 Multispectral fluorometer (exploded view). Shown is a sample of fluorescence-dyed beads (Molecular Probes, Inc.) in a cuvette. The side of the instrument has been removed to display the instrument configuration, and the insert demonstrates the manner in which excitation light is split.

instrument to be used for kinetic studies as well as being employed in a detection scheme.

Figure 2 demonstrates how the data can be viewed with this instrument. The samples shown were fluorescence-dyed beads (Transfluorospheres 505/515 and 488/560, Molecular Probes Inc., Eugene, OR) optimized for two different sets of excitation absorption and fluorescence emission maxima. The beads are measured in a 1 cm $\times$ 1 cm quartz cuvette (see Fig. 1), at a concentration of $\sim 10^8$/mL in water. Slightly different ranges of excitation and fluorescence, which are adjustable by the operator, are shown for the two samples. The entire two-dimensional spectra were acquired in approximately 1 s (limited in this case by the readout time of the detector).

The second instrument developed is a combined Raman spectroscope and high-sensitivity (laser-induced) fluorescence instrument (Fig. 3). The current design employs a helium-neon laser as an excitation source, which is convenient and relatively inexpensive. The light is focused onto the target with a microscope objective lens, for increased sensitivity. The target itself is mounted on a kinematic base, which guarantees reproducibility of positioning upon a change of target, and allows for ease and accuracy in positioning of the target. The optical path of the instrument is folded in such a manner that detection can be performed either with direct backscatter of the incident



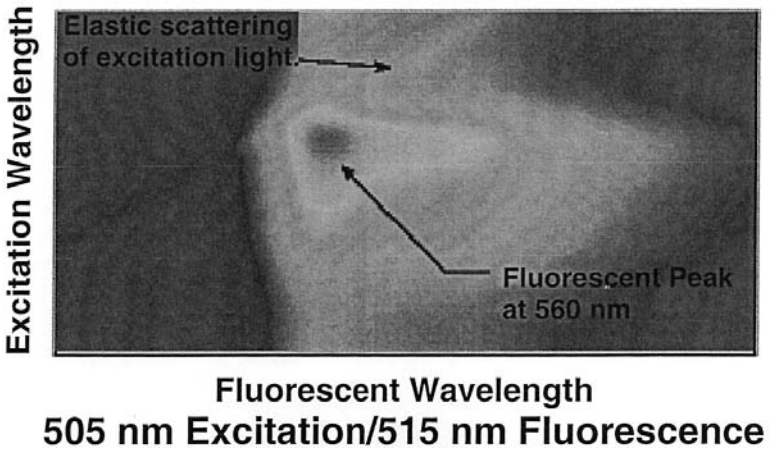


Figure 2 Multispectral data taken with the MUFS instrument. Samples are two varieties of fluorescence-dyed beads (Molecular Probes Inc.) suspended at a concentration of ~10^8/mL in water and placed in a quartz cuvette.

light, or at small angle. This geometry is optimal in that Raman scatter intensity can be shown to be highly peaked in the forward or backscatter direction. The scattered light is then passed through a holographic notch filter (Kaiser Optics, Inc.) centered on the emission of the helium-neon laser and then focused into the 0.25-M spectrometer (CVI, Inc., Albuquerque, NM) and detected, as with the MUFS instrument, with a back-thinned

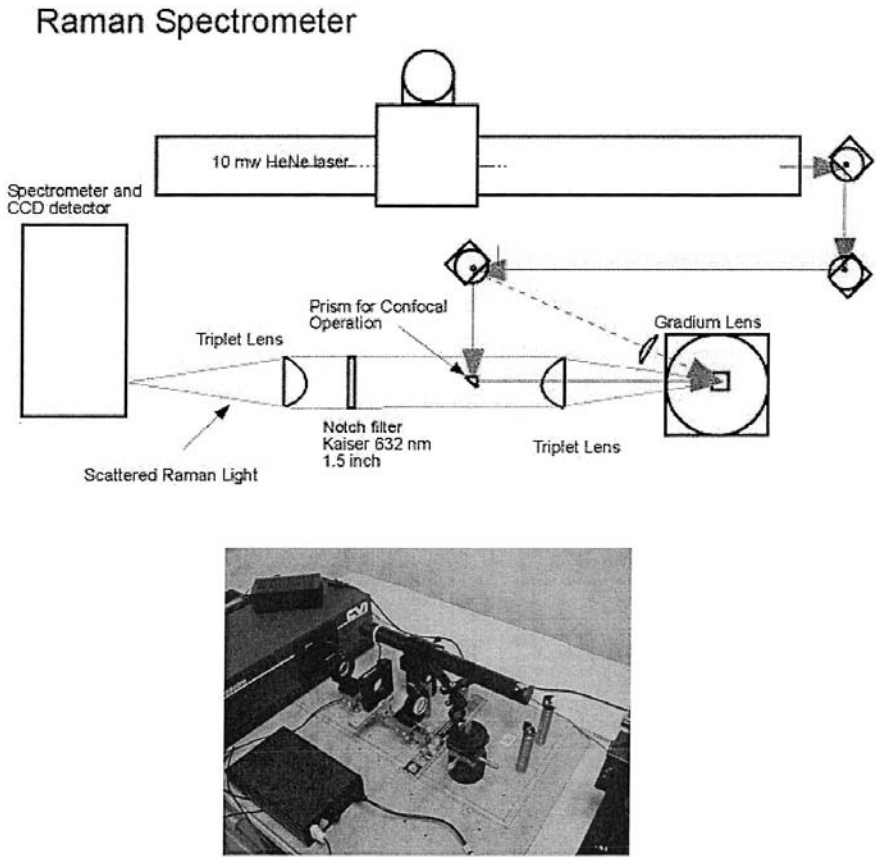


Figure 3 Raman/high-sensitivity fluorescence spectrometer, optical design and system.

thermoelectrically cooled CCD (Hamamatsu, Inc.). Using this instrument, the specific Raman spectra for several samples have been demonstrated (Fig. 4). In addition to Raman spectroscopy, this instrument, through minor adjustment of the spectrometer, is also appropriate for measurement on fluorescence-labeled targets with a high degree of sensitivity. A major consideration in the design of both instruments was flexibility in choice of target configuration (cuvette, glass slide, etc), which allows for maximum flexibility in the design of laboratory protocols and sample preparation.

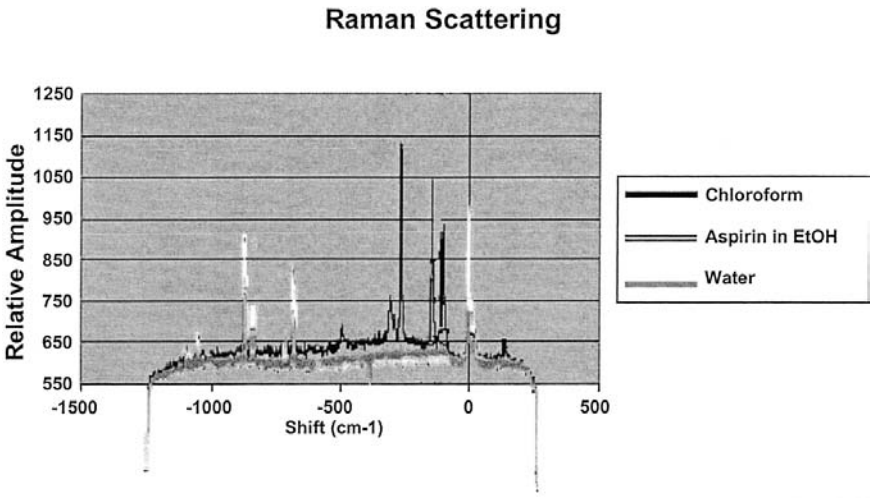


Figure 4 Raman spectra from Raman/fluorescence detection system. The samples, which consisted of chloroform, aspirin in ethanol, and water, were all analyzed in quartz cuvettes.

III. ANALYSIS TECHNIQUES

The optical detection schemes have three components: the laboratory assay/protocols for preparation of samples, the instrumentation associated with the particular detection scheme, and the numerical methods associated with analysis of the data that results in detection, concentration estimates, and discrimination. This last component is the one most ignored in the design of instrumentation (as seen by the paucity of tools available from even major vendors of such equipment).

We are interested in detection and discrimination of fluorescence signatures in natural backgrounds. To achieve this we require a quantitative measure of difference between different spectral signatures. This is obtainable through an application of multivariate analysis. After obtaining reference spectra of known signatures under controlled conditions, unknown fluorescence signature, ${}^{u}S$, is expressed in terms of known reference spectra ${}^{i}S$:

$$ {}^{u}S = \sum_{i} {}^{i}c \cdot {}^{i}S + \delta $$

The multivariate problem is then to find the "concentrations," ${}^{i}c$, that minimize the residual, δ, or expressing the problem in terms of the generalized least-squares problem:

$$\delta^2 = \left| \sum_i {}^{i}c \cdot {}^{i}S - {}^{u}S \right|^2$$

which is readily solved by employing methods such as singular valued decomposition (SVD) (27). Additional information concerning the details of the spectra can be exploited through biasing or heuristic methods. A measure of difference is readily obtained. Fluorescence signatures ${}^{1}S$ and ${}^{2}S$ are written then in terms of each other as:

$${}^{1}S_{i,j} = C \cdot {}^{2}S_{i,j} + {}^{1,2}\delta_{i,j}$$

For the two-dimensional spectra using the inner product and minimization of the residual ${}^{1,2}\delta$, in a least-squares sense, leads to:

$$C_{max} = \frac{\sum_{i,j} {}^{1}S_{i,j} {}^{2}S_{i,j}}{\sum_{i,j} ({}^{2}S_{i,j})^2}$$

C_{max} is identical to the "concentration" similar to the values SVD would yield from known ${}^{2}S$. The residual thus defined, δ, is a measure of the "orthogonal" difference between ${}^{1}S$ and ${}^{2}S$.

As an example of the application of this technique we have taken spectra of fetal bovine serum (FBS) spiked with sodium salicylate, a convenient fluorescent compound, across a wide range of concentrations. Figure 5 shows the fluorescence signatures from FBS, salicylate in phosphate-buffered saline (PBS), and FBS spiked with salicylate to the same concentration. This shows the relative position of the fluorescence peaks. The dominant peak for the FBS is near an excitation wavelength of 290 nm, typical for proteins. For these concentrations of salicylate, the contribution to the optical extinction is significant. Figure 6 shows the application of the orthogonal difference to samples with an order-of-magnitude-lower concentration of salicylate. Shown is the signature of salicylate in FBS (from which the fluorescence contribution of the FBS has been subtracted), the signature of salicylate in PBS at the same concentration, and finally the orthogonal difference between the two. This last plot shows the degree and location of the distortion of the salicylate fluorescence due to the interaction with the FBS. Since the serum protein concentration is large enough to cause nonlinear affects and extinction of the fluorescence, it is this type of nonlinear

spectral distortion that is presumed to pose a major problem for in situ optical measurements. To test the validity of this concern, measurements were made of salicylate in FBS through serial dilution over three decades of concentration. The sample at a concentration of 7.8 μg/mL was then used as the "known" spectrum in multivariate analysis and concentrations were computed using this standard for all samples. The concentration estimates thus obtained are shown in Fig. 7.

Estimates were calculated for data sets obtained using a long-path-length cell (1-cm path) and a short-path-length cell (0.1-mm path). The latter is similar to a measurement on first surface (i.e., glass microscope slide). As can be seen, the concentration estimates for the samples are reasonably good over at least two (and for the short-path-length measurement, 3) orders of magnitude in concentration. This result, at first glance, appears surprising until one realizes that in these measurements the concentration of the protein

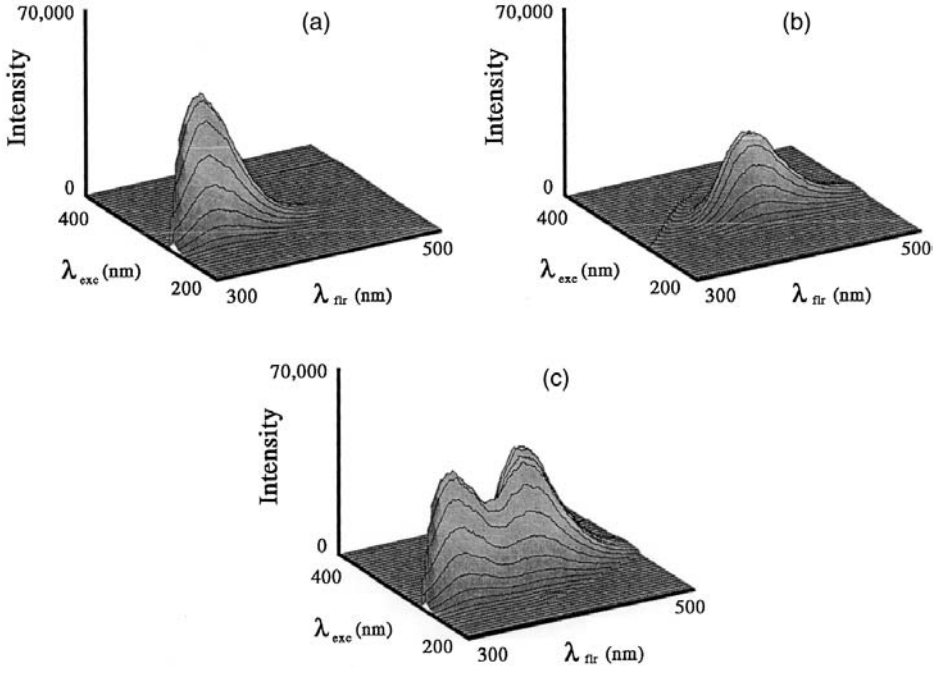


Figure 5 Multispectral fluorescence signatures of FBS (a), 125 μg/mL salicylate in PBS (b), and 125 μg/mL salicylate in FBS (c). First-order scattered light is masked to show fluorescence alone.

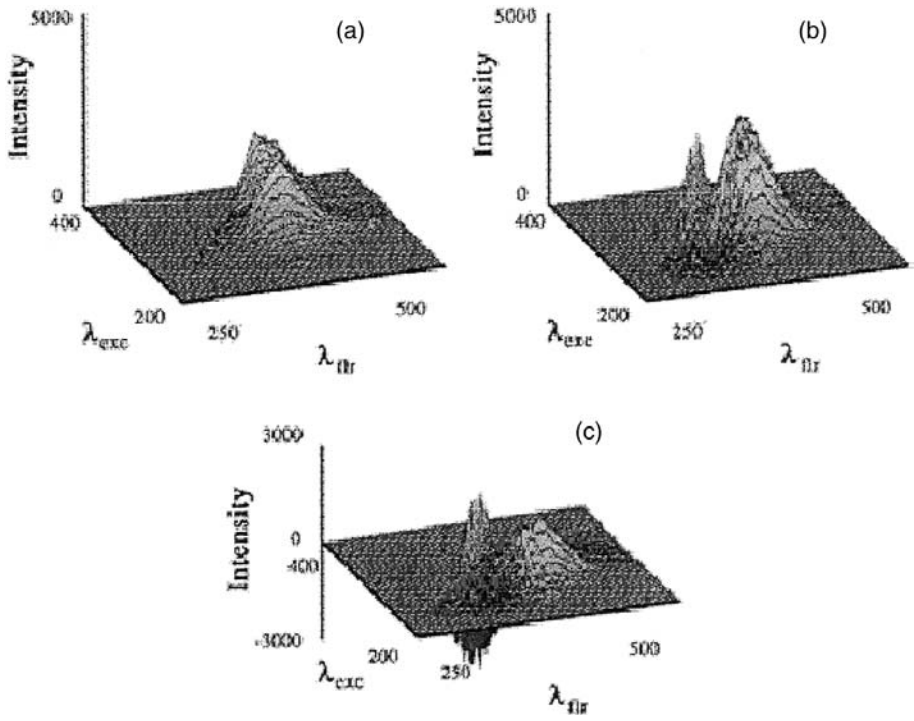


Figure 6 Demonstration of spectral distortion due to the optically dense matrix, FBS. Multispectral fluorescence of: 7.8 μg/mL salicylate in FBS with the FBS contribution subtracted (a), 7.8 μg/mL salicylate in PBS (b), and the orthogonal difference (c).

in the FBS is essentially constant, thus so is the spectral distortion. This means that the effects due to the FBS can be accounted for as just an additional part of the instrument response. The cause of the dropoff in the long-path-length measurements can be accounted for by nonlinear effects due to the salicylate itself at high concentration. Figure 8 shows the results of absorption measurements taken with the long-path-length cell of: FBS, FBS with salicylate at 125 μg/mL (where the turnover in the curve for the long-path-length in Fig. 8 occurs), and the difference between the two curves. As can be seen, at these concentrations the salicylate accounts for a significant part of the absorption near the peak fluorescence, near 300 nm.

The above example shows the utility of multivariate analysis, and we are in the process of developing automated tools for application of these methods in concert with the instruments being developed for this project.

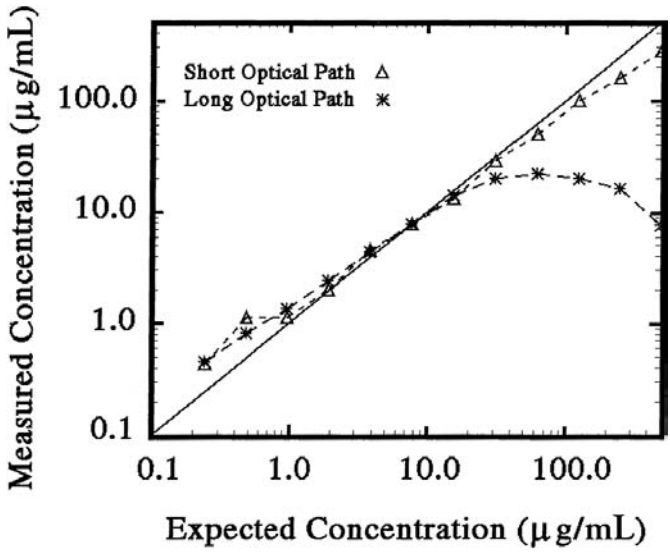


Figure 7 Multivariate concentration estimates for salicylate in undiluted FBS. The solid line indicates expected values, and estimates from short- and long-path-length cells are indicated.

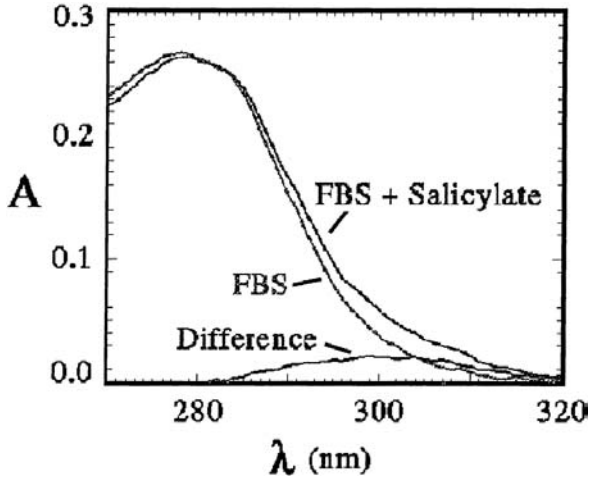


Figure 8 Contribution of salicylate to the absorption. Shown are the absorption for FBS, FBS with 125 μg/mL salicylate, and the difference between the two.

IV. MEASUREMENTS OF PrP^{Sc}

Our initial goals in measuring the fluorescence spectrum of the protein were to calculate fluorescence cross-sections and determine whether there were qualitative differences in signatures between the untreated and the PK-treated protein, as well as differences between PK-treated PrP^{Sc} from hamster and mouse. Quantitative estimates of the differences between spectral signatures were obtained by calculating the orthogonal differences between pairs of spectra (10). Figure 9 shows the spectra from untreated and PK-treated samples of PrP^{Sc} from 263K-scrapie-strain-infected hamsters, and the orthogonal difference between the two spectra is shown. Figure 10 shows the spectra for PK-treated PrP^{Sc} from mouse (ME7) and hamster (263K), and the orthogonal difference calculated from the two spectra shown. Figures 9 and 10 are surface plots of the spectra scaled to the same peak intensity of 10^4 counts (relative), along with the orthogonal differences calculated for the pairs of spectra shown. The data shown in these figures are not corrected for excitation lamp intensity or detector response. The data are displayed in this manner because normalization for excitation intensity increases the background noise along with the signal, and hence no additional information is conveyed. In the spectra and orthogonal differences shown, the first-order scattered light (where the signal obeys $\lambda_{flr} = \lambda_{exc}$) has been removed to display the information in the fluorescence alone. There are two important points to note in both Figs. 9 and 10: first, that there is a significant amount of fluorescence from the protein; second, in both cases the orthogonal differences were ~10% of the overall signal strength, which is more than enough to allow one to distinguish between signals by standard multivariate methods (10,27,28).

The fluorescence cross-section for the protein establishes the limits of detectability for a given instrument. We have performed calculations to estimate the fluorescence cross-section for PrP^{Sc} using the signatures from all four samples. Spectra were corrected for excitation intensity and detector response so that an estimate of the fluorescence cross-section for the protein at an excitation wavelength of 290 nm (near the peak fluorescence for tryptophan) could be calculated numerically. The value obtained for the integrated fluorescence cross-section at 290 nm excitation is $\sim 10^{-17}$ cm^2/molecule. Figure 11 shows the differential fluorescence cross-sections at 290 nm excitation as a function of fluorescence wavelength. These values are very similar (differing only by a factor of two) indicating that the fluorescence cross-section is determined by properties common to all the samples. This is most likely their amino acid sequence homology. There is evidence suggesting that scrapie strains differ in conformation, polymeric states, and/or ligand associations (29). Evidence for the existence of multiple conformations of

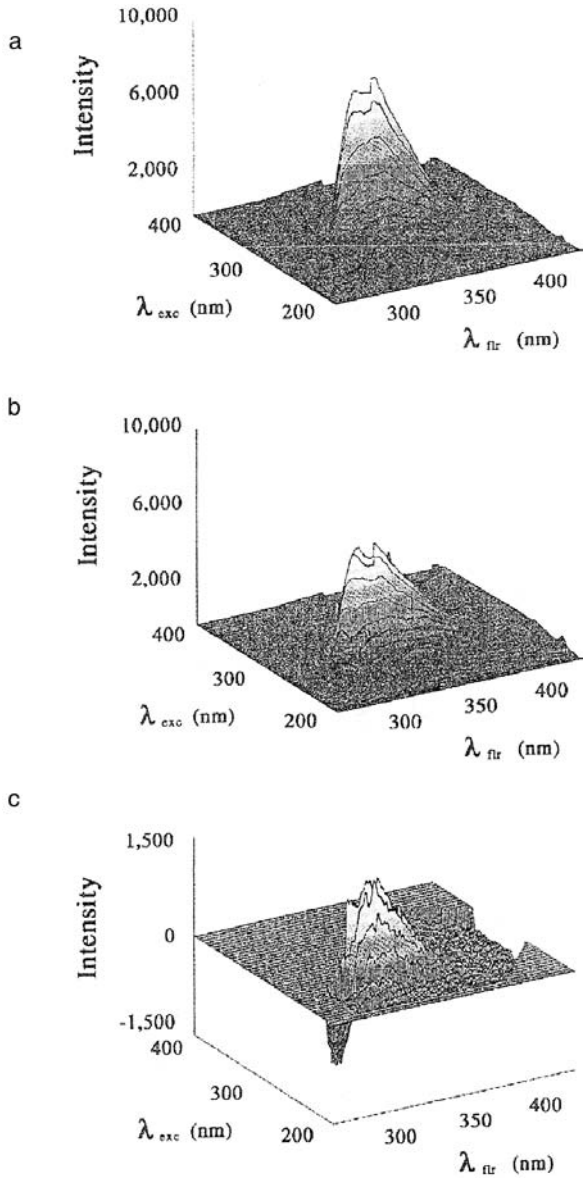


Figure 9 Spectra of PrP^{Sc} from 263K-infected hamsters in the absence (a) or presence (b) of PK treatment, and the orthogonal difference calculated between the two spectra (c).

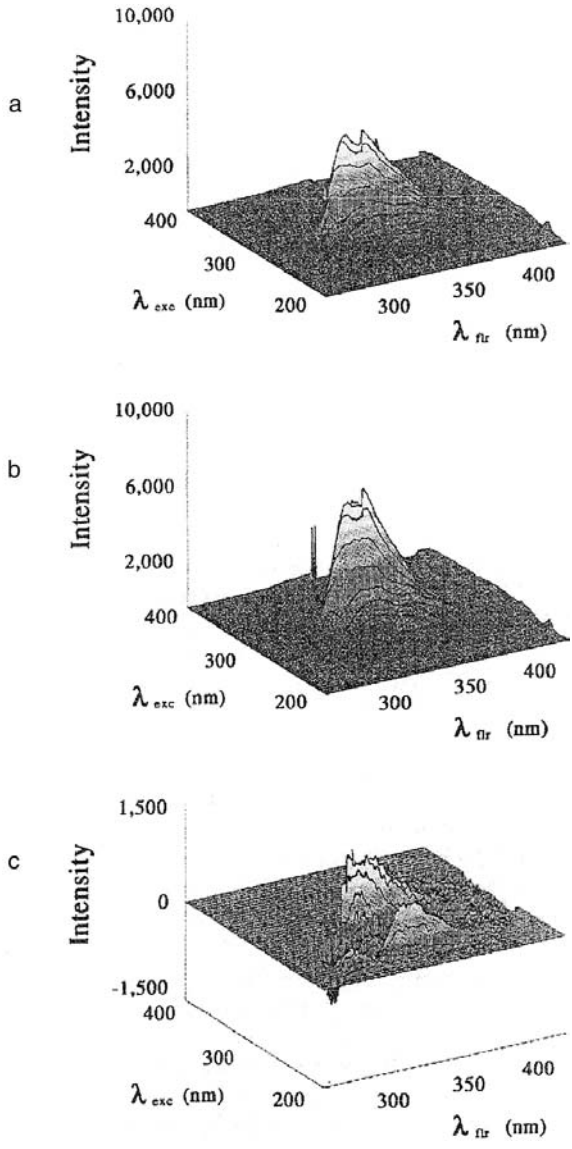


Figure 10 Spectra of PK-treated PrP^{Sc} from 263K-infected hamsters (a), ME7-infected mice (b), and the orthogonal difference calculated between the two spectra (c).

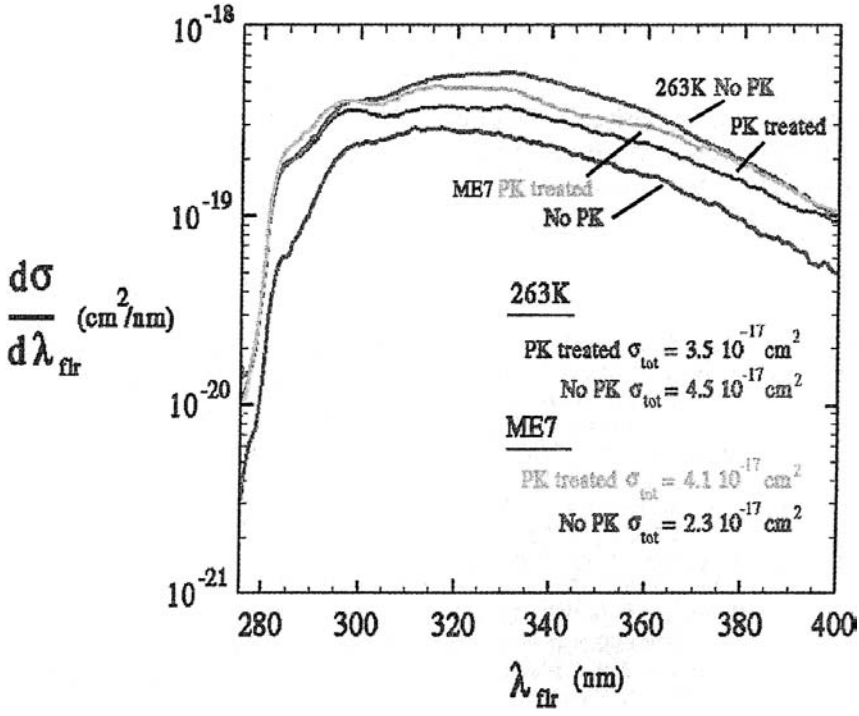


Figure 11 Differential and total fluorescence cross-section (s_{fl}) for untreated and PK-treated PrP^{Sc} from 263K-infected hamsters and ME7-infected mice calculated at an excitation wavelength of 290 nm.

PrP^{Sc} has come from studies on the differing N-terminal cleavage sites by PK (30) and from conformational-dependent immunoassay studies that used selective antibody binding to different scrapie strains (31). However, the calculated cross-section should only be taken as an order-of-magnitude estimate since it is limited by the accuracy of the estimate of protein concentration in the samples. The method of purification of PrP^{Sc} takes advantage of the physical properties of the abnormal form of the protein, in particular its tendency to aggregate. As a result, the samples in the absence of PK treatment contain PrP^{Sc} and extraneous material that copurifies with the protein. The differences in spectral shape observed between untreated and PK-treated samples in Fig. 9 may be due to: the elimination of the extraneous material following digestion with PK, a shift in signature of PrP^{Sc} due to cleavage of amino acid residues by PK, and/or a shift in fluorescence due to the association of the PrP^{Sc} with the extraneous material.

The fluorescence cross-section values are an order of magnitude larger than what could be accounted for from the aromatic residues alone. Typically, the molecular fluorescence cross-section for molecules such as tryptophan is 10^{-21}–10^{-19} cm^2/molecule (32). Therefore, mouse and hamster PrP^{Sc}, which have 30 aromatic residues per molecule (1), might be expected to have a cross-section as high as 10^{-18} cm^2/molecule. The 10-fold greater fluorescence values we have obtained can be accounted for by the presence of nonaromatic residues. Furthermore, the orthogonal difference observed between PK-treated PrP^{Sc} samples from hamster and mouse (Fig. 10) is of interest with regard to protein conformations. There are relatively small differences in the PrP sequence between these two species. Sequence comparisons (1) show differences in 16 of the 254 residues, or approximately 94% homology, with only a minority involving aromatic amino acids (only two of the 16), which are the dominant source of the fluorescence. These results suggest that conformational differences between these proteins, along with spatial interactions between specific aromatic and nonaromatic amino acids, play an influential role in contributing to both the significant amount of fluorescence detected and the distinctive spectral signatures.

In the next series of studies, spectra were taken of preparations of PrP^{Sc} from various species: sheep (scrapie), hamster (263K), mouse (ME7), and deer (CWD). While the computational protocol followed that published in previous work (7,10), the goal was to establish expected power in the differences (i.e., signal-to-noise ratio) and obtain a qualitative measure of overall shapes.

Spectra were taken with identical settings on the spectrometer, and exposures, with similar estimated protein concentrations. The two-dimensional spectra were masked (computationally) to restrict comparison between spectra to the spectral region dominated by the fluorescence alone (i.e., first- and second-order elastic scattered light was masked out). Orthogonal differences were computed pairwise for PK-treated versus untreated PrP, and PrP from different species (PK-treated and untreated). Differences are plotted as scaled images with identical range of values corresponding to $\pm 10\%$ of the maximum fluorescence level from the instrument (which is an order of magnitude above the background level for the detector).

Figure 12 shows comparisons, orthogonal differences, between PK-treated and untreated samples from the same species (e.g., treated vs. untreated ME7). Although not evident in these black-and-white figures, a color pallet is used to represent the visible spectrum, with red indicating most positive (highest) values and blue most negative (lowest). Note that the background field, which indicates where we have masked out the elastic scattered light and is by definition zero, denotes the zero contour level of the image, so that differences in the solid-colored field indicate gross relative

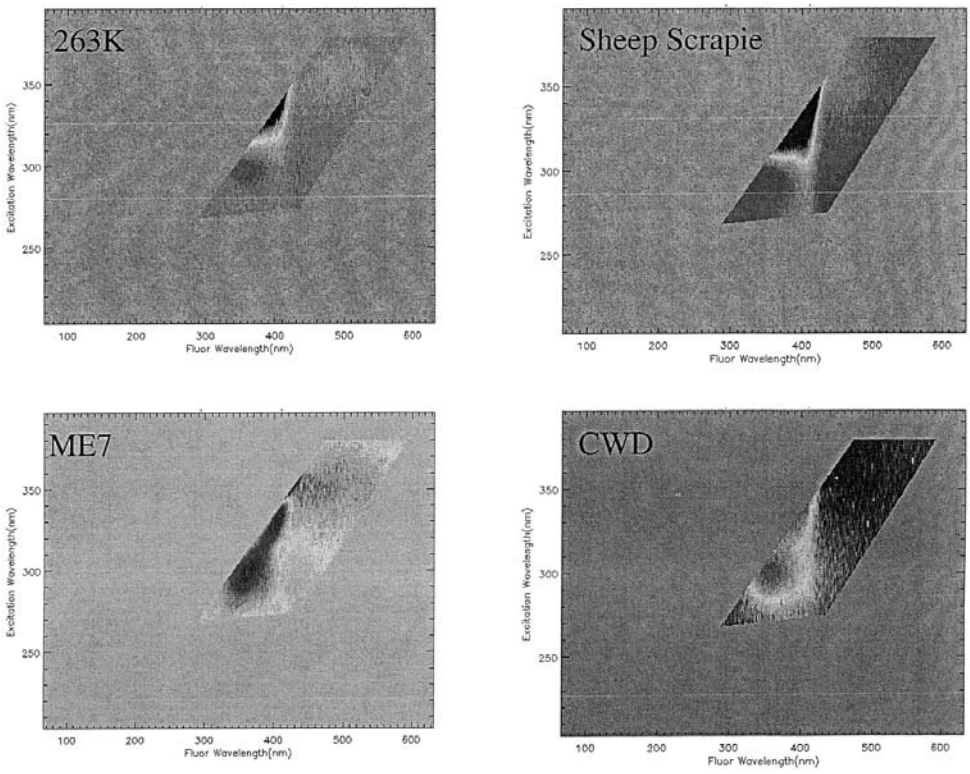


Figure 12 Orthogonal differences of spectra taken from PK-treated versus untreated samples of scrapie (sheep), ME7 (mouse), 263K (hamster), and CWD (mule deer) PrP^{Sc}.

asymmetry in the figure. For the small changes caused by the PK treatment alone, we are able to see significant differences in the spectral signatures of the samples.

Figure 13 shows orthogonal differences computed between samples from different species. There are two important things to note: First is that in all cases the full scale of the color pallet appears. This indicates that similar amounts of power are available to distinguish between samples. The second is that by visual inspection alone the patterns seen are clearly different, leading one to believe that this technique has potential as a means of distinguishing between different strains of PrP^{Sc}. Figure 14 shows the same analysis applied to samples that were first PK-treated. Again

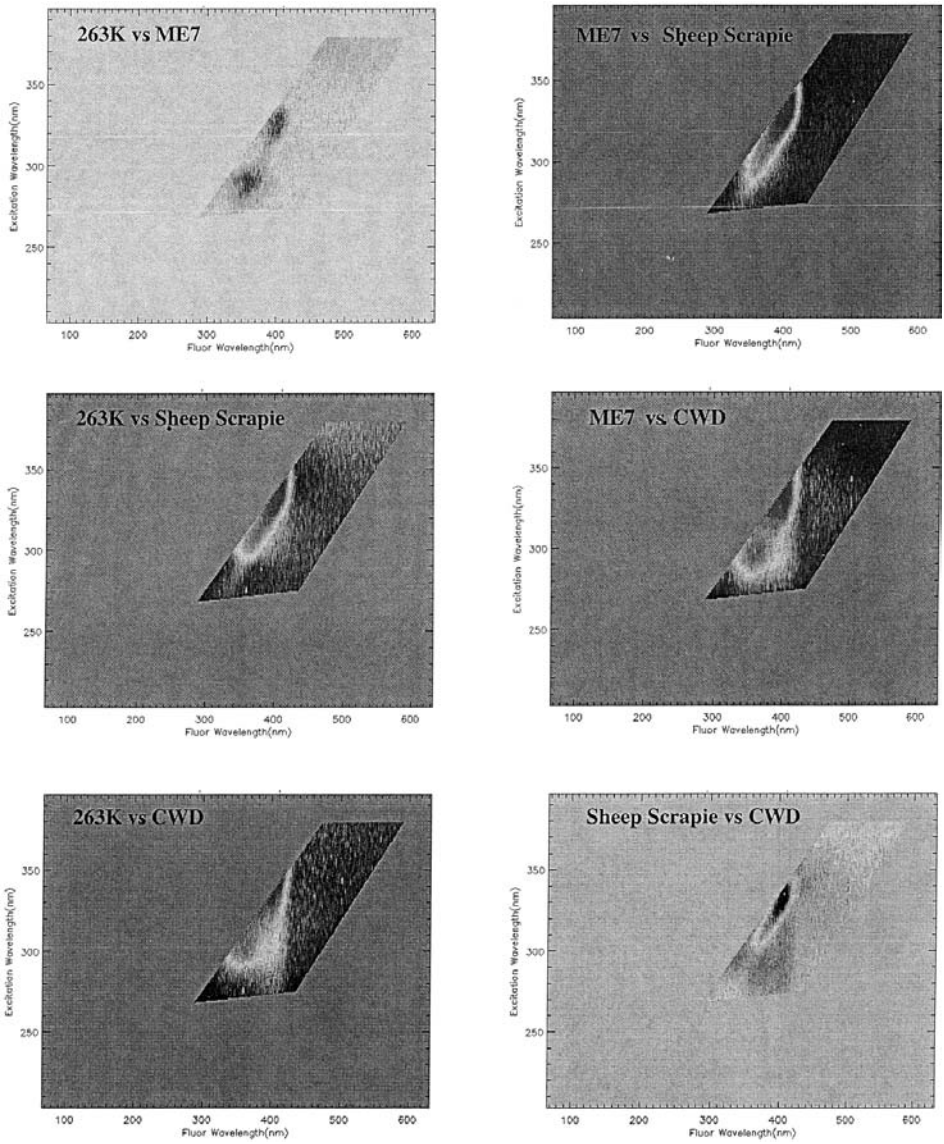


Figure 13 Orthogonal differences computed as comparisons between different species: mouse (ME7), hamster (263K), sheep (scrapie), and mule deer (CWD). Samples have not been treated with PK, and cutoff values on scaling are $\pm 10\%$ maximum fluorescence.

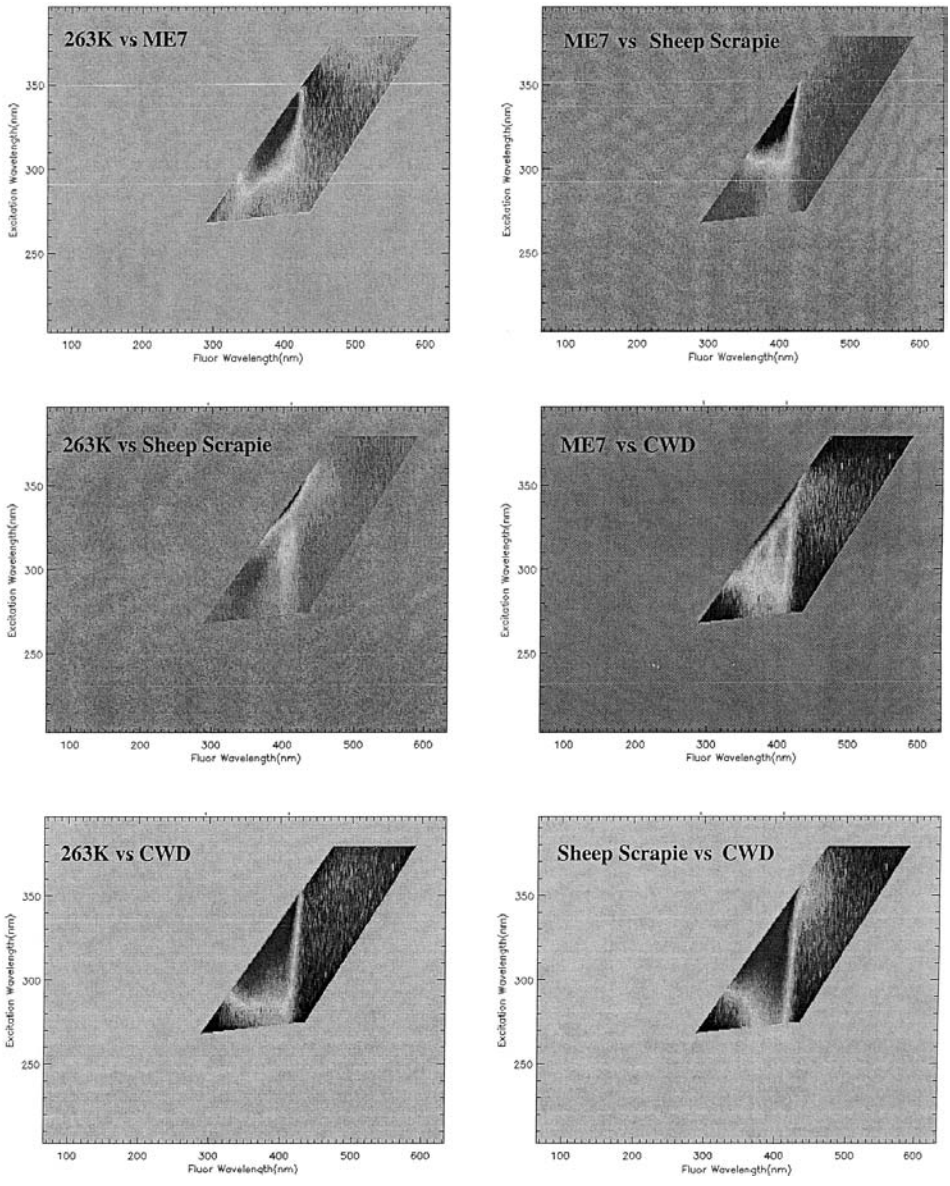


Figure 14 Orthogonal differences of PK-treated samples, computed as comparisons between different species: ME7 (mouse), 263K (hamster), scrapie (sheep), and CWD (mule deer). Cutoff values on scaling are $\pm 10\%$ maximum fluorescence.

there is significant power available for discrimination and clear differences in the shape of the spectral information. The power levels and relative shapes of these orthogonal differences are consistent with data previously published for ME7 and 263K.

V. DISCUSSION AND FUTURE DIRECTIONS

These studies clearly demonstrate the potential applications that fluorescence spectroscopy (i.e., MUFS) has for direct, nondestructive detection of proteins and other compounds. In addition, various novel approaches to optical tagging have been made possible owing to recent advances in optical technology.

In an effort to increase the sensitivity of detection and improve the signal-to-noise ratio, various approaches may be used to enhance Raman scatter. For example, it may be possible to use colloidal gold, and silver enhancement, as Raman tags for IgG or FAB fragments in immunoassays. Conventional Raman spectroscopy usually lacks sufficient sensitivity for use as a readout method for immunoassays. Surface-enhanced Raman spectroscopy (SERS), on the other hand, has demonstrated its ability to detect picomole to femtomole amounts of materials adsorbed on several types of roughened metal surfaces (33). Among the metallic substrates used for SERS are metal nanoparticles (e.g., gold and sliver colloids). Impressively, recent reports have shown that certain nanoparticle substrates can yield SERS intensities comparable to or even exceeding those from fluorescence (34,35). Raman scatter is normally weaker than fluorescence by several orders of magnitude. Enhanced Raman can be comparable and has the advantage that, unlike fluorescence, it does not saturate. The main issues that need to be addressed for this method to be of use are total signal strength and appropriate target geometry. The enhancement of Raman scatter by colloidal gold and/or silver is due to the proximity of the target molecule (in our case the FAB fragment) to the colloidal particle and the size of the colloidal particle. Silver enhancement, in which the colloidal gold is used as a nucleation site for precipitation of silver, can be used to examine the dependence of signal on particle size. Several characteristics of SERS suggest it may be of value as a readout method (33,36,37). First, Raman bands are generally 10–100 times narrower than most fluorescence bands, minimizing the potential overlap of different labels in a given spectral region. Second, the optimum excitation wavelength for SERS is not strongly dependent on the adsorbed molecule, allowing the use of a single excitation source for multiple species. Third, Raman scattering is not sensitive to humidity or affected by oxygen and other quenchers, facilitating applica-

tions in a variety of environments. Finally, the SERS signal is less subject to photobleaching, potentially enabling one to signal average for extended time periods to lower the limit of detection.

Surprisingly, there have been only a few reports on the application of SERS for detection in immunoassays. These approaches used a sandwich-type assay, which coupled surface and resonance enhancements. Rohr et al. (38) used labeled detection antibodies and roughened silver films coated with a capture antibody, and Dou et al. (23,24) exploited the adsorption on silver colloids of an enzymatically amplified product. Ni et al. (39) described an alternative approach using SERS together with labeled immunogold colloids as a readout method. This technique did not require resonance enhancement or enzymatic amplification. Instead, it involved the immobilization of capture antibodies on a gold surface, the use of the immobilized antibodies to capture antigens from solution, and indirect Raman detection via gold nanoparticles labeled with both antibodies and intrinsically strong Raman scatterers (i.e., Raman reporter molecules: 4-mercaptobenzoic acid, 2-naphthalenethiol, and thiophenol). A different Raman scatterer was used to label extrinsically each of the different immunogold colloids, with the presence of different antigens detected by the characteristic Raman bands of the labels. This approach also took advantage of long-wavelength excitation operative for gold colloids (34,40,41), which minimizes native fluorescence and photobleaching complications.

Recently, Schultz et al. (42) reported the use of colloidal silver plasmon-resonant particles (PlrPs) as optical reporters in typical biological assays. PlrPs are ultrabright, nanosized optical scatterers, which scatter light elastically and can be prepared with a scattering peak at any color in the visible spectrum. PlrPs can be used as indicators for single-molecule detection; that is, the presence of a bound PlrP in a field of view can indicate a single binding event. As typically prepared, PlrPs have a scattering cross-section of 10^{-10} cm^2; therefore, under epi-illumination (100W halogen) with a $\times 100$ lens (0.9 numerical aperture), a single PlrP will deliver approximately 10^7 photons in 1 s to the detector. Compared with other optical-labeling entities under the same illumination conditions, the 80-nm PlrP scattering flux is equivalent to that from 5 million individual fluorescein molecules—1000-fold that provided from a 100-nm fluorosphere (Molecular Probes; data not shown) or $>10^5$-fold that from typical quantum dots (43,44). Proteins such as antibodies can be conjugated to PlrPs by techniques developed for gold colloids and these novel biological labels can be used to replace less sensitive optical detection systems.

As an alternate approach to the use of colloidal gold for enhanced Raman, optical gratings may be used to supply the enhanced Raman effect. In this scheme the grating would be used as the support for the target, supplying the additional resonant modes that couple to the excitation and vibrational

modes of the target molecule to supply the enhanced scatter. This approach, if feasible, has two extremely attractive attributes. First, it would reduce the preparation, since there would not be any need for the wet chemistry used in labeling the IgG with the colloidal gold. Second, Raman scatter is highly peaked in intensity for direct forward or backscatter, and the use of an optical grating gives one the ability to "tune" the preferred direction of scatter by matching the wave vector of the incident light with expected Raman signature and the structure of the grating. This adds a great deal of flexibility to choice of detection geometry, which would have otherwise been constrained by the normal preference for forward/backscatter.

There is a tremendous need for the rapid detection of prion diseases and other biological pathogens to ensure public health and safety. Recent outbreaks of bacterial contamination, as well as concerns about the possibility of BSE-contaminated beef products, have resulted in new and higher consumer expectations and government standards. Furthermore, a reliable assay for PrP in tissues and body fluids would be of clinical importance to permit monitoring for the presence of infectivity in humans, thereby ensuring the integrity of the donor blood supply, as well as facilitating the early clinical diagnosis of CJD. Optical sensing technology provides a novel, nondestructive method for the analysis of biological samples. Moreover, these methods are amenable to data fusion whereby the information generated by multiple techniques is combined to yield the final, overall spectral characterization and identification of a protein and/or compound. Multispectral techniques, alone or in combination with an immunoassay platform, will provide a highly sensitive, specific, and rapid detection scheme for PrP in biological fluids.

ACKNOWLEDGMENTS

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REFERENCES

1. Pocchiari M. Prions and related neurological diseases. In: Baum H, Azzi A, Jordan VC, Ozawa T, Rice-Evans C, eds. Molecular Aspects of Medicine. New York: Elsevier Science Ltd., 1994, pp 195–291.
2. Gabizon R, McKinley MP, Prusiner SB. Purified prion proteins and scrapie

infectivity copartition into lysosomes. Proc Natl Acad Sci USA 1987; 84: 4017–4021.
3. Himert H, Diringer H. A rapid and efficient method to enrich SAF protein from scrapie brains of hamsters. Biosci Rep 1984; 2:165–170.
4. Prusiner SB, Bolton D, Groth DF, Bowman KA, Cochran SP, McKinley MP. Further purification and characterization of scrapie prions. Biochemistry 1982; 21:6942–6950.
5. Kascsak RJ, Tonna-DeMasi M, Fersko R, Rubenstein R, Carp RI, Powers JM. The role of antibodies to PrP in the diagnosis of transmissible spongiform encephalopathies. In: Brown F, ed. Transmissible Spongiform Encephalopathies—Impact on Animal and Human Health. Basel, Karger, 1993, pp 141–151.
6. Kascsak RJ, Fersko R, Pulgiano D, Rubenstein R, Carp RI. Immunodiagnosis of prion diseases. Immunol Invest 1997; 26:259–268.
7. Rubenstein R, Gray PC, Wehlburg CM, Wagner JS, Tisone GC. Detection and discrimination of PrP^{Sc} by multi-spectral ultraviolet fluorescence. Biochem Biophys Res Commun 1998; 246:100–106.
8. Wagner JS, Trahan MW, Nelson WE, Tisone GC, Preppernau BL. How intelligent chemical recognition benefits from multivariate analysis and genetic optimization. Comp Phys 1996; 10:114–118.
9. Kyte J. Structure in Protein Chemistry. New York & London: Garland Publishing Inc., 1995, pp 404–418.
10. Gray PC, Shokar IR, Rosenthal SE, Tisone GC, Wagner JS, Rigdon LD, Siragusa GR, Heinen RJ. Distinguishability of biological material by use of ultraviolet multispectral fluorescence. Appl Optics 1998; 37:6037–6041.
11. Naik VM, Chetty I. Vibrational spectroscopic studies of L, D-dipeptides. Spectrosc Lett 1996; 29:1719–1731.
12. Fleury F, Ianoul A, Berjot M, Feofanov A, Alix AJP, Nabiev I. Camptothecin-binding site in human serum albumin and protein transformations induced by drug binding. FEBS Lett 1997; 411:215–220.
13. Tuma R, Russell M, Rosendahl M, Thomas GJ Jr. Solution conformation of the extracellular domain of the human tumor necrosis factor receptor probed by Raman and UV-resonance Raman spectroscopy: structural effects of an engineered PEG linker. Biochemistry 1995; 34:15150–15156.
14. Li P, Chen XG, Shulin E, Asher SA. UV resonance Raman ground and excited state studies of amide and peptide isomerization dynamics. J Am Chem Soc 1997; 119:1116–1120.
15. Nafie LA. Infrared and Raman vibrational optical activity: theoretical and experimental aspects. Annu Rev Phys Chem 1997; 48:357–386.
16. Vargek M, Freedman TB, Nafie LA. Improved backscattering dual circular polarization Raman optical activity spectrometer with enhanced performance for biomolecular applications. J Raman Spectrosc 1997; 28:627–633.
17. Wilson G, Hecht L, Barron LD. Vibrational Raman optical-activity of alpha-helical and unordered poly (L-lysine). J Chem Soc Faraday Trans 1996a; 92:1503–1509.

18. Wilson G, Hecht L, Barron LD. Residual structure in unfolded proteins revealed by Raman optical activity. Biochemistry 1996b; 35:12518–12525.
19. Wilson G, Ford SJ, Cooper A, Hecht L, Wen ZQ, Barron LD. Vibrational Raman optical activity of alpha-lactalbumin: comparison with lysozyme, and evidence for native tertiary folds in molten globule states. J Mol Biol 1995; 254:747–760.
20. Chen Y-Q, Kraut J, Callender R. pH-dependent conformational changes in *Escherichia coli* dihydrofolate reductase revealed by Raman difference spectroscopy. Biophys J 1997; 72:936–941.
21. Andrew CR, Fraczkiewicz R, Czernuszewicz RS, Lappalainen P, Saraste M, Sanders-Loehr J. Identification and description of copper-thiolate vibrations in the dinuclear Cu_A site of cytochrome C oxidase. J Am Chem Soc 1996; 118: 10436–10445.
22. Schulze HG, Greek LS, Blades MW, Bree AV, Gorzalka BB, Turner RFB. Neurotransmitter measurement with a fiber-optic probe using pulsed ultraviolet resonance Raman spectroscopy. In: Priezzhev AV, Asakura T, Leif RC, Katzir A, eds. Optical diagnostics of biological fluids and advanced techniques in analytical cytology. Proc SPIE-Int Soc Opt Eng 2982: 1997, pp 180–188.
23. Dou X, Takama T, Yamaguchi Y, Yamamoto H, Ozaki Y. Enzyme immunoassay utilizing surface-enhanced Raman scattering of the enzyme reaction product. Anal Chem 1997a; 69:1492–1495.
24. Dou X, Yamaguchi Y, Yamamoto H, Doi S, Ozaki Y. Quantitative analysis of metabolites in urine by anti-Stokes Raman spectroscopy. Biospectroscopy 1997b; 3:113–120.
25. Berger AJ, Itzkan I, Feld MS. Near infrared Raman spectroscopy of human whole blood and serum. Proc. SPIE-Int Soc Opt Eng 1997a; 2982:87–90.
26. Berger AJ, Itzkan I, Feld MS. Feasibility of measuring blood glucose concentration by near-infrared Raman spectroscopy. Spectrochim Acta A 1997b; 53:287–292.
27. Golub GH, Van Loan CF. Matrix Computations. Baltimore, MD: Johns Hopkins University Press, 1989.
28. Press B, Flannery P, Teukolsky SA, Vettering WT. Numerical Recipes: The Art of Scientific Computing. New York: Cambridge University Press, 1986.
29. Telling GC, Parchi P, DeArmond SJ, Cortelli P, Montagna P, Gabizon R, Mastrianni J, Lugaresi E, Gambetti P, Prusiner SB. Evidence for the conformation of the pathogenic isoform of the prion protein enciphering and propagating prion diversity. Science 1996; 274:2079–2082.
30. Bessen RA, Marsh RF. Distinct PrP properties suggest the molecular basis of strain variation in transmissible mink encephalopathy. J Virol 1994; 68: 7859–7868.
31. Safar J, Wille H, Itri V, Groth D, Serban H, Torchia M, Cohen FE, Prusiner SB. Eight prion strains have PrP^{Sc} molecules with different conformations. Nature Med 1998; 4:1157–1165.

32. Measures RM. Laser Remote Sensing Fundamentals and Applications. Melbourne, FL: Krieger Publishing Co., 1992.
33. Garrell RL. Surface-enhanced Raman spectroscopy. Anal Chem 1989; 61: 401A–411A.
34. Kneipp K, Kneipp H, Manoharan R, Hanlon EB, Itzkan I, Dasari RR, Feld MS. Extremely large enhancement factors in surface-enhanced Raman scattering for molecules on colloidal gold clusters. Appl Spectrosc 1998; 52: 1493.
35. Nie S, Emory SR. Probing single molecules and single nanoparticles by surface-enhanced Raman scattering. Science 1997; 275:1102–1106.
36. Lyon LA, Keating CD, Fox AP, Baker BE, He L, Nicewarner SR, Mulvaney SP, Natan MJ. Raman spectroscopy. Anal Chem 1998; 70:341R.
37. Vo-Dinh T. Surface-enhanced Raman spectroscopy using metallic nano-structures. Trends Anal Chem 1998; 17:557–563.
38. Rohr TE, Cotton T, Fan N, Tarcha PJ. Immunoassay employing surface-enhanced Raman spectroscopy. Anal Biochem 1989; 182:388–395.
39. Ni J, Lipert RJ, Dawson GB, Porter MD. Immunoassay readout method using extrinsic Raman labels absorbed on immunogold colloids. Anal Chem 1999; 71:4903–4908.
40. Kneipp K, Dasari RR, Wang Y. Near infrared surface-enhanced Raman scattering (NER SERS) on colloidal silver and gold. Appl Spectrosc 1994; 48: 951–954.
41. Zeman EJ, Schatz GC. An accurate electromagnetic theory study of surface enhancement factors for Ag, Au, Cu, Li, Na, Al, Ga, In, Zn and Cd. J Phys Chem 1987; 91:634–642.
42. Schultz S, Smith DR, Mock JJ, Schultz DA. Single-target molecule detection with nonbleaching multicolor optical immunolabels. Proc Natl Acad Sci USA 2000; 97:996–1001.
43. Chan WC, Nie S. Quantum dot bioconjugates for ultrasensitive nonisotopic detection. Science 1998; 281:2016–2018.
44. Bruchez M, Moronne M, Gin P, Weiss S, Alivisatos AP. Semiconductor nanocrystals as fluorescent biological labels. Science 1998; 281:2013–2015.

13

Detection of the Prion Protein in Blood Using a Fluorescence Immunoassay

Mary Jo Schmerr
Agricultural Research Service, U.S. Department of Agriculture, Ames, Iowa, U.S.A.

Andrew Alpert
PolyLC, Inc., Columbia, Maryland, U.S.A.

I. INTRODUCTION

Transmissible spongiform encephalopathies (TSEs) of humans and animals are relatively rare neurodegenerative diseases (1–4). Scrapie, a TSE in sheep, is the oldest-known member of this family and was first described over 200 years ago (5). Another TSE found in the United States and Canada, chronic wasting disease, has been described in mule deer (6) and then later in Rocky Mountain elk (7). The recent outbreak of a TSE in cattle, bovine spongiform encephalopathy (BSE), in the United Kingdom has caused considerable concern because of the transmission to humans (8,9).

Many experiments have been done to determine which tissues and fluids including blood, contain the infectious agent of this disease (10–15). Generally, brain and central nervous system (CNS) tissue are the most infectious and are the tissues used to test for BSE in cattle in Europe (16). Lymphoid tissue, spleen, and tonsil also are infectious but at a lower level than brain and CNS tissue (12). The results from studies on blood have varied widely. A recent study has demonstrated that BSE can be transmitted

by blood transfusion in sheep (17). A systematic study has shown that fractions from blood are infectious in a rodent model but at a rather low infection rate (18). Although the blood was fractionated in this study to determine which fractions contained infectivity, no attempt was reported to isolate to isolate the putative agent of these diseases, the abnormal prion protein. Recent experiments have shown that the abnormal prion protein does not appear to replicate in the blood but in the follicular dendritic cells (19) in the spleen and lymphoid tissue. Because the abnormal prion protein is insoluble in biological buffers and probably not very soluble in the blood matrix, it is likely to bind to cells or plasma lipids. Also, the concentration in the blood is relatively low compared to CNS tissue and brain. These properties represent a challenge that needs to be met by any testing system. As a result, analytical tests for the abnormal prion protein have been elusive. Some of the conventional analytical tests that are available are Western blot (16,20) and plate assays (21). Both assays have been used successfully for brain, CNS tissues, and lymphoid tissue. There has been one report of the use of Western blot to detect the abnormal prion protein in urine (22) but no reported success with other fluids such as blood. Practical tests for prion disease need to be based on fluids that are easier to obtain than tissue samples and can be obtained repeatedly with minimally invasive procedures. A key fluid is blood.

Development of an assay for blood requires new approaches and better methods of concentration of the abnormal prion protein. One of the difficulties in the development of a new technology in prion research is to obtain appropriate samples in replicate to test the various parameters. It is problematic to obtain enough blood for replicates from preclinical sheep. Commercial producers do not want to bleed larger volumes of blood from their sheep, and at research installations relatively small herds of sheep are kept because of the costs involved. In addition, it is necessary to follow the sheep through their lifetime to determine the best time to test blood for sheep scapie. These same problems are also true for research on chronic wasting disease in the cervid population. Test development requires a major coordination effort among field veterinarians, wildlife biologists, and the laboratory developing a test. Several important factors in sample procurement are involved, including the amount of blood, proper collection tubes, and shipping conditions. Finally, meticulous record keeping is required to ensure that the final correlation of results is meaningful.

Our research has centered on developing methods to solubilize and extract the prion protein from fluids and to develop a robust testing system with enough sensitivity to detect the abnormal prion protein in picogram quantities.

A. Solubilization and Extraction of the Prion Protein

Conventional methods to concentrate and purify the abnormal prion protein use a series of centrifugation steps including treatment with ionic detergents such as sarcosine and deoxycholate and proteases, specifically proteinase K. These protocols are time-consuming. Initially, these same protocols were used to prepare sheep brain testing in an immunoassay. After concentration, the samples were solubilized by boiling in sodium dodecyl sulfate (SDS). Since SDS interferes in immunoassays, a lengthy protocol that included precipitation of the SDS with guanidine HCl followed by precipitation of the protein with cold ethanol was used to remove the SDS. The efficiency of recovering the abnormal prion protein was low and difficult to resolubilize. Consequently, other methods were sought to remove the SDS.

A new approach was developed using hydrophilic interaction chromatography (23). After solubilization in SDS, the samples were subjected to high-performance liquid chromatography (HPLC) using either a PolyWAX LP column or PolyHYDROXYETHYL Aspartamide column (PolyHYDROXYETHYL A) (PolyLC, Inc., Columbia, MD). Samples were applied to the column in 95% acetonitrile, containing 0.1% TFA, 50 mM hexafluoro-2-propanol (HFIP) (mobile phase A). After isocratic elution for 10 min, the abnormal prion protein was eluted with a linear gradient from 0–100% B over 15 min. Mobile phase B was an aqueous solution of 0.1% TFA, with 50 mM HFIP. The abnormal prion protein eluted at 100% B. A column profile of the run using a scrapie-infected brain sample is shown in Fig. 1 using absorbance at 280 nm. Fractions were collected from all of the peaks showing absorbance at 280 nm and analyzed by dot blots with antibody to the prion protein. The fractions that reacted with specific antibody to the prion protein were fractions at 24–25 min. These fractions were collected on subsequent runs and iodinated. Reactivity against the iodinated prion protein was also found in these fractions in a capture assay. The recovery from the two columns was estimated by injecting iodinated prion protein. The recoveries were ~70% from the PolyWAX LP column and 95% from the PolyHYDROXYLETHYL Aspartamide column. However, after ~10 runs the HPLC columns needed to be cleaned because the backpressure on the column became quite high, presumably owing to accumulation of uneluted solutes. To circumvent this problem, solid-phase cartridges (SPE) packed with the PolyHYDROXYLETHYL A material were used (PolyLC, Inc., Columbia, MD). These inexpensive cartridges could be discarded after a single use, preventing carryover of retained material to the next sample.

It was noted that the abnormal prion protein was very slightly soluble in the mobile phase A. Brain samples needed to be diluted to prevent

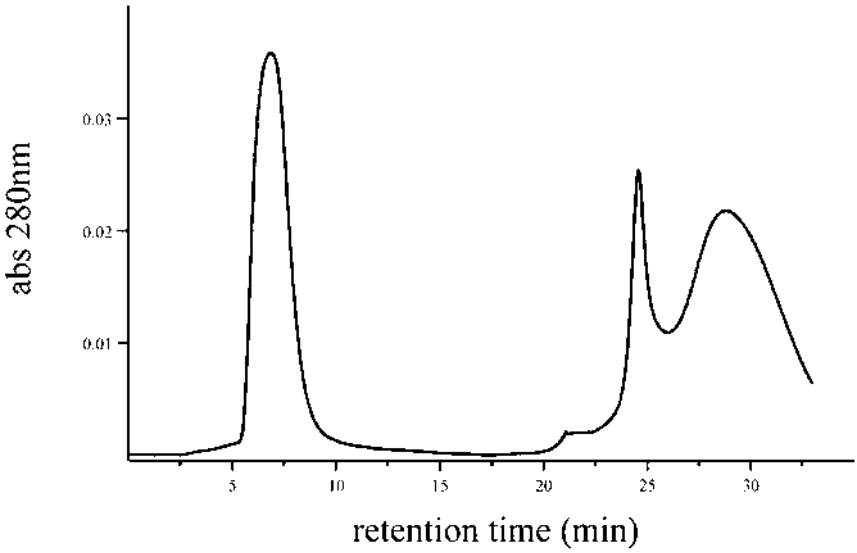

Figure 1 Hydrophilic interaction chromatography of the abnormal prion protein. Column: PolyHYDROXETHYL A (5 μm, 300 Å), 200 × 4.6 mm, flow rate of 0.5mL/min. The solid line represents the absorbance at 280 nm.

precipitation of abnormal protein before application to the SPE cartridge. Success in preparing the abnormal prion protein from brain led us to apply this approach successfully with lymphoid tissues that contain smaller amounts of abnormal prion. It should be noted that not all animals or humans infected with a TSE have deposition of the abnormal prion protein in lymphoid tissue. Generally in sheep with susceptible genotypes (24–30), chronic wasting disease in cervids (31), and variant Creutzfeldt-Jakob disease (32), there is deposition in lymphoid tissue of infected individuals. However, in BSE, deposition of the abnormal prion protein in lymphoid tissue has not been observed (33).

The buffy coat fraction from blood was chosen because both it and the plasma contain infectivity (18). In this same study, plasma was found to contain infectivity as well. Following our success with brain and lymphatic tissue, we adapted this extraction protocol to blood. Buffy coats were isolated from blood in the usual manner. The buffy coats were frozen and thawed and treated with DNase. This was followed by proteinase K treatment that digests the normal protein, rapidly. The abnormal prion protein is partly resistant to this enzyme. The organic solvent, HFIP, has been reported (34) to solubilize the abnormal prion protein and was used

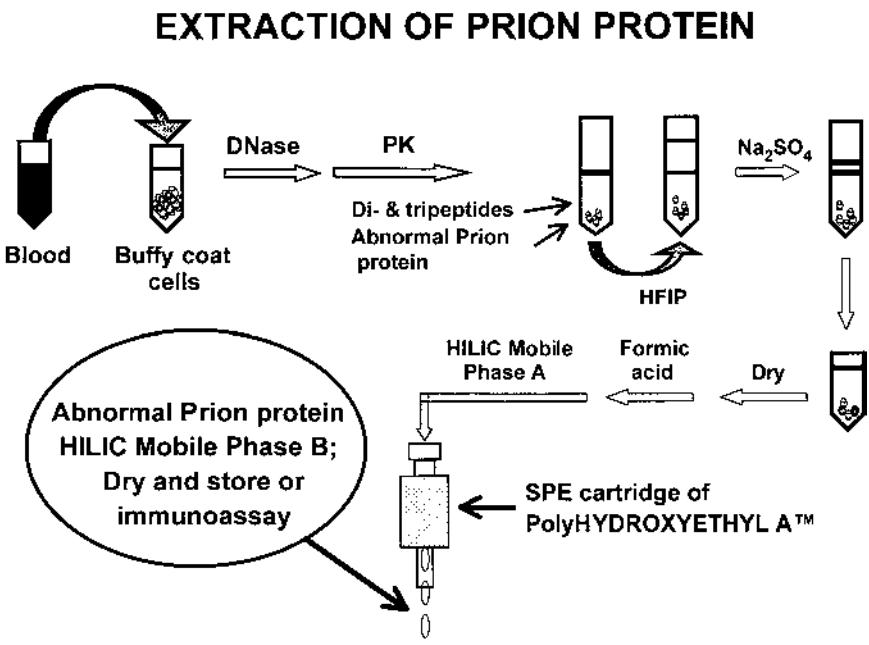

Figure 2 Flowchart that represents the process steps for preparation of the abnormal prion protein from blood.

here for that purpose. While HFIP is miscible with water, it is possible to cause a phase separation by the addition of sodium sulfate. The abnormal prion protein partitioned into the lower organic phase that was collected and dried. The dried sample was resuspended in formic acid and then mobile phase A and applied to a SPE cartridge of PolyHYDROXETHYL A. The cartridge was washed and the abnormal prion protein was eluted with mobile phase B. The eluted fractions were collected, dried, and store at 4°C until they were analyzed. A flowchart showing this process is shown in Fig. 2. The detailed protocol has been described (35).

II. FLUORESCENCE IMMUNOASSAY (FIA)

A. Labeling of Peptides

Five peptide sequences that were identified as antigenic sites on the prion protein were evaluated (36). (See Table 1.) For each peptide, a cysteine was added to the NH_2 terminal residue. The thiol group of cysteine was used as a

Table 1 Peptides from the Prion Protein Used in the Fluorescence Immunoassay

Peptide sequence	Amino acid position
GQGGGTHNQWNKPSL-NH_2	89–103
KTNMKHVAGAAAAGAVVGGLG-NH_2	106–126
NDWEDRYYRENMYR-NH_2	142–154
RYPNQVYYRPVDRYSNQNNFVHD-NH_2	155–177
RESQAYYQRGASVIL-NH_2	218–232

reactive site to attach a fluorescent label, either fluorescein or Oregon Green. This ensured the specificity of labeling and limited the number of labels per peptide. The labeled peptides were purified using a conventional C-18 column suitable for peptide applications. We also had all of the peptides synthesized by a commercial company (Multiple Peptide Systems, San Diego, CA) that produced the fluorescein-labeled peptide using a gamma butyric acid link with fluorescein.

B. Antibodies

Antibodies were made to each of the peptides in rabbits. The polyclonal antibodies were affinity-purified on a column of the corresponding immobilized peptide. These antibodies were titered against dilutions of the fluorescent-labeled peptide. A dilution of the antibody that bound 50% of the fluorescein-labeled peptide was used. For one of the peptides (amino acid residues 218–232), three lots of antibodies have been produced and each lot performs similarly.

III. DETECTION METHODS

A. Free-Zone Capillary Electrophoresis

Immunoassay systems that use free-zone capillary electrophoresis have been developed. Schultz et al. (37) used fluorescein-labeled insulin and a monoclonal antibody to insulin to perform their studies. Other capillary electrophoresis (CE) methods using sandwich assays with either an anti-immunoglobulin or protein G had been reported (38,39). These studies demonstrated that fluorescence immunoassay systems using CE with laser-induced fluorescence as a testing platform could be quite sensitive. In the first attempts to produce an assay for the prion protein, we used a sandwich assay approach. Rabbit antibodies against the prion protein peptide

(amino acid positions 89–103) were mixed with the prepared sample. The signal reagent was fluorescein labeled anti–rabbit IgG. If only the normal form was present, it was digested by the proteinase K and there was no reaction. However, if the abnormal prion was present in the reaction, a peak was observed. This system was cumbersome and not very reproducible. These problems were overcome by using fluorescent-labeled peptides of the abnormal prion protein. When corresponding antibodies were added to the assay, two peaks were obtained in the electropherogram (Fig. 3), one corresponding to the peptide bound by the antibody and one for the free peptide. Initially borate buffer, pH 9.0, was used as the separating buffer. This caused the antibody-bound, fluorescent-labeled peptide to give a fairly complicated electropherogram with several peaks because of the separation of the immunoglobulins based on their glycosylation. When a 0.2M Tricine buffer, pH 8.0, containing the detergent 0.1% *N*-octylglucoside and 0.1% bovine serum albumin (BSA) was used, the electropherogram was much simpler (38). If the internal diameter (I.D.) of the

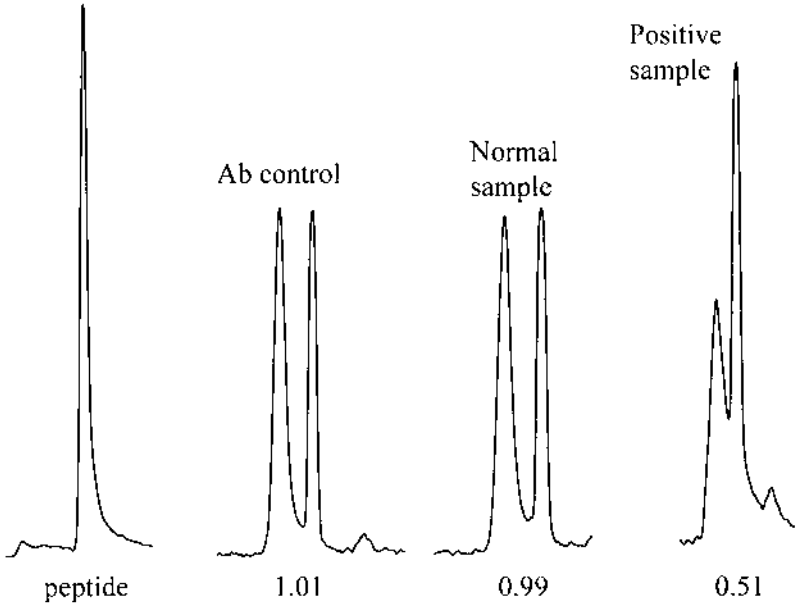

Figure 3 Electropherograms representing analysis of the fluorescent peptide, the antibody control, a normal sample, and a sample from a scrapie-infected sheep. The numbers at the bottom of each electropherogram are the ratio of (bound/free) peptide peak heights. Running time was 3 min.

capillary was 20–25 μm, the separation was quicker and had better resolution than when a 50-μm I.D. was used.

The initial assay development was done on the P/ACE 5500 (Beckman Coulter, Fullerton, CA) (40,41). Recent work has been performed on the P/ACE MDQ (Beckman Coulter, Fullerton, CA). This instrument is more sensitive and has the capacity to handle several more samples and can cool the samples to the prescribed temperature. Although the analysis takes 3 min, preparation of the capillary adds ~9 min to the run time. Prior to analysis, the initial equilibrium of the immunoassay takes ~16 h. The ratio of the immunocomplex peak and the free peptide changes over time (Fig. 4). To correct for this change the samples are run bracketed by antibody controls.

B. HPLC Size Exclusion

There are reports of immunoassays using size exclusion chromatography (SEC) to separate the bound labeled antigen from the antigen com-

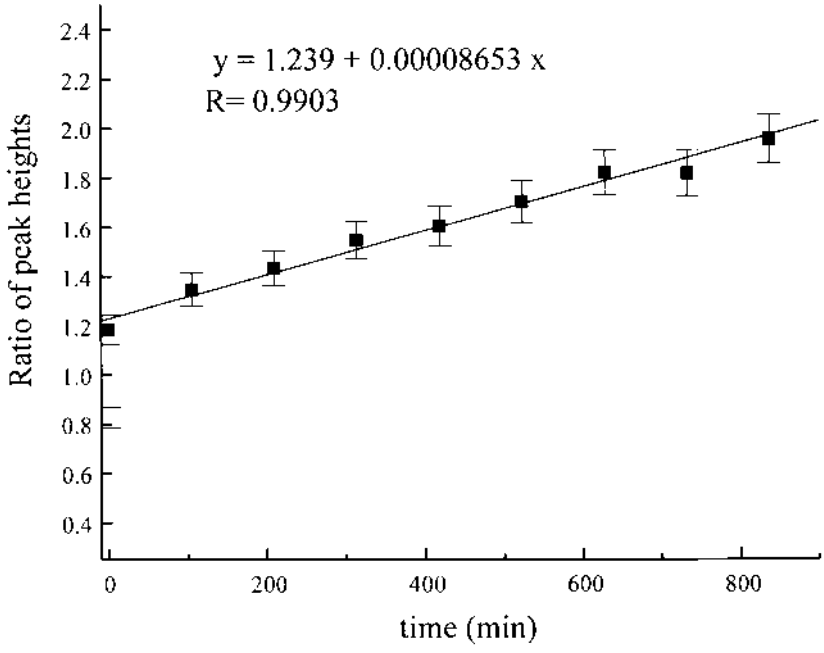

Figure 4 Curve representing the increase in the ratio of the antibody complex to free peptide.

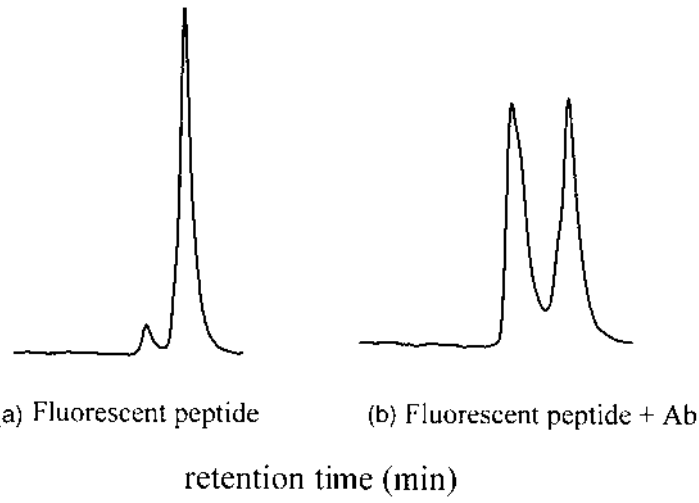

Figure 5 Chromatograms representing HPLC-size exclusion (a) of the free peptide and (b) of the antibody-bound peptide and the free peptide. Column: Poly-HYDROXETHYL A (5 μm, 500 Å), 100 × 4.6 mm flow rate 0.25 mL/min mobile phase 0.1M potassium phosphate pH 7.0. Heterogeneity in the chromatogram represents heterogeneity in the peptide.

plexed to antibody (42–46). We assessed this separation method on a Poly-HYDROXYETHYL A column in the SEC mode using laser-induced fluorescence (Picometrics, Ramonville, France) for detection. Several column sizes were evaluated as well as two pore sizes of 200 and 500 Å. A 100 × 4.6 mm column with a 500-Å pore size was used; the resolution of the free peptide from the bound peptide was almost baseline resolved in 6 min (Fig. 5). Similar results were obtained with a narrow bore column (100 × 2.1 mm) using one-fourth as much sample. The peptide was labeled with Oregon Green to determine if the pH of the running buffer affected the sensitivity. Oregon Green does not have a specific pH optimum for fluorescence emission while the optimum for fluorescein is pH of 9.0 or above, the running buffer was pH 7.0. We found little difference in the sensitivity of the dyes. This method needs further development but is very promising as a test platform for the FIA.

IV. ASSAY SENSITIVITY

The prion peptide that was used in these measurements was the peptide at amino acid positions 218–232, in the prion protein sequence (see Table 1). A

stock solution of the fluorescent-labeled peptide is made up to a concentration of 1 mg/mL. This peptide is diluted 1/200,000 for use in the FIA. The peptide solution is then diluted by a factor of 2 by the addition of antibody and sample. Approximately 2 nL of the combined sample is analyzed in the capillary. This is equivalent to 2.26 attomoles of fluorescent peptide. The concentration of the antibody is adjusted so that one-half of the fluorescent peptide is bound giving a ratio of the antibody-bound peptide/free peptide equal to 1.0. The concentration of the bound fluorescent-labeled peptide is ~0.55 attomoles. If one-half of the bound fluorescent-labeled peptide is displaced by the competitor, the prion protein, theoretically 0.25 attomoles could be measured in this assay. This is equivalent to ~0.25 pmoles/mL. When the corresponding unlabeled peptide was used as a competitor in the assay, the measured values for the amount of the unlabeled was slightly higher, ranging from 0.5 to ~2.0 pmoles/mL. Although the sample volume analyzed on the HPLC size exclusion was 5000 times more than on capillary

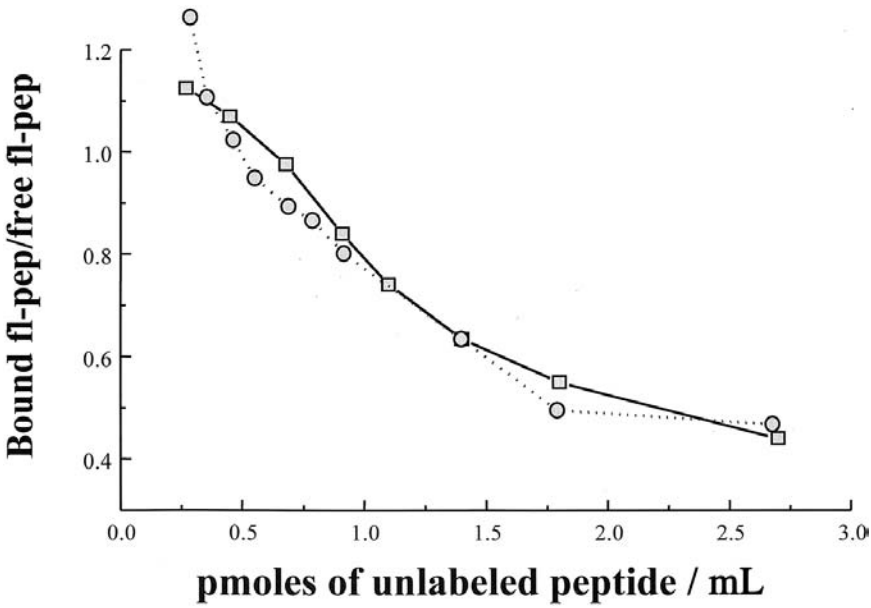

Figure 6 Curves representing the concentration of the unlabeled peptide versus the bound versus free ratio in the FIA. □—□ Represents the competition using HPLC size exclusion for testing. ○···○ Represents the competition using capillary electrophoresis as the testing platform.

electrophoresis, the measurements from both techniques had an excellent correlation (Fig. 6). The HPLC method differed from the CE method in that the concentration of the antibody in the fluorescence immunoassay needed to be ~1.5 times greater to achieve a ratio of 1.0 for the bound versus the free fluorescent-labeled peptide (Fig. 7). This did not appear to affect the sensitivity. When a bovine recombinant prion protein (rPrP) was used as a competitor in the assay, the amount necessary for detection ranged from ~75 pmoles to ~350 pmoles/mL (Fig. 8). This is ~75 times more required for competition than the unlabeled peptide. This difference may be due to the greater size of the rPrP that results in steric hindrance in binding to the antibody. Another explanation for this discrepancy may have resulted from the manner in which the rPrP was produced. The last four amino acids on the carboxyl terminus were not included in the recombinant PrP sequence. These amino acids were present on the peptide when the antibodies were produced to the peptide. As a result, some of the epitopes are missing and the antibodies had lower reactivity for the recombinant PrP. Similarly, when

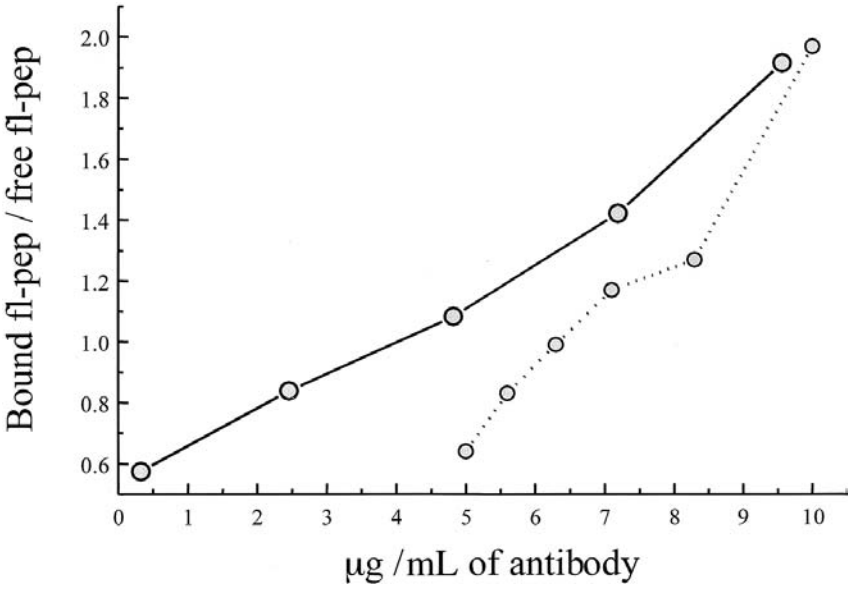

Figure 7 Curves representing the concentration of the antibody versus the bound versus the free ratio in the FIA. □—□ Represents the competition using HPLC size exclusion for testing. ○···○ Represents the competition using capillary electrophoresis as the testing platform.

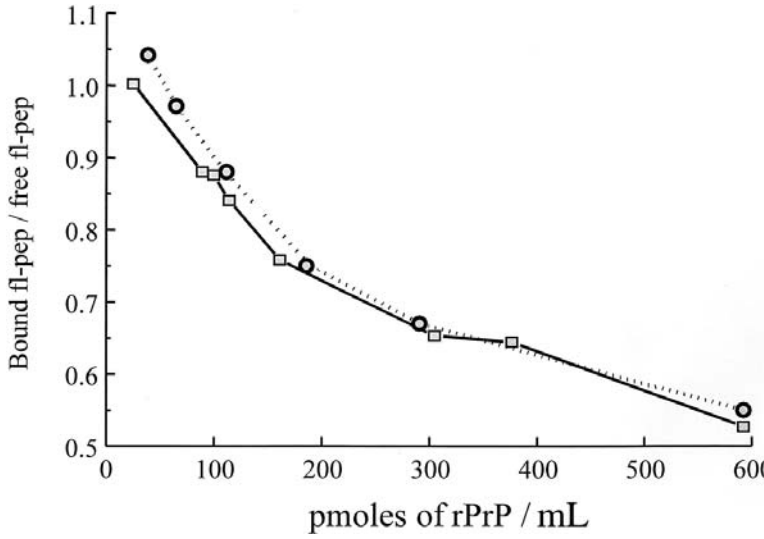

Figure 8 Curves representing the concentration of bovine recombinant prion protein versus the bound versus free ratio in the FIA. □—□ Represents the competition using HPLC size exclusion for testing. ○···○ Represents the competition using capillary electrophoresis as the testing platform.

substitutions at the carboxyl terminus of the peptide sequence are made to change the sheep peptide sequence to that of the human sequence, there is some loss in reactivity of the antibody made to the sheep sequence with the human fluorescent-labeled peptide.

The amount of blood used for testing ranges from 15 to 20 mL. After concentration, approximately one-half of the prepared sample is used for testing. The sample is concentrated ~1500 times. The assay can detect ~10 pg of the abnormal prion protein in the blood. This sensitivity is ~10–20 times that of Western blot. Animals with a TSE infection, either preclinical or clinical, have been found to have abnormal prion levels of 20 pg/mL or higher in the FIA. Thus, this assay has the necessary sensitivity for detection of infection in individual animals or humans.

V. BIOLOGICAL CORRELATIONS

After developing this protocol for extracting and testing for the abnormal prion protein in the blood, we can now address the relevant biology.

A. Control Sheep

A study was done with sheep blood that came from sheep flocks certified to be free of scrapie for a period of 5 years. The ratios for one of these flocks of 57 sheep is shown in Fig. 9. As can be seen in this figure, all of the sheep had ratios above 0.70. Most of the ratios were greater than 0.85. In addition, all of the other "normal" sheep tested as well as 20 sheep from New Zealand had ratios that were similar, with most of the ratios being greater than 0.85 (data not shown).

B. Correlation of Sheep That Are Positive on Lymphoid Biopsy

Sheep testing positive lymphoid tissue were also positive in the blood assay. These sheep were bled when they were ~7–15 months of age and tested by the protocol that was developed for the blood assay. When these sheep reached 15–18 months of age, a submandibular lymph node was surgically removed and tested for the presence of the prion protein by immuno-

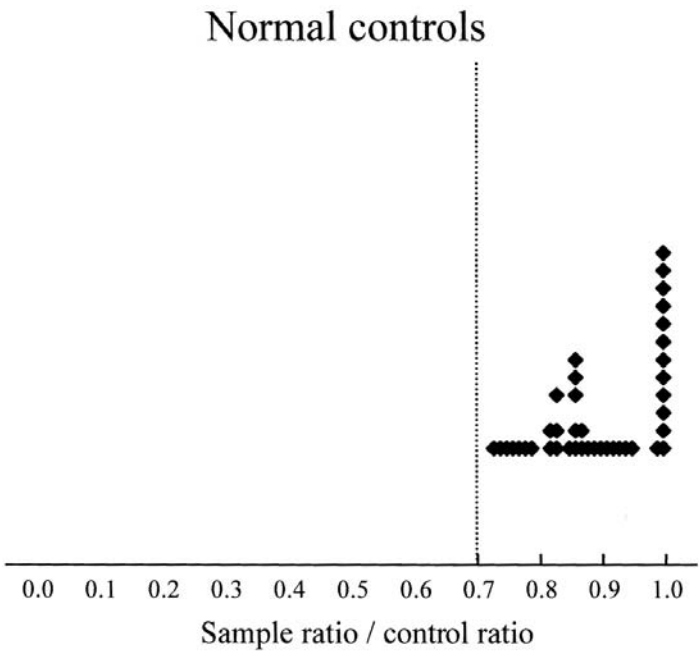

Figure 9 Represents the ratio of the bound peptide/free peptide of an individual sheep. The line drawn at 0.7 represents the value chosen as a cutoff.

Table 2 Correlation of the Blood Assay with Positive Lymphoid Tissue for Preclinical Sheep

Sheep #	Mean	Standard deviation	Coefficient of variance × 100
1	0.46	0.0748	16.3
2	0.47	0.0618	13.2
3	0.368	0.0342	9.3
4	0.438	0.0268	6.1
5	0.428	0.0832	19.4
6	0.515	0.0403	7.8
7	0.545	0.0320	5.9
8	0.523	0.0760	14.5
9	0.403	0.0838	20.8
10	0.560	0.0797	14.2

histochemistry. For these 10 sheep that were lymphoid tissue positive, each sample prepared from these sheep was run in duplicate and two CE runs were obtained for each duplicate. The mean and standard deviation was obtained for each of these values and the coefficient of variance was calculated (see Table 2). A t-test was performed on the samples and the sample ratios were significantly different from the cutoff value of 0.7 with 95% confidence level. Generally, the abnormal prion protein is detected in the blood of sheep before the lymphoid tissue is generally found to be positive (12–18 months of age). These 10 sheep are being held until they develop clinical scrapie.

Table 3 Percent of the Antibody Control of Sheep Tested at ~12–18 Months of Age Before Showing Signs of Clinical Scrapie

Sheep #	Antibody control (%)
1	16.4
2	8.9
3	63.7
4	41.3
5	64.2
6	58.0
7	64.0
8	61.3
9	61.0
10	64.0

C. Correlation of Positive Results in the Blood Assay and the Later Development of Clinical Disease

Ten asymptomatic sheep that were tested by blood assay from 12 to 18 months of age were kept under observation. These sheep have now died and have been confirmed to have scrapie. In Table 3, the results of the blood assay are shown.

VI. CONCLUSIONS

A process has been developed that can be used to detect the abnormal prion in the blood of infected individuals. The fluorescence immunoassay used for testing has advantages over solid-phase immunoassay systems used in other prion testing:

1. The assay takes place in free solution. This permits free rotation of the molecules allowing for the maximum number of possible conformations and increasing the chance for effective interactions.
2. The assay is a direct binding assay and both the bound antigen and the free antigen are measured. Other immunoassays measure only the bound form of analyte, which is frequently a relatively small percentage of the analyte in question.
3. The sensitivity is limited by the detector and is not based on signal amplification, which has its own unique problems.
4. The stoichiometry is related to actual binding rather than an amplified signal.
5. The actual measurements are very close to the calculated measurements.

With further improvement and development, this process for extracting, concentrating, and testing has the potential to become a preclinical diagnostic test for prion diseases. For example, additional fluorescent dyes that have higher excitation coefficients and/or molar extinction coefficients will be evaluated. These could improve the sensitivity of detection. The dyes must not interfere in the antigenicity of the peptide or change the migration rate in the separation technique significantly. The test platform using HPLC-SEC needs more development and could be easily adapted for use with an autosampler. This method then would have the potential for high throughput of samples. Large numbers of replicate samples that have become available only recently are needed to ascertain the reproducibility of the assays. Experiments have been designed and are underway at present to address this issue. Additional numbers

are needed to address the predictability of the assay for development of clinical disease or to differentiate a TSE from another neurological disease (e.g., testing cerebrospinal fluid to distinguish between Creutzfeldt-Jakob disease and Alzheimer's disease). This process for testing for the abnormal prion protein may also be adapted to test commercial materials and medical products for contamination. With further development, this immunoassay has the potential to be a sensitive and economical method to test for infection with a TSE and may have many other applications beyond those of TSEs.

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REFERENCES

1. Safar J, Prusiner SB. Molecular studies of prion diseases. Prog Brain Res 1998; 117:421–434.
2. Aguzzi A, Weissmann C. Prion diseases. Haemophilia 1998; 4:619–627.
3. Weissmann C. The Ninth Datta Lecture. Molecular biology of transmissible spongiform encephalopathies. FEBS Lett 1996; 389:3–11.
4. Mabbott NA, Bruce ME. The immunobiology of TSE diseases. J Gen Virol 2001; 82:2307–2318.
5. Pattison IH. Fifty years with scrapie: a personal reminiscence. Vet Rec 1988; 123:661–666.
6. Williams ES, Young S. Chronic wasting disease of captive mule deer: a spongiform encephalopathy. J Wildlife Dis 1980; 16:89–98.
7. Williams ES, Young S. Spongiform encephalopathy of Rocky Mountain elk. J Wildlife Dis 1982; 18:465–471.
8. Bruce ME, Will RG, Ironside JW, McConnell I, Drummond D, Suttie A, McCardle L, Chree A, Hope J, Birkett C, Cousens S, Fraser H, Bostock CJ. Transmissions to mice indicate that "new variant" CJD is caused by the BSE agent. Nature 1997; 389:498–501.

9. Hill AF, Desbruslais M, Joiner S, Sidle KC, Gowland I, Collinge J, Doey LJ, Lantos P. The same prion strain causes vCJD and BSE. Nature 1997; 389:448–450.
10. Dickinson AG, Meikle VM, Fraser H. Genetical control of the concentration of ME7 scrapie agent in the brain of mice. J Comp Pathol 1969; 1:15–22.
11. Hadlow WJ, Eklund CM, Kennedy RC, Jackson TA, Whitford HW, Boyle CC. Course of experimental scrapie virus infection in the goat. J Infect Dis 1974; 129:559–567.
12. Hadlow WJ, Kennedy RC, Race RE. Natural infection of Suffolk sheep with scrapie virus. J Infect Dis 1982; 146:657–664.
13. Tamai Y, Kojima H, Kitajima R, Taguchi F, Ohtani Y, Kawaguchi T, Miura S, Sato M, Ishihara Y. Demonstration of the transmissible agent in tissue from a pregnant woman with Creutzfeldt-Jakob disease. N Engl J Med 1992; 327:649.
14. Race R, Ernst D, Jenny A, Taylor W, Sutton D, Caughey B. Diagnostic implications of detection of proteinase K–resistant protein in spleen, lymph nodes, and brain of sheep. Am J Vet Res 1992; 53:883–889.
15. Radebold K, Chernyak M, Martin D, Manuelidis L. Blood borne transit of CJD from brain to gut at early stages of infection. BMC Infect Dis 2001; 1:20.
16. Oesch B, Doherr M, Heim D, Fischer K, Egli S, Bolliger S, Biffiger K, Schaller O, Vandevelde M, Moser M. Application of Prionics Western blotting procedure to screen for BSE in cattle regularly slaughtered at Swiss abattoirs. Arch Virol Suppl 2000; 16:189–195.
17. Houston F, Foster JD, Chong A, Hunter N, Bostock CJ. Transmission of BSE by blood transfusion in sheep. Lancet 2000; 356:999–1000.
18. Brown P, Rohwer RG, Dunstan BC, MacAuley C, Gajdusek DC, Drohan WN. The distribution of infectivity in blood components and plasma derivatives in experimental models of transmissible spongiform encephalopathy. Transfusion 1998; 38:810–816.
19. Bruce ME, Brown KL, Mabbott NA, Farquhar CF, Jeffrey M. Follicular dendritic cells in TSE pathogenesis. Immunol Today 2000; 21:442–446.
20. Bolton DC, McKinley MP, Prusiner SB. Molecular characteristics of the major scrapie prion protein. Biochemistry 1984; 23:5898–5906.
21. Grathwohl KU, Horiuchi M, Ishiguro N, Shinagawa M. Sensitive enzyme-linked immunosorbent assay for detection of PrP(Sc) in crude tissue extracts from scrapie-affected mice. J Virol Methods 1997; 64:205–216.
22. Shaked GM, Shaked Y, Kariv-Inbal Z, Halimi M, Avraham I, Gabizon R. A protease-resistant prion protein isoform is present in urine of animals and humans affected with prion diseases. J Biol Chem 2001; 276:31479–31482.
23. Alpert AJ. Hydrophilic-interaction chromatography for the separation of peptides, nucleic acids and other polar compounds. J Chromagtogr 1990; 499:177–196.
24. Hunter N, Moore L, Hosie BD, Dingwall WS, Greig A. Association between

natural scrapie and PrP genotype in a flock of Suffolk sheep in Scotland. Vet Rec 1997; 140:59–63.
25. van Keulen LJ, Schreuder BE, Meloen RH, Mooij-Harkes G, Vromans ME, Langeveld JP. Immunohistochemical detection of prion protein in lymphoid tissues of sheep with natural scrapie. J Clin Microbiol 1996; 34:1228–1231.
26. Schreuder BE, van Keulen LJ, Vromans ME, Langeveld JP, Smits MA. Tonsillar biopsy and PrPSc detection in the preclinical diagnosis of scrapie. Vet Rec 1998; 142:564–568.
27. van Keulen LJ, Schreuder BE, Vromans ME, Langeveld JP, Smits MA. Pathogenesis of natural scrapie in sheep. Arch Virol Suppl 2000; 16:57–71.
28. Andreoletti O, Berthon P, Marc D, Sarradin P, Grosclaude J, van Keulen L, Schelcher F, Elsen JM, Lantier F. Early accumulation of PrP(Sc) in gut-associated lymphoid and nervous tissues of susceptible sheep from a Romanov flock with natural scrapie. J Gen Virol 2000; 81:3115–3126.
29. Jeffrey M, Martin S, Thomson JR, Dingwall WS, Begara-McGorum I, Gonzales L. Onset and distribution of tissue prp accumulation in scrapie-affected suffolk sheep as demonstrated by sequential necropsies and tonsillar biopsies. J Comp Pathol 2001; 125:48–57.
30. Jeffrey M, McGovern G, Martin S, Goodsir CM, Brown KL. Cellular and subcellular localisation of PrP in the lymphoreticular system of mice and sheep. Arch Virol Suppl 2000; 16:23–38.
31. Sigurdson CJ, Williams ES, Miller MW, Spraker TR, KI O'Rourke, Hoover EA. Oral transmission and early lymphoid tropism of chronic wasting disease PrPres in mule deer fawns (*Odocoileus hemionus*). J Gen Virol 1999; 80:2757–2764.
32. Wadsworth JD, Joiner S, Hill AF, Campbell TA, Desbruslais M, Luthert PJ, Collinge J. Tissue distribution of protease resistant prion protein in variant Creutzfeldt-Jakob disease using highly sensitive immunoblotting assay. Lancet 2001; 358:171–180.
33. Will RG, Ironside JW. Oral infection by the bovine spongiform encephalopathy prion. Proc Natl Acad Sci USA 1999; 96:4738–4739.
34. Wille H, Zhang GF, Baldwin MA, Cohen FE, Prusiner SB. Separation of scrapie prion infectivity from PrP amyloid polymers. Mol Biol 1996; 259:608–621.
35. MJ Schmerr, Alpert AJ. Method and Kit for Extracting Prion Proteins. US Patent #6,150,172.
36. Vorberg I, Pfaff E, Groschup MH. The use of monoclonal antibody epitopes for tagging PrP in conversion experiments. Arch Virol Suppl 2000; 16:285–290.
37. Schultz NM, Huang L, Kennedy RT. Capillary electrophoresis-based immunoassay to determine insulin content and insulin secretion from single islets of Langerhans. Anal Chem 1995; 67:924–929.
38. Cole LJ, Kennedy RT. Selective preconcentration for capillary zone electrophoresis using protein G immunoaffinity capillary chromatography. Electrophoresis 1995; 16:549–556.

39. Heegaard NH. Determination of antigen-antibody affinity by immunocapillary electrophoresis. J Chromagtogr A 1994; 680:405–412.
40. Schmerr MJ, Jenny A. A diagnostic test for scrapie-infected sheep using a capillary electrophoresis immunoassay with fluorescent-labeled peptides. Electrophoresis 1998; 19(3):409–414.
41. Schmerr MJ, Jenny AL, Bulgin MS, Miller JM, Hamir AN, Cutlip RC, Goodwin KR. Use of capillary electrophoresis and fluorescent labeled peptides to detect the abnormal prion protein in the blood of animals that are infected with a transmissible spongiform encephalopathy. J Chromatogr A 1999; 853:207–214.
42. Önnerfjord P, Eremin SA, Emneus J, Marko-Varga G. High sample throughput flow immunoassay utilising restricted access columns for the separation of bound and free label. J Chromatogr A 1998; 800:219–230.
43. Önnerfjord P, Eremin SA, Emneus J, Marko-Varga G. A flow immunoassay for studies of human exposure and toxicity in biological samples. J Mol Recognit 1998; 11:182–184.
44. Bereczkim A, Horvath V, Horvai G. Immunoassay-based determination of phenobarbital using size-exclusion chromatography. J Chromatogr B Biomed Sci Appl 2000; 749:215–223.
45. Fernando SA, Sportsman JR, Wilson GS. Studies of the low dose "hook" effect in a competitive homogeneous immunoassay. J Immunol Methods 1992; 151:27–46.
46. Jensen PE, Moore JC, Lukacher AE. A europium fluoroimmunoassay for measuring peptide binding to MHC class I molecules. J Immunol Methods 1998; 215:71–80.

14

Prion Detection and Application to the Safety of Biological Products

Jeanette L. C. Miller,* Christopher J. Stenland, Stephen R. Petteway, Jr., and Douglas C. Lee
Bayer Corporation, Research Triangle Park, North Carolina, U.S.A.

I. INTRODUCTION

A. General Pathogen Safety of Biological Products

The class of pharmaceutical products referred to as biologicals include not only those products obtained from animal tissues (e.g., human plasma), but also biotechnology products derived from recombinant and transgenic sources that may require the use of animal-derived materials during their production. While biological products have had an excellent safety record with regard to pathogens, some biological products have transmitted pathogens, including HIV and HCV (1–3). Because animal-derived materials are sometimes used in the production of biological products, the potential exists for the transmission of animal pathogens. Therefore, precautions must be taken during the manufacture of biological products to avoid the potential for transmission of infectious diseases. These precautions include careful acquisition of the raw materials used in the manufacture of the product as well as careful control of the manufacturing process itself.

In general, methods used to ensure the pathogen safety of biological products can be grouped into three basic categories: screening, inactivation, and clearance. Raw material screening is an up-front approach that includes plasma donor screening, to reduce the probability of introducing pathogens

* *Current affiliation*: Meridian Bioscience Inc., Cincinnati, Ohio, U.S.A.

into the manufacturing process. Inactivation methods may be either chemical or physical and are designed to reduce a pathogen's ability to infect a host or to replicate. Clearance methods are processes that physically remove pathogens from the product purification stream. Some inactivation and clearance methods are intrinsic to the manufacturing process while others are specifically designed and incorporated into the process to improve pathogen safety margins. For the manufacture of all biological therapeutics the goal is to implement several of these complementary methods, while ensuring that the biological activity of the product is not altered, and that the product yield is not affected significantly. This chapter focuses on therapeutic proteins that are isolated from human plasma as a paradigm for assessing the pathogen safety of biological products.

B. TSEs: Nonconventional Pathogens and the Safety of Human-Plasma-Derived Therapeutics

Whether they are derived from plasma, recombinant, or transgenic sources, one characteristic all biological therapeutics have in common is the potential to be contaminated with pathogens. In most cases, potential contamination is associated with pathogenic agents typically referred to as conventional pathogens, such as viruses or bacteria. However, nonconventional pathogens, such as those associated with the transmissible spongiform encephalopathies (TSEs), must be considered as well. The TSE agents are referred to as nonconventional pathogens because their physical characteristics and infectivity properties differ significantly from those of conventional pathogens (see below).

TSEs are a class of mammalian neurodegenerative diseases that include scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, and classic or sporadic Creutzfeldt-Jakob disease (sCJD) in humans. In addition, recent events in Europe have raised concerns that BSE has crossed into the human population resulting in a new form of human CJD, commonly referred to as variant CJD (vCJD). As a group, TSEs are characterized by progressive dementia, ataxia, amyloid plaque formation, and spongiform degeneration in the central nervous system (CNS) (4–7). The highest infectivity titers are typically found in brain and other CNS tissues. However, low levels of infectivity have been detected in skeletal muscle (8), spleen (9,10), lymph nodes (11,12), blood buffy coats (13), and plasma (14,15). Recent experimental evidence demonstrated that low levels of TSE infectivity can be present in blood or blood fractions derived from experimental rodent and sheep models (14, 16,17). Although there is no epidemiological evidence for any TSE transmissions through biological products (18–22), manufacturers of plasma-derived and other biological products have been proactive in researching and assessing the potential risk of TSE transmission.

Every member of the TSE family has at least one feature in common, the presence of a conformational isoform of the prion protein that is associated with the disease state. In the disease state, the normal cellular prion protein, PrP^{c} (see Table 1) undergoes a conformational change that results in the alteration of its secondary and tertiary structures. These structural alterations result in a biologically distinct form of the prion protein commonly referred to as PrP^{Sc} in animals infected with scrapie, or PrP^{CJD} in humans infected with CJD (Table 1). Although they are chemically identical, $PrP^{Sc/CJD}$ and PrP^{c} are physically distinct protein isoforms. $PrP^{Sc/CJD}$ is less soluble in aqueous buffers (23,24) and more resistant to proteinases (25) than its PrP^{c} counterpart. $PrP^{Sc/CJD}$ tends to spontaneously aggregate in aqueous solutions and it is the major component of the CNS amyloid plaques associated with TSE diseases (26). The presence of $PrP^{Sc/CJD}$ is closely associated with TSE infectivity in infected animals and humans. In fact, there is a close correlation between the presence of $PrP^{Sc/CJD}$ and TSE infectivity (25,27–29). The relationship between $PrP^{Sc/CJD}$ and TSE infectivity has led to the "protein-only" hypothesis of TSE infectivity, which maintains that this form of the prion protein is an essential component of the TSE infectious agent (30). Researchers have exploited this relationship by using the in vitro detection of PrP^{Sc} as an indicator for the presence of TSE infectivity (31,32).

Table 1 Abbreviations Used to Define Different Forms of the Prion Protein

Abbreviation	Definition	Comments
PrP	Prion protein (proteinaceous infectious particle)	Term coined by S. Prusiner (30)
PrP^{c}	Cellular PrP (~33–35 kDa)	Nonpathogenic, normal, ubiquitous protein of unknown function; proteinase-sensitive
PrP^{Sc}	Scrapie form of PrP (~33–35 kDa)	Pathogenic protein; natural in sheep or experimentally adapted to rodents
PrP^{RES}	Proteinase-resistant form of PrP^{Sc} (~27–30 kDa)	Distinguishes PrP^{Sc} from PrP^{c}
PrP^{vCJD}	Variant Creutzfeldt-Jakob disease form of PrP	Human pathogenic form; may be associated with BSE
PrP^{sCJD}	Sporadic Creutzfeldt-Jakob disease form of PrP	Human pathogenic form; spontaneous conversion
PrP^{GSS}	Gerstmann-Sträussler-Scheinker form of PrP	Human pathogenic form; familial

The general approaches (screening, inactivation, and clearance) used to increase safety relative to conventional pathogens could potentially be applied to all pathogens; however, not all of these methods are effective or practical with respect to TSEs. Currently no reliable TSE test is available that is sensitive and practical enough to use as a screening assay for TSE detection in raw materials such as blood and plasma donations. However, at the level of donor screening for CJD, there are recommended guidelines for donation deferrals based on donor-supplied information such as family medical history and time spent in specific countries with high incidence rates of BSE. There are also guidelines that address the sourcing of raw materials used in the processing of biological products. These "risk" assessments typically take into account the animal species, the tissue type, and the country from which the raw material originated. The goal of this approach is to minimize the use of animal-derived raw materials from sources that have a higher likelihood of harboring TSE infectivity. Currently, several assays have the potential to be adapted to screen some raw materials for the presence of the pathogenic form of PrP. At this time, however, the use of screening has not become standard practice in the biological products industry owing to the lack of sufficiently sensitive, robust, and rapid assays.

With regard to pathogen inactivation methods, TSE agents are significantly more resistant to the classic technologies that are effective for conventional pathogens such as viruses. For example, certain viruses are susceptible to pasteurization (33), dry heat (34), ultraviolet exposure (35), and solvent-detergent exposure (36–38), none of which have been demonstrated to be effective at inactivating TSE agents (39,40). Inactivation technologies that target nucleic acids are particularly ineffective, which has led most scientists to the conclusion that TSE agents do not contain an essential nucleic acid component. Methods such as treatment with strong base or autoclaving can significantly decrease the titer of TSE agents, but they are detrimental to the function of the pharmaceutical agents of a proteinaceous nature, i.e., biological products. Therefore, manufacturers of biological products currently rely on clearance techniques to assess and reduce the theoretical potential for TSE disease transmission.

Clearance studies are used to evaluate the capacity of manufacturing processes such as plasma protein purification processes to partition TSE infectivity and to estimate the degree to which processing can remove infectivity from the product stream. In general, a scaled-down process step is challenged by the exogenous addition of a known amount of the pathogen of interest, and then the pathogen is tracked to determine whether it partitions into a waste or a product stream. Currently, clearance studies are the most reliable methods for ensuring the pathogen safety of biologicals with respect to TSEs since screening and inactivation methods are either not

available or not effective. To perform meaningful TSE clearance studies, several elements must be thoroughly considered: scaling down of the manufacturing step, characterization of the agent to be used as the spiking material (i.e., source of TSE infectivity), and the type of assay used to track the infectious agent.

II. COMPONENTS OF A TSE CLEARANCE STUDY

Clearance studies are designed to predict the potential for partitioning of a given pathogen in a selected manufacturing process and for providing an estimate of the relative removal of the pathogen with respect to the protein product of interest. The process step of interest may be an integral part of the manufacturing process, or it may be a step that is being designed specifically for the removal of pathogens.

Clearance studies are comprised of two major components: the process scale-down studies and the TSE partitioning studies (Fig. 1). Scaling down the manufacturing process is necessary because performing clearance studies with infectious agents at the manufacturing site or at the manufacturing scale cannot be done owing to safety reasons and general practicality. In the scale-down studies, an accurate model of the manufacturing process of interest is created at a small scale (bench scale) so it can be performed

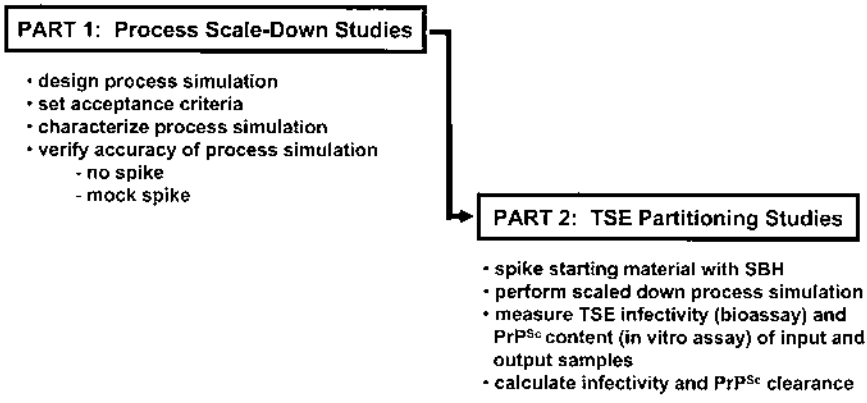

Figure 1 General components of a TSE clearance study. Clearance studies are comprised of two major components: process scale-down studies and partitioning studies. Typically, independent spiked process simulations are performed multiple times, and these replicates are averaged to estimate the amount of clearance for a given process step. SBH, scrapie brain homogenate.

routinely in laboratory setting. Next, the TSE partitioning studies are performed on the scaled-down process to measure pathogen clearance in the pathogen-spiked laboratory simulation.

A. Process Scale-Down Studies

To perform a TSE clearance study, the first set of experiments is designed to generate a bench-scale simulation of the manufacturing process of interest, and then characterize it to verify that it is comparable to the manufacturing scale (Fig. 1). The scale-down studies initially require the review of production documents associated with the manufacturing process, and then the effort shifts to performing the process in the laboratory and confirming the accuracy of the scale-down model. Scaling down an existing manufacturing step requires that a large-scale process, which may use materials weighing 100–1000 kg, be constrained to a bench-scale simulation measured in grams. Process parameters such as starting materials, buffers, temperature, pH adjustments, addition rates of reagents, incubation times, sample storage time and conditions, and other factors should be preserved whenever possible in the bench-scale model.

Although many process operations, such as temperature and pH adjustment or the addition of a reagent to a specified final concentration, can be scaled down with little difficulty, some process operations are size- and geometry-dependent. Such processes include mixing, centrifugation, filtration, and chromatography. When faced with size- and geometry-dependent processes, a best approximation of the manufacturing scale is selected and optimized. For example, during the manufacture of plasma-derived therapeutics, low-viscosity fluids are often mixed using an external propeller or paddles. However, mixing fluids at the bench scale may be performed using a magnetic stir bar and a stir plate instead. As long as the small-scale solution is adequately mixed and it meets established acceptance criteria (discussed below), it is assumed that the accuracy of the scale-down is not significantly affected by these differences.

Centrifugation at the production scale is another example of a geometry-dependent process that may be a challenge to scale down. Typically, production centrifugation utilizes a flow-through configuration. However, in the laboratory, centrifugation is usually performed in a standard centrifuge that has a "dead-end" configuration. A careful and systematic investigation of parameters such as g-force and centrifugation time is performed to ensure an accurate reproduction of the centrifugation forces used in manufacturing. Other size- and geometry-dependent operations include filtration and chromatographic separations. Filtration is scaled by the volume of the process solution relative to the filter surface area. For chromatographic scale-downs,

the two parameters that should remain invariant are the bed height and the linear flow rate. However, because the resin volume has changed, sample loads and volumes will be different. For a more comprehensive review of how to scale down chromatography processes, refer to the *Handbook of Process Chromatography* (41).

For experimental investigations of the bench-scale model to be valid, acceptance criteria for key operating parameters that are based on the documented operating characteristics of the large-scale manufacturing process should be established. These criteria are used to confirm the accuracy of a scaled-down process simulation. Characterization of the scale-down may include examining parameters such as the amount or activity of selected target and/or contaminating proteins, measuring pH, or mass recovery. A variety of analytical techniques, such as turbidity and conductivity measurements, simple UV/VIS measurements (e.g., A_{280}), SDS-PAGE, and immunonephelometry, may be used to evaluate the performance of the bench-scale model. If properly designed and executed, the bench-scale model should yield characterization data that meet the acceptance criteria and represent an accurate model of the manufacturing process.

The initial characterization of the bench-scale model is done by comparing the manufacturing data to the scaled-down process, which is performed using starting material that does not contain a pathogen spike. Once the researchers are confident that the simulation is accurate, the bench-scale process is challenged with a mock spike and the characterization process is repeated. Typically, the mock spike is a vehicle control consisting of the milieu in which the pathogen resides. For TSE clearance studies performed at Bayer Corporation, the mock spike is normal brain homogenate from uninfected hamsters. Using this method, most discrepancies in the scale-down that may be due to the spiking material can be distinguished from the process of scaling down.

B. TSE Partitioning Studies

Once the scale-down meets the predetermined characterization acceptance criteria, the effort shifts to performing the partitioning studies (Fig. 1). For these experiments, the starting material is spiked with a known amount of pathogen. The process is performed using the parameters established in the scale-down studies, and the input sample and resulting fractions are measured for the presence of the pathogen. Typically, pathogen clearance is assessed relative to the fraction that contains the therapeutic protein product of interest. Therefore, clearance would be defined as the difference in the amount of pathogen detected in the input sample and the product-containing fraction.

III. WHAT TO MEASURE IN A TSE CLEARANCE STUDY

A. TSE Infectivity: In Vivo Assays as Direct Measurements of Infectivity

Traditionally, TSE infectivity has been tracked through process simulations by performing animal bioassays on the input material and the resulting fractionation samples. The bioassay is a direct measure of infectivity, and based on an end-point dilution and the inoculum volume, the titer can be estimated and expressed as an ID_{50}/mL (42,43). In theory, the bioassay has the ability to detect 1.0 infectious unit per milliliter (IU/mL); however, the definition of what constitutes a single infectious unit for TSEs remains undefined.

TSE bioassays are typically performed in rodents (e.g., mice, hamsters, and guinea pigs) owing to (1) the relative short incubation times (8–16 months) compared to larger mammals (several years), (2) the availability of large numbers of animals, and (3) the fact that the rodent models are well characterized. Performing bioassays for TSEs is a greater challenge than for conventional pathogens since bacteria and viruses require much shorter incubation times (days to weeks) and they can be propagated in vitro. For a typical TSE clearance study, end-point titration bioassays are performed on the spiked input material and the output samples from each process step. In general, the processed samples are serially diluted in log increments, and aliquots of each dilution are intracranially injected into anesthetized weanlings. Animals are observed on a regular basis for clinical signs of disease. If any animal is believed to have succumbed to the disease, a necropsy may be performed to confirm the presence of spongiotic changes and amyloid plaques in the animal's CNS. Infectivity titers, or the measure of the dose that causes infection in 50% of the test animals (ID_{50}) for a given sample, are calculated using either the Reed-Muench (44) or Spearman-Kärber method (45). To calculate a clearance value, the number of logs of infectivity detected in the input and output samples are normalized to take into account any differences in sample volumes. Next, the number of logs of infectivity detected in the product-containing fraction is subtracted from the number of logs detected in the input sample, resulting in a log clearance value.

Until recently, TSE detection relied solely on the animal bioassay. Although bioassays are sensitive and are a direct measure of infectivity, they require significant resources such as specialized facilities and personnel. Furthermore, they can take more than a year to complete. This can limit the number of biological processes that can be readily assessed for TSE clearance and the number of replicates that can be performed, thus reducing statistical confidence in the data.

B. TSE Markers: In Vitro Assays as Indirect Measurements of Infectivity

An alternative to using bioassays to track infectivity is to use an in vitro assay to measure a surrogate marker for infectivity. Consequently, many researchers have endeavored to identify a reliable molecule for tracking TSE infectivity, and to develop appropriate in vitro analytical assays for such markers. In general, in vitro assays are more rapid, have higher throughput, and are more economical than bioassays. Regardless of the type of analytical assay used for clearance studies, it must have the ability to detect relative titer differences between samples.

While several candidates have been proposed as clinical markers for TSEs, by far the most widely accepted marker for TSE infectivity is the pathogenic form of the prion protein, PrP^{Sc}. As discussed earlier, PrP^{Sc} is closely associated with TSEs and is required for TSE infectivity (25,46,47). Several research groups have successfully demonstrated a relationship between PrP^{Sc} concentration and TSE infectivity titer. Experiments using protein separation methods such as isoelectric focusing (28), density gradients (27,48), size exclusion chromatography (49), and purification of cellular fractions (29) showed that TSE infectivity copurifies with PrP^{Sc}. Additional experiments demonstrated a relationship between the degradation of PrP^{Sc} and a reduction in TSE infectivity (25). Infectivity studies using PrP gene knockout animals demonstrated a clear requirement for PrP expression for disease transmission and propagation (47,50). Finally, research from Bayer Corporation's laboratory has demonstrated a direct relationship between the partitioning of PrP^{Sc} and TSE infectivity in clearance studies used to assess plasma and biotechnology manufacturing processes (51,52).

There are numerous in vitro assay formats that can be used to detect PrP^{Sc}, including the Western blot assay, ELISA, DELFIA, capillary electrophoresis, and multispectral UV fluorescence (reviewed in Ref. 53). Regardless of the in vitro assay format used, at least three issues with respect to PrP^{Sc} must be considered. First, the assay must be able to distinguish between PrP^{Sc} and its normal, nonpathogenic, cellular counterpart, PrP^{C}. Second, matrix effects that might interfere with the assay's ability to detect PrP^{Sc} must be minimized. Third, the assay must be sensitive enough to detect low titers of PrP^{Sc}.

C. Bayer Corporation's In Vitro Assay for PrP^{Sc}

To facilitate the quantitative detection of TSE partitioning in plasma fractionation processes, Bayer's Pathogen Safety and Research group has

developed a PrP^{Sc}-specific Western blot assay that is based on end-point dilution quantitation (51). Once a rudimentary PrP^{Sc}-specific Western blot assay was designed, an in-depth development phase ensued to systematically optimize assay parameters including sample preparation, reagent specifications, and incubation times and temperatures.

For the purposes of this discussion, sample preparation as performed in Bayer's laboratory can be divided into two stages: (1) treatment with proteinase K and (2) centrifugation. First, aliquots of the undiluted samples are treated with proteinase K to eliminate PrP^{C} and other contaminating proteins that may interfere with the detection of PrP^{Sc}. This is necessary because PrP^{C} and PrP^{Sc} have identical molecular weights (33–35 kDa) and currently no antibodies are available that can distinguish between these two forms of the protein. Treatment with proteinase K is a standard method for eliminating PrP^{C}, which is digested by this enzyme, and for converting PrP^{Sc} to PrP^{RES} (Fig. 2). Proteinase K removes 60–90 amino acids from the N-terminus of PrP^{Sc}, generating PrP^{RES} (27–30 kDa), which has a molecular weight slightly lower than PrP^{Sc} and PrP^{C} (25). To identify optimal digestion conditions that produced PrP^{RES} without overdigesting or underdigesting the samples, a number of parameters were tested, including enzyme concentration (0–500 μg/mL), incubation time (5–120 min), and incubation temperature (21–37°C).

After treatment with proteinase K, the second stage of sample preparation is a centrifugation step. Since PrP^{RES} is insoluble and readily sedimentable, the samples are centrifuged to concentrate PrP^{RES} in the pellet. The

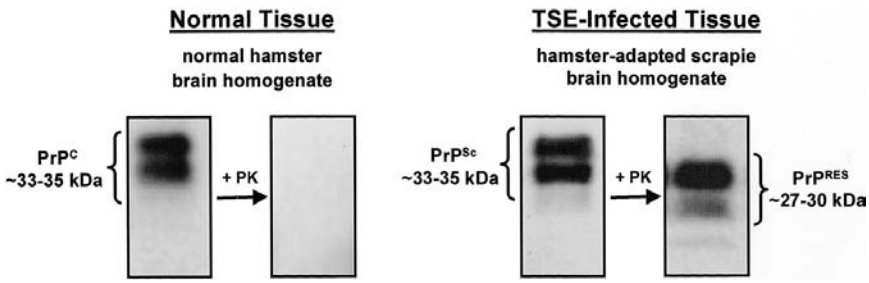

Figure 2 Differentiating between PrP^{c}, PrP^{Sc}, and PrP^{RES} using proteinase K and the Western blot assay. PrP^{c} and PrP^{Sc} have identical molecular weights and are indistinguishable in Western blots unless samples are pretreated with proteinase K. Proteinase K eliminates PrP^{c} and converts PrP^{Sc} to its protease-resistant core referred to as PrP^{RES}. The multiple bands observed are due to differences in the prion protein's glycosylation states. PK, proteinase K.

supernatants are discarded and the pellets are resuspended in sample buffer for Western blot analysis. Besides increasing the detection limit by concentrating PrP^{RES} in the pellet, centrifugation provides an additional purification step for PrP^{RES} by removing nonspecific proteins that may interfere with the assay. After heating, each sample is serially diluted in 0.5-log increments in sample buffer and loaded on 12% SDS-PAGE gels. Western blot assays are performed using the PrP-specific monoclonal antibody 3F4, a goat antimouse alkaline phosphatase conjugate, and the chemiluminescent substrate 1,2-dioxetane. The detection end-point is defined as the last sample in the serial titration that exhibits a PrP^{RES}-specific signal (Fig. 3).

A major advantage of the Western blot format over other in vitro formats is the electrophoretic separation step that precedes immunological detection. The Western blot image for PrP^{RES} demonstrates a distinct "fingerprint" of three protein bands of molecular weights ranging from 27 kDa to 30 kDa (Fig. 3). This characteristic triplet is due to the protein's multiple glycosylation states. In addition to this protein fingerprint, the Western blot takes advantage of both antibody specificity and the proteinase K resistance of $PrP^{Sc/RES}$ to ensure specificity. To further demonstrate signal specificity, a 3F4-specific competitive peptide is routinely used to confirm the identities of the protein bands (51). The Western blot therefore provides multiple independent indications for confirming the presence (or absence) of $PrP^{Sc/CJD}$.

Currently, Bayer's refined PrP-specific Western blot assay can detect PrP^{RES} in <10 ng of hamster brain tissue, which is equivalent to ≤ 5 pg of recombinant PrP. This level of PrP^{RES} detection corresponds to an infectivity titer of $\leq 10^2$ IU/mL, as determined using the rodent bioassay. PrP^{RES}

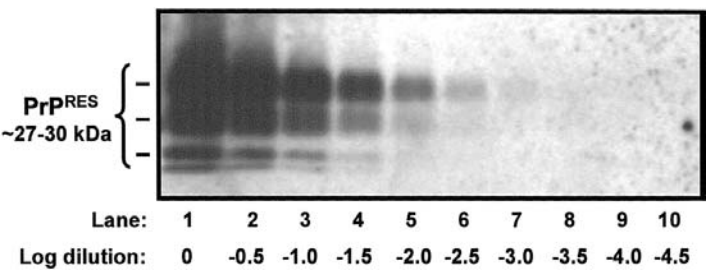

Figure 3 Western blot assay demonstrating end-point dilution of PrP^{RES}. The PrP^{RES} detection end-point is defined as the last sample in the half-log titration series that contains a visible PrP^{RES} signal. In this example, the starting material (lane 1) contains 0.01% crude hamster SBH and the last visible signal is in lane 7, which corresponds to a dilution of -3.0 logs.

detection using serial titrations and Western blot assays is reproducible and robust, and the average difference observed for the limit of detection between the bioassay and Bayer's Western blot assay is ~1.5 logs. This Western blot assay is at least as sensitive as other assays described in the literature, including dot blot (31), various ELISA systems (32,54,55), capillary electrophoresis (56,57), and SIFT (58,59), as well as other Western blot systems (60,61). Other groups have reported Western blot detection systems for detecting PrP in brain homogenates ranging from 2.5-μg brain equivalents (61) to 0.3-mg brain equivalents (60). Western blot systems using spleen as the starting material demonstrated detection of $PrP^{Sc/RES}$ in as little as 0.3 mg material following treatment with collagenase (62). Finally, Safar and co-workers (54) describe a conformation-dependent ELISA reportedly able to distinguish between PrP conformers from various scrapie strains with the capacity to detect <1 ng/mL PrP^{Sc}.

Bayer's Western blot assay is robust, reproducible, and quantitative over a 6.0-log dynamic range, and can be used to determine the relative differences in PrP^{Sc} content between samples. Although in vitro assays for $PrP^{Sc/RES}$ do not measure TSE infectivity directly, such in vitro assays have been used to qualitatively estimate infectivity. It should be noted that while PrP^{Sc} has been shown to be reliable for predicting the partitioning of TSE infectivity, in some cases where TSE infectivity is low, PrP^{Sc} could not be detected owing to the sensitivity limits of the assay. Consequently, the lack of PrP^{Sc} detection does not necessarily indicate the absence of infectivity. This is expected since in vitro assays cannot detect PrP^{Sc} in the more dilute samples where the bioassay can still detect infectivity. Nevertheless, in the context of protein purification from the biological resources, the Western blot assay provides a reliable means by which the magnitude of PrP^{Sc} partitioning can be used to estimate infectivity partitioning. Bayer has used this assay to predict the partitioning of TSEs by measuring the removal of PrP^{Sc} in plasma processing simulations (51,52). In addition, Bayer's assay has been validated independently and has been made commercially available through BioReliance Corporation.

IV. SPIKING MATERIAL FOR TSE CLEARANCE STUDIES

A. Source of the Spiking Material

When selecting a source of TSE-infected material to use as spiking agent, at least three criteria must be considered. First, the material should have sufficient titer so that it can be readily monitored using either in vitro assays or bioassays. Second, it should be well characterized with respect to infectivity titer and/or PrP^{Sc} content. Characterization of the spiking material

will simplify subsequent data analysis and prevent the production of ambiguous results. Third, the source of the spiking agent should be readily available or relatively easy to produce, since limited availability of the spiking material could preclude performing enough experiments to obtain statistical relevance.

In theory, infected human plasma would be the most appropriate spiking source for analyzing TSE clearance for human plasma protein purification processes, but this is not feasible. Although there is evidence in experimental animal models (rodents and sheep) that TSE infectivity can be present in plasma and other blood fractions (14,16,17), TSE infectivity has not been demonstrated in human plasma or blood. Plasma from infected rodents could be used, but the TSE titers are too low to monitor using current in vitro assays for TSE markers such as PrP^{Sc}, and the amounts of infectivity are too low to be measured reliably. Since brain tissue from rodents experimentally infected with scrapie typically has high TSE infectivity and PrP^{Sc} titers, rodent brain homogenates have been routinely used as the spiking source for process clearance studies. Because of these high levels of PrP^{Sc}, it is relatively straightforward to measure the output fractions from clearance studies using in vitro techniques.

Brain homogenates derived from experimental strains of rodent-adapted scrapie are also preferred for spiking studies because this source of TSE is well characterized. These experimental models have been studied extensively, and a significant amount of data is available regarding titers, incubation times, and disease characteristics to support their use. Rodents that have been adapted to experimental strains of TSEs typically exhibit clinical signs within relatively short incubation times (months) compared to rodents challenged with natural isolates of TSEs (years). For example, when using the hamster-adapted 263K strain of sheep scrapie, infectivity results can be obtained in 9–12 months for samples with low titers. In contrast, the first passage of human CJD to naive mice does not typically elicit clinical signs in these rodents and therefore provides little quantitative information regarding infectivity. For instance, when sCJD brain homogenate is injected intracranially into wild-type mice, they do not demonstrate clinical signs of CJD (63). Only after several more passages via intracranial inoculation does it become possible to observe clinical signs. In fact, after several passages in mice, the amount of infectivity would represent the titer of the adapted mouse agent and would not represent the original human inoculum. This difficulty in transmitting TSEs between different species of animals has been described in the literature as the "species barrier" (64).

A third rationale for using experimental strains of scrapie-adapted rodent brain tissues as the spiking material for TSE clearance studies is that

they are readily available and can be produced in relatively large quantities in the laboratory. These experimental strains have been used for years and researchers can readily stockpile tissues, thus producing enough material to maintain laboratory stocks for research. In comparison, the occurrence of human TSE diseases is a relatively infrequent event and therefore, the infected tissues are not readily available.

B. Preparation of the Spiking Material

TSE infectious particles are likely to be heterogeneous with respect to characteristics such as size and charge distribution (27,28,30). Therefore, the method used to generate the PrP^{Sc} spiking material that will be used in clearance studies must be considered, because different preparation methods could influence the PrP^{Sc} size and charge distribution. This could result in a PrP^{Sc} spike with a population distribution that does not necessarily reflect its original characteristics. The common brain PrP^{Sc} preparations used in TSE clearance studies include crude homogenates, microsomal fractions, and scrapie associated fibrils (SAFs). In addition, clarified homogenates, cellular fractions, and caveolae-like domains (CLDs) can be used (Table 2). Each of these preparations has its advantages and disadvantages with regard to its method of preparation, as well as its relative value as a spiking material in clearance studies.

Crude brain homogenates are simple to produce and, because they require a minimal amount of manipulation, they are consistent and reliable (Table 2). In general, brain tissue is thoroughly dispersed in a buffer using mechanical homogenization (51). In a crude homogenate, the prions are more likely to remain in their native state, containing the original populations of PrP^{Sc} and maintaining PrP^{Sc} and infectivity titers. However, all other brain components are still present in this preparation; therefore, it has the potential to affect the performance of a bench-scale, plasma-processing step. A clarified version of the crude homogenate can be prepared by removing cellular debris after homogenization. The major disadvantages of using clarified homogenates are that clarification may remove some populations of prions and the PrP^{Sc} and/or TSE infectivity titers are reduced.

To generate a microsomal preparation of PrP^{Sc}, the infected brain material is homogenized in buffer and then subjected to differential centrifugation (65,66). The resulting fraction contains the PrP^{Sc} associated with the cellular lipid fraction, the population hypothesized to reflect the protein's physiological state in the blood plasma fraction. As described for clarified preparations, the microsomal fraction may be lacking relevant populations of PrP^{Sc} and some infectivity may be lost (Table 2). A more

Table 2 Comparison of Different Preparations of TSE Spiking Materials

Spike preparation	Description	Advantages	Disadvantages	Ref.
Crude brain homogenate	Brain tissue is homogenized (dounce and/or mechanical homogenizer) in a simple buffer solution	Minimal sample manipulation. Simple and rapid preparation. Contains heterogeneous population of prions. Prions are associated with the cellular lipid components (similar to physiological conditions).	May contain large aggregates insoluble material. Complex sample, which has the potential to affect performance of the scaled-down process simulation.	51
Clarified brain homogenate	Crude brain homogenate is subjected to low-speed centrifugation to remove cellular debris.	Minimal sample manipulation. Simple and rapid preparation. No large aggregates of insoluble materials.	Centrifugation may remove populations of prions (e.g., large infectious particles). Prion titers are reduced.	
Microsomal preparation	Crude brain homogenate is subjected to differential separation methods to collect the prions associated with the cellular lipid fraction.	Partially purified fraction of prions. Prions are associated with the cellular lipid components (similar to physiological conditions). Prions can be concentrated.	Significant amount of sample manipulation. Method may remove populations of prions (e.g., large infectious particles).	77
Scrapie-associated fibrils (SAF)	PrP^{RES} is purified from brain homogenate that has been treated with proteinase and is detergent-extracted.	Highly purified fraction of prions. Prions can be concentrated	Significant amount of sample manipulation. Method may alter the physiological nature of the prion.	67
Isolated cells	Minced brain tissue is treated with proteinases and the cellular fraction is isolated.	Prions are associated with the cellular lipid components (similar to physiological conditions).	Intact cells are not likely to exist in plasma.	14
Caveolae-like domains (CLDs)	Crude brain homogenate is treated with detergent and fractionated on a sucrose gradient to collect the CLDs.	Partly purified fraction of prions. Prions can be concentrated. Prions are associated with the cellular lipid components (similar to physiological conditions).	Significant amount of sample manipulation. Method may remove populations of prions (e.g., non-membrane-bound infectious particles).	68

highly purified spiking material is the SAF preparation, which is processed using a combination of differential centrifugation and detergent extractions and includes protease and nuclease digestions (67). SAF preparations have the potential to yield a highly concentrated and purified form of PrP^{Sc} that retains a significant amount of infectivity. However, because the lipids have been removed, the physicochemical properties of native PrP^{Sc} are not maintained. The isolation of intact cells from infected brain tissue (14) and the isolation of CLDs (68) produce two other distinct preparations containing PrP^{Sc}.

In the majority of the published clearance studies, researchers have used either crude brain homogenates or microsomal preparations as their spiking materials. For Bayer's standard plasma process clearance studies, crude brain homogenate prepared from hamsters infected with the 263K strain of scrapie is used. Some researchers use more purified preparations such as microsomal fractions because the PrP^{Sc} has been concentrated and some of the brain tissue impurities have been removed. Regardless of which material is used, care must be taken to ensure that the spiking preparation does not significantly interfere with the normal performance of the scaled-down process simulation. Typically, this is controlled by performing mock clearance studies in which the process simulation is spiked with a sample of normal (i.e., not infected) brain homogenate that has been prepared in the same manner as the infected spiking material. Process characterization tests are performed to determine whether this mock spike affects the performance of the scaled-down process. If the mock spike interferes with the process, then a lower concentration of spiking material may be necessary or the process cannot be assessed for TSE removal.

V. CLEARANCE RESULTS FOR BIOLOGICAL PRODUCTS

A. Bayer's Plasma Fractionation Clearance Studies

The objectives of Bayer's clearance studies were to determine how PrP^{Sc} and TSE infectivity partition during the course of isolation of therapeutic proteins from human plasma and to demonstrate the utility of PrP^{Sc} as a marker for tracking TSE infectivity. Both animal bioassays and a PrP-specific Western blot assay were used to assess plasma fractionation processes for their ability to remove TSE infectivity and PrP^{Sc} from product streams. For each process analyzed, a minimum of three independent fractionations was performed, and each sample set was assessed at least twice using the Western blot assay. Currently seven different plasma fractionation processes have been examined (52). Each plasma intermediate

used as the starting material for a specific process was spiked with crude hamster scrapie brain homogenate containing a known amount of infectivity and PrP^{Sc}, and each fractionation step was performed independently in the scaled-down format. Table 3 includes data from Bayer's plasma fractionation clearance studies using hamster scrapie as the spiking material.

The first set of Bayer plasma protein fractionation processes examined comprises the "Cohn trunk" of the human plasma protein purification process. These are the basic processes from which Bayer plasma proteins are derived. The Cohn scheme of protein purification is comprised of several processing steps that involve various combinations of the following methods to facilitate the precipitation of different groups of proteins: (1) addition of alcohol, (2) temperature adjustments, and (3) pH adjustments.

As the first step, cryoprecipitation is separate from the Cohn scheme (Fig. 4). At this step, frozen pooled plasma is thawed and the precipitated material is separated from the supernatant (effluent). The resulting effluent is the starting solution for the next process in the Cohn trunk, the fraction I separation. The effluent produced from the fraction I separation is the starting material for the fraction II + III separation, and so on through the fraction IV-1 and fraction IV-4 separations (Fig. 4). Conventionally, clearance is defined as the difference between the PrP^{Sc} or infectivity detected in the prove sample and the product-containing fraction, which is usually the effluent. In these initial studies, the levels of PrP^{Sc}/infectivity clearance were defined as nominal (<2.0 logs), intermediate (2.0–4.0 logs), or substantial (>4.0 logs). Cryoprecipitation demonstrated nominal clearance for both PrP^{Sc} and infectivity with respect to the effluent. Fraction I also showed nominal clearance for PrP^{Sc}; however, infectivity clearance was not measured. The fraction II + III and fraction IV-4 separations each demonstrated substantial clearance. According to the classification scheme described above, the fraction IV-1 separation demonstrated substantial clearance for PrP^{Sc} and intermediate clearance for infectivity.

A second set of plasma processes was also examined. These purification steps are product-specific and branch off of the primary Cohn purification scheme (Fig. 4). For the purification of factor VIII a 3% polyethylene glycol (PEG) precipitation is performed where initial cryoprecipitate paste is resuspended and incubated with PEG. The resulting precipitate is discarded and the effluent is further processed to factor VIII. TSE partitioning studies for this step demonstrated an intermediate clearance of PrP^{Sc} and TSE infectivity with respect to factor VIII. For the fraction III separation, which is the final purification step for Bayer's immunoglobulin product, the paste from the fraction II + III separation is resuspended and subjected to an

Table 3 Survey of PrP^{Sc} and TSE Infectivity Clearance Result from Plasma-Processing Studies

Process step	Assay method	Animal strain	Inoculum	Reduction factor (log)	Therapeutic proteins	Ref.
Precipitation						
Cryoprecipitation	WB	Hamster 263K	Microsomal fraction	< 1.0	lg, alb, thr, FII, FIX, FX	66
Cryoprecipitation	WB	Hamster 263K	Brain homogenate	1.0	lg, α_1-PI, AT, alb	52
Cryoprecipitation	Bioassay	Hamster 263K	Brain homogenate	1.0	lg, α_1-PI, AT, alb	52
Cryoprecipitation	Bioassay	Hamster 263K	Intact brain Cells	2.0	lg, α_1-PI, AT, alb	14
Cryoprecipitation	CDI	Hamster 263K	Brain homogenate	< 1.0		69
Cryoprecipitation	CDI	Hamster 263K	Microsomal fraction	< 1.0		69
Cryoprecipitation	CDI	Hamster 263K	Caveolae-like domains	< 1.0		69
Cryoprecipitation	CDI	Hamster 263K	Purified PrP^{Sc}	2.4		69
Fraction I + II + III precipitation	WB	Hamster 263K	Microsomal fraction	1.3	alb	66
Fraction I + III precipitation	WB	Hamster 263K	Microsomal fraction	≥3.7	lg	66
Fraction I separation	WB	Hamster 263K	Brain homogenate	1.1	lg, α_1-PI, AT, alb	51
Fraction II + III separation	WB	Hamster 263K	Brain homogenate	≥4.7	α_1-PI, AT, alb	52
Fraction II + III separation	Bioassay	Hamster 263K	Brain homogenate	6.0	α_1-PI, AT, alb	52
Fraction III separation	WB	Hamster 263K	Brain homogenate	≥4.3	lg	52
Fraction III separation	Bioassay	Hamster 263K	Brain homogenate	5.3	lg	52
Fraction IV precipitation	WB	Hamster 263K	Microsomal fraction	≥3.0	alb	66
Fraction IV-1 Separation	WB	Hamster 263K	Brain homogenate	≥4.2	alb	52
Fraction IV-1 separation	Bioassay	Hamster 263K	Brain homogenate	3.7	alb	52
Fraction IV-4 separation	WB	Hamster 263K	Brain homogenate	≥4.1	alb	52
Fraction IV-4 separation	Bioassay	Hamster 263K	Brain homogenate	7.2	alb	52
Cryoprecipitate 3% PEG separation	WB	Hamster 263K	Brain homogenate	3.0	FVIII	52
Cryoprecipitate 3% PEG separation	Bioassay	Hamster 263K	Brain homogenate	2.2	FVIII	52
Fraction IV-1 paste 11.5% PEG separation	WB	Hamster 263K	Brain homogenate	≥4.9	α_1-PI	52

Table 3 (continued)

Process step	Assay method	Animal strain	Inoculum	Reduction factor (log)	Therapeutic proteins	Ref.
Fraction IV-1 paste 11.5% PEG separation	Bioassay	Hamster 263K	Brain homogenate	≥5.4	α_1-PI	52
9% ethanol precipitation (ppt I)	CDI	Hamster 263K	Brain homogenate	< 1.0		69
9% ethanol precipitation (ppt I)	CDI	Hamster 263K	Microsomal fraction	< 1.0		69
9% ethanol precipitation (ppt I)	CDI	Hamster 263K	Caveolae-like domains	< 1.0		69
9% ethanol precipitation (ppt I)	CDI	Hamster 263K	Purified PrP^{Sc}	3.1		69
25% ethanol precipitation (ppt II + III)	CDI	Hamster 263K	Brain homogenate	3.6		69
25% ethanol precipitation (ppt II + III)	CDI	Hamster 263K	Microsomal fraction	3.0		69
25% ethanol precipitation (ppt II + III)	CDI	Hamster 263K	Caveolae-like domains	3.1		69
25% ethanol precipitation (ppt II + III)	CDI	Hamster 263K	Purified PrP^{Sc}	4.0		69
38% ethanol precipitation (ppt IV)	CDI	Hamster 263K	Brain homogenate	≥4.1		69
38% ethanol precipitation (ppt IV)	CDI	Hamster 263K	Microsomal fraction	≥4.5		69
38% ethanol precipitation (ppt IV)	CDI	Hamster 263K	Caveolae-like domains	≥4.1		69
38% ethanol precipitation (ppt IV)	CDI	Hamster 263K	Purified PrP^{Sc}	≥4.6		69
Zinc precipitation + Al $(OH)_3$ adsorption	WB	Hamster 263K	Microsomal fraction	1.0	FVIII, fbrg	66
Filtration						
Fraction V depth filtration (Seitz KS80)	WB	Hamster 263K	Microsomal fraction	≥4.9	alb	66
Fraction V depth filtration (CUNO)		Hamster 263K	Microsomal fraction	2.3	alb	66
Membrane filtration (0.22 μm)	WB	Hamster 263K	Microsomal fraction	< 1.0	alb[a]	66
Fraction II depth filtration (Seitz 200)	WB	Hamster 263K	Microsomal fraction	≥2.8	lg	66

(*continued on next page*)

Table 3 (continued)

Process step	Assay method	Animal strain	Inoculum	Reduction factor (log)	Therapeutic proteins	Ref.
Membrane filtration (0.45 μm /0.22 μm)	WB	Hamster 263K	Microsomal fraction	1.0	FVIII	66
Membrane filtration (NMW cut-off 10 kD)	Bioassay	Mouse ME7	Microsomal preparation in lysolecithin	≥4.6	aprotinin[b]	71
Ultrafiltration filter (35 nm)	Bioassay	Mouse ME7	Microsomal fraction	4.93	alb[c]	72
Ultrafiltration filter (15nm)	Bioassay	Mouse ME7	Microsomal fraction	> 5.87	alb[c]	72
Ultrafiltration filter (10 nm)	Bioassay	Mouse ME7	Microsomal fraction	> 3.80	alb[c]	72
Column chromatography						
DEAE Tyopearl 650 M chromatography	WB	Hamster 263K	Microsomal fraction	3.1/≥3.5	FVIII/fibrg	66
DEAE-Sepharose chromatography	WB	Hamster 263K	Microsomal fraction	3.0	FIX	66
Heparin-Sepharose chromatography	WB	Hamster 263K	Microsomal fraction	1.4	FIX	66
S-Sepharose chromatography	WB	Hamster 263K	Microsomal fraction	2.9	thr	66
Ion-exchange chromatography[d]	Bioassay	Mouse ME7	Microsomal preparation in lysolecithin	5.2	aprotinin[b]	71
Ion-exchange chromatography[d]	Bioassay	Mouse ME7	Microsomal preparation in lysolecithin	4.0	aprotinin[b]	71
DEAE-cellulose	WB	Hamster 263K	Microsomal fraction	1.7/3.0	lg, alb/ thr, FII, FVIII, FIX, FX	66
Sephacryl chromatography	Bioassay	Hamster 263K	Brain homogenate	4.3	hGH	78
Other						
Methanol extraction	Bioassay	Mouse ME7	Microsomal preparation in lysolecithin	≥4.4	aprotinin[b]	71

Abbreviations: α_1-PI = alpha-1 proteinase inhibitor; alb = albumin; AT = antithrombin III; CDI = conformation-dependent Immunoassay; fibrg = fibrinogen; FII = factor II; FVIII = factor VIII; FIX = factor IX; FX = factor X; hGH = human growth hormone; lg = immunoglobulin; PEG = polyethylene glycol; ppt = precipitate; thr = thrombin; WB = Western blot assay.

[a] Human alb present at 45 mg/ml.

[b] Protein derived from bovine lung.

[c] Human alb (20 mg/mL) used as model solution.

[d] Chromatography resin not reported.

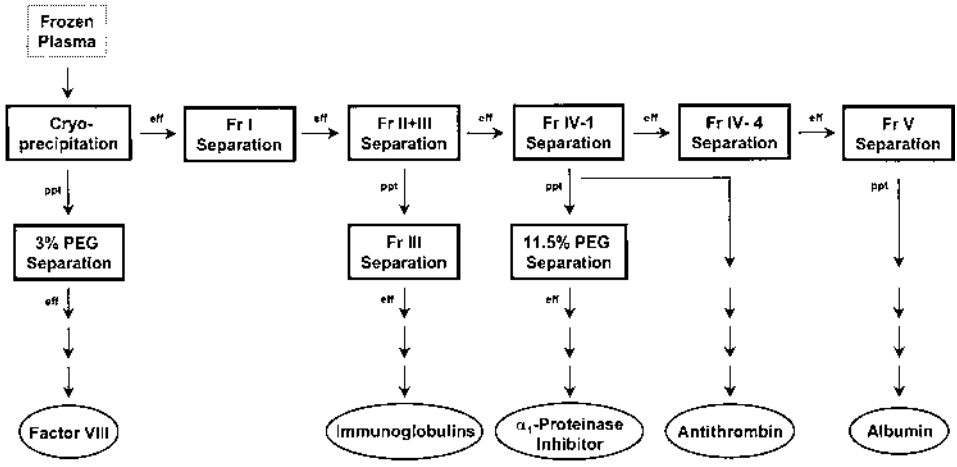

Figure 4 General plasma-processing steps used at Bayer Biological Products Division for the production of human therapeutic proteins. The solid boxes represent individual plasma-processing steps and the ovals represent the therapeutic protein targets. eff, effluent; Fr, fraction; PEG, polyethylene glycol; ppt, precipitate.

additional ethanol precipitation. The resulting precipitate is discarded and the effluent is processed to immunoglobulins. Substantial clearance of PrP^{Sc} and TSE infectivity was observed in partitioning studies with respect to immunoglobulins. Finally, to isolate α_1-proteinase inhibitor from plasma, the paste derived from the fraction IV-1 separation is resuspended and incubated with 11.5% PEG. The clearance studies for this PEG step demonstrated a substantial amount of clearance for both PrP^{Sc} and TSE infectivity. In several of the experiments described, $PrP^{Sc/RES}$ was not detected using the Western blot assay, although infectivity was detected using the bioassay. This was expected, since the in vitro Western blot assay is less sensitive than the in vivo bioassay, and cannot detect PrP in some low-titer samples.

Regardless of whether the clearance observed for a plasma protein purification step was nominal, intermediate, or substantial, there was always a direct relationship between the partitioning of infectivity (determined using animal bioassays) and PrP^{Sc} (determined using Western blot assays) (52). These results support the relationship between PrP^{Sc} and infectivity that had been previously established and demonstrate that PrP^{Sc} detection can be used to predict partitioning of TSE infectivity during plasma purification processes for the isolation of therapeutic proteins, as well as during processes for isolating other biological products.

B. Plasma Fractionation Clearance Studies from Other Groups

Three other research groups have published studies on prion partitioning for plasma fractionation using Cohn-Oncley processes (Table 3). The spiking materials used in all three studies were derived from hamster scrapie brains (263K), but they were prepared and spiked using different methods. In the Brown et al. study (14), a trypsinized cellular fraction was spiked into whole human blood. The plasma was collected from this sample and used as the starting material for partitioning studies. Using the animal bioassay, Brown and co-workers observed varying levels of TSE infectivity clearance for four plasma fractionation steps (Table 3). Partitioning of PrP^{Sc} was not measured in this study. Foster and co-workers used a microsomal preparation, and spiked a variety of plasma intermediates used in their Cohn-Oncley processes (66). They also examined clearance in some filtration and chromatography steps. Partitioning of PrP^{Sc} was measured using a Western blot assay; however, TSE infectivity was not measured (Table 3). Baron (69) compared four different preparations of spiking materials that contained PrP^{Sc} in its least purified form to a highly purified form. PrP^{Sc} partitioning was analyzed using a conformation-dependent immunoassay, but TSE infectivity partitioning was not evaluated.

Despite the differences in the fractionation processes and the spiking materials used in these studies, the trends for TSE infectivity and PrP^{Sc} partitioning were similar. In general, the data from all three groups indicate a progressive increase in clearance as the plasma fractionation processes proceed toward the isolation of human albumin (Table 3 and Figure 4). This trend correlates well with the data from Bayer's partitioning studies. There is also an indication that a greater amount of clearance is observed when a highly purified form of PrP^{Sc} is used as the spiking material (69). However, it seems that a less purified spiking material such as crude homogenate presents a greater challenge to the system and therefore may represent a worst-case scenario for plasma fractionation clearance studies.

C. Independent Plasma Fractionation Steps vs. a Series of Coupled Steps

The majority of TSE clearance studies have been performed on isolated plasma protein purification steps. However, plasma manufacturing processes are normally performed as a purification series where the fractionation product from one step is immediately processed through the subsequent processing steps. Simply summing clearance from individual steps could

potentially overestimate the total clearance value in relation to a specific protein product, by ignoring the possibility that there could be PrP^{Sc} populations that may be resistant to a particular partitioning mechanism. To address this question, clearance studies were performed in which a sample of pooled plasma was spiked with SBH and five consecutive steps of the plasma fractionation process were performed in a series. In this experimental format, each step was coupled to the previous step, in that the starting material for one step was produced from the previous step. Therefore, any PrP^{Sc} or infectivity that was not removed from the product stream by one processing step would be available for partitioning in the subsequent processing step.

The steps examined in this series included cryoseparation, fraction I separation, fraction II + III separation, fraction III separation, and fraction IV-1 separation, which lead to the isolation of immunoglobulins, ATIII, PPF, albumin, and α_1-proteinase inhibitor (Fig. 4). Results indicated that PrP^{Sc} and infectivity that was not removed by one fractionation step was removed by a subsequent step (70). Also, the extent of the PrP^{Sc} and infectivity clearance in the coupled fractionation was comparable to the single step clearance observed previously (Table 3). This provided evidence that a hypothetical, clearance-resistant population of PrP^{Sc} was not present, and that the removal of PrP^{Sc} by a series of processing steps can be additive. A recent study suggests that the major factors that affect PrP^{Sc} partitioning in plasma fractionation steps include pH, salt content, and the presence of ethanol (70).

D. Clearance Studies for Biological Products That Are Not Derived from Plasma

Although most of the TSE partitioning studies to date have been performed on processes used for the production of therapeutics from human plasma, some work has been done to address pathogen safety with respect to TSEs for non-plasma-derived biological products, including recombinant proteins. For example, the production of Bayer's recombinant factor VIII provides an example of the numerous actions that manufacturers take to reduce the risk of disease transmission by a recombinant product. The raw materials and the manufacturing processes for producing recombinant factor VIII have been carefully assessed for pathogen reduction and have been designed to incorporate several methods for reducing potential pathogen safety risks. TSE spiking studies on process simulations have demonstrated that two of Bayer's recombinant factor VIII chromatography steps have the potential to remove TSE infectivity and PrP^{Sc}.

One of the earliest TSE clearance studies for biological products was published by Golker and co-workers for aprotinin derived from bovine lung tissue (71). In this study, the researchers used detergent-solubilized brain homogenates obtained from a mouse-adapted strain of scrapie (ME7) and measured infectivity by end-point titration. The researchers analyzed four different processing steps, one methanol extraction, two ion-exchange chromatography steps, and a membrane filtration step (Table 3). For every step analyzed, the authors reported a significant level of TSE infectivity clearance.

E. Survey of Clearance Data

A survey of TSE clearance data for various biological therapeutic proteins is provided in Table 3. Although various spiking agents and preparations were used in these studies, only two analysis strategies were employed: (1) measurement of the removal of the prion proteins or (2) measurement of the removal of TSE infectivity. Some of the steps that were analyzed were intentionally designed and/or implemented in manufacturing schemes for the sole purpose of pathogen removal. The most notable of this particular group includes several filtration steps. However, the majority of manufacturing steps that were analyzed for their capacity to clear TSEs were initially implemented for the purpose of isolating the target protein. The fact that prions were removed from the product stream was coincidental.

A careful review of Table 3 reveals some patterns, especially for the plasma precipitation steps. Cryoprecipitation was performed by different groups using multiple assay methods and consistently resulted in ≤ 1 log of clearance relative to the effluent. The fractionation IV-1 and fractionation IV-4 steps consistently demonstrated significant TSE clearance for all available data sets. The only apparent difference between Foster's fraction I + II + III (66) and Lee's fraction II + III (52) precipitation steps was the input material. The starting material for fraction I + II + III was cryopoor (cryoprecipitated) plasma but the starting material for fraction II + III was the fraction I effluent, which was devoid of the fraction I and cryoprecipitate fractions. Therefore, the starting materials had slightly different milieus; however, the precipitation conditions for both of these steps were equivalent (20% ethanol, pH 6.7, -5°C). The differences in starting materials may account for the different clearance values observed between the two studies.

Several filtration studies are available for comparison. As expected, the smaller pore membrane filters demonstrated some capacity to remove prions. In fact, in the studies available for review, increasing clearance

was observed as the membrane pore size decreased (72,73). In contrast, the depth filtrations described by Foster et al., which were associated with purification steps, appeared to be dependent on the filter pad/support matrix that was used. Column chromatography using ionic mechanisms is becoming increasingly popular for biological product purification at the production scale. Ion chromatography depends predominately on the charge of the particle in solution, and the net charge of the protein depends on its isoelectric point and the buffer conditions. Owing to the complex carbohydrates attached to the prion protein, a wide range of isoelectric points has been reported for this protein (28). Chromatographic methods are typically optimized during the process development phase and are specifically designed for the recovery of the target protein, not for prion removal. Because of the complexity of the prion protein's physicochemical nature, as well as the complexities of chromatographic separations, general conclusions about the clearance potential of the prion protein and/or TSE infectivity cannot be made and they must be determined experimentally.

VI. MODEL STUDIES

All of the clearance data described thus far were generated using spiking materials derived from scrapie-infected rodents. Use of rodent models has raised several concerns regarding its accuracy in predicting the partitioning of TSE agents from other species, specifically humans. A number of differences between mammalian TSE agents have been identified, including species selectivity or species barrier, the primary sequence of the prion protein, and other unidentified factors involved in infectivity and prion replication (74). For instance, despite a high degree of prion sequence homology at the amino acid level across mammalian species (75), small changes in amino acid sequences may alter the physicochemical characteristics of the prion protein between species. These characteristics could influence the partitioning of a particular species' prion protein and the associated infectivity.

To determine whether the hamster 263K strain is an accurate model for predicting the clearance of human TSEs, partitioning of the pathogenic form of the prion protein associated with human variant CJD (PrP^{vCJD}), sporadic CJD (PrP^{sCJD}), and Gerstmann Sträussler Sheinker disease (PrP^{GSS}) was measured in three plasma purification processes (76). Because sheep containing human transgenes may become an important future source of biopharmaceuticals, a brain homogenate from scrapie-infected sheep was tested also. For these studies, the cryoseparation, 3% PEG, and

11% PEG steps were analyzed (Fig. 4). The appropriate plasma intermediates were spiked with brain homogenates from patient samples or infected sheep, and the partitioning of PrP^{vCJD}, PrP^{sCJD}, PrP^{GSS}, and sheep PrP^{Sc} was measured using Western blot assays (76). Regardless of whether partitioning was nominal, intermediate, or substantial, the relative partitioning of human and sheep prions was consistent with the partitioning of hamster PrP^{Sc}.

Although small differences in the primary structure may change the higher-order structure of the prion protein, these results demonstrate that five different strains of prion protein partition in a similar manner during plasma fractionation processes. This indicates that the differences between hamster, sheep, and human prion isoforms do not significantly influence PrP clearance in these processes, and that hamster PrP^{Sc} can be used to reliably predict the partitioning of both human and sheep prion protein. Also, because there is a well-established correlation between the clearance of hamster PrP^{Sc} and the clearance of hamster infectivity , it is reasonable to conclude that human and sheep infectivity will partition similarly to hamster infectivity.

VII. SUMMARY AND CONCLUSIONS

Plasma and biotechnology product manufacturing provides for a means to remove pathogens as a result of fractionation and purification processes, resulting in a reduced risk for the transmission of genuine or theoretical pathogens. The demonstration that viral pathogens are inactivated or removed during manufacturing provides a significant margin of safety for plasma-derived or biotech products. Therefore, demonstrating the potential for plasma or biotech manufacturing processes to remove PrP^{Sc}/TSE infectivity establishes an empirically determined margin of safety for this theoretical risk.

In Bayer Corporation's studies, the identification of individual process steps capable of effective (4 logs or greater) and reproducible removal of PrP^{Sc}/TSE infectivity from the many process steps involved in plasma product manufacture required the development of a rapid in vitro assay to detect PrP^{Sc}. Although the assay specifically detects the presence of PrP^{Sc}, digestion with proteinase K to eliminate PrP^{c} results in a banding pattern on the Western blot characteristic of PrP^{RES}. Quantitation using end-point dilution analysis applied to the Western blot assay was selected because the characteristic PrP^{RES} banding pattern provides assurance that the signal is due to the presence of PrP^{Sc}. However, any in vitro assay that specifically detects PrP^{Sc} could be used for this application. The Western blot assay

proved to be reproducible and quantitative over a 6-log dynamic range. Partitioning could be determined by spiking a scaled-down process step with PrP^{Sc} and tracking it throughout the process. In most cases, PrP^{Sc} was removed from the process because it partitioned into a fraction that was eliminated from downstream manufacturing processes. Process steps capable of effective PrP^{Sc} partitioning could be identified in several weeks as opposed to many months or up to a year with the bioassay. However, it was important to demonstrate that the PrP^{Sc} partitioning determined by Western blot correlated with TSE infectivity partitioning determined by bioassay. The results of comparative partitioning studies showed a strong correlation between the partitioning of PrP^{Sc} determined by Western blot and TSE infectivity determined by bioassay. These results support the application of a PrP^{Sc} in vitro assay as a predictor of TSE infectivity partitioning during plasma or biotechnology fractionation and purification steps. When applied to the evaluation of PrP^{Sc} partitioning as a result of fractionation or purification, the assay identified individual process steps capable of partitioning 1 to >5 logs.

If PrP^{Sc}/TSE infectivity were present in plasma, partitioning would occur over the entire process. Therefore, in addition to identifying individual process steps capable of PrP^{Sc} partitioning, it was important to demonstrate that PrP^{Sc} spiked into plasma could be removed by a series of process steps. Several important questions concerning spiking and partitioning experiments were addressed by the studies reported here. Removal was additive over the coupled process steps examined, demonstrating that PrP^{Sc} that was not removed by one process step could be removed by another, and confirming that the addition of individual process steps could predict the overall capacity of a manufacturing process to reduce PrP^{Sc} load. Owing to the heterogeneous nature of PrP^{Sc} preparations, there was a concern that a fraction of PrP^{Sc} may be resistant to removal. However, these studies showed that no fraction of the PrP^{Sc} spike was found to be resistant to removal by precipitation/centrifugation, and the removal of PrP^{Sc} by precipitation/centrifugation was independent of the brain homogenate preparation used as the spiking material. This result was consistent with experiments showing that precipitation/centrifugation is an efficient means of removing PrP^{Sc} regardless of whether the spiking material was brain homogenate or purified PrP^{Sc}.

To date, published studies have investigated the partitioning and removal of rodent-adapted TSE infectivity and PrP^{Sc} either as an endogenous component of blood or as spiked brain homogenates. Regardless of whether the scaled-down process steps were conducted with endogenous TSE infectivity from the blood of infected animals or homogenates of infected brain, the results demonstrate that plasma fractionation processes

have a significant potential to remove $PrP^{Sc/TSE}$ infectivity. This comparison suggests that partitioning and removal of $PrP^{Sc/TSE}$ infectivity determined by spiking studies is similar to the removal of endogenous TSE infectivity from the blood of infected animals.

Of primary interest is the potential for plasma and biotechnology manufacturing processes to remove the human forms of TSE. Owing to the biochemical similarities between animal and human PrP/infectivity, both species of PrP^{Sc} could be expected to partition in the same way during fractionation or purification. Nonetheless, it was important to demonstrate that the partitioning of animal PrP^{Sc} and human PrP^{CJD} was similar. The results of the studies summarized here demonstrate that hamster PrP^{Sc}, sheep PrP^{Sc}, and human PrP^{sCJD}, PrP^{vCJD}, and PrP^{GSS} all partition in a similar manner regardless of the partitioning step. Therefore, precipitation/centrifugation spiking studies conducted with rodent-adapted PrP^{Sc} are predictive of the partitioning and removal of human PrP^{CJD}. Because we have demonstrated that PrP^{Sc} partitioning correlates with TSE infectivity partitioning, it also follows that rodent-adapted TSE infectivity partitioning will be predictive of human CJD infectivity partitioning.

The studies discussed here demonstrate the utility and relevance of applying in vitro assays for the detection of $PrP^{Sc/CJD}$ to identify plasma and biotechnology manufacturing process steps that are capable of removing animal and human $PrP^{Sc/CJD}$. The $PrP^{Sc/CJD}$ assay provides a practical means for performing a rapid and through analysis of potential TSE removal. Importantly, many separate partitioning experiments can be conducted in a relatively short time frame to establish the reproducibility of partitioning and removal steps.

REFERENCES

1. Klein RS, Friedland GH. Transmission of human immunodeficiency virus type 1 (HIV-1) by exposure to blood: defining the risk. Ann Intern Med 1990; 113:729–730.
2. Gurtler L. Blood-borne viral infections. Blood Coagul Fibrinolysis 1994; (suppl 3):S5–S12.
3. Tabor E. The epidemiology of virus transmission by plasma derivatives: clinical studies verifying the lack of transmission of hepatitus B and C viruses and HIV type 1. Transfusion 1999; 39:1160–1168.
4. Prusiner S. Prion encephalopathies of animals and humans. Dev Biol Standardization 1993; 80:31–44.
5. Prusiner S, Hsiao K. Human prion diseases. Ann Neurol 1994; 35:385–395.

6. Prusiner SB. Prions. Proc Natl Acad Sci USA 1998; 95:13363–13383.
7. Kitamoto T, Tateishi J. Human prion disease and human prion protein disease. Curr Top Microbiol Immunol 1996; 207:27–34.
8. Bosque PJ, Ryou C, Telling G, et al. Prions in skeletal muscle. Proc Natl Acad Sci USA 2002; 99:3812–3817.
9. Kimberlin R, Walker CA, Fraser H. The genomic identity of different strains of mouse scrapie is expressed in hamsters and preserved on reisolation in mice. J Virol 1989; 70:2017–2025.
10. Rubenstein R, Carp RI, Ju W, et al. Concentration and distribution of infectivity and PrP^{sc} following partial denaturation of a mouse-adapted and a hamsters-adapted scrapie strain. Arch Virol 1994; 139:301–311.
11. Kuroda Y, Jr CJG, Amyx HL, Gajusek DC. Creutzfeldt-Jakob disease in mice: persistent viremia and preferential replication of virus in low-density lymphocytes. Infect Immun 1983; 41:154–161.
12. O'Rourke KI, Baszler TV, Parish SM, Knowles DP. Preclinical detection of PrP^{sc} in nictitating membrane lymphoid tissue of sheep. Vet Rec 1998; 142: 489–491.
13. Manuelldis EE, Gorgacz EJ, Manelidis L. Viremia in experimental Cruetzfeldt-Jakob disease. Science 1978; 200:1069–1071.
14. Brown P, Rohwer RG, Dunstan BC, MacAuley C, Gajdusek DC, Drohan WN. The distribution of infectivity in blood components and plasma derivatives in experimental models of transmissible spongiform encephalopathy. Transfusion 1998; 38:810–816.
15. Diringer H. Sustained viremia in experimental hamster scrapie. Arch Virol 1984; 82:105–109.
16. Brown P, Cervenakova L, McShane LM, Barber P, Rubenstein R, Drohan WN. Further studies of blood infectivity in an experimental model of transmissible spongiform encephalopathy, with an explanation of why blood components do not transmit Creutzfeldt-Jakob disease in humans. Transfusion 1999; 39:1169–1178.
17. Houston F, Foster JD, Chong A, Hunter N, Bostock CJ. Transmission of BSE by blood transfusion in sheep. Lancet 2000; 356:999–1000.
18. Robinson MM. Transmissible encephalopathies and biopharmaceutical products. Dev Biol Standardization 1996; 88:237–241.
19. Brown P. Transmission of spongiform encephalopathy through biological products. Dev Biol Standardization 1998; 93:73–78.
20. Ricketts MN. Is Creutzfeldt-Jakob disease transmitted in blood. Can Med Assoc J 1997; 157:1367–1370.
21. Esmonde TFG, Will RG, Slattery JM, et al. Creutzfeldt-Jakob disease and blood transfusion. Lancet 1993; 341:205–207.
22. Heye N, Hensen S, Muller N. Creutzfeldt-Jakob disease and blood transfusion. Lancet 1994; 343:298–299.
23. Gabizon R, McKinley M, Prusiner S. Purified prion proteins and scrapie infectivity copartition into liposomes. Proc Natl Acad Sci USA 1987; 84:4017–4021.

24. Meyer RK, McKinley MP, Bowman KA, Braunfield MB, Barry RA, Prusiner SB. Separation and properties of cellular and scrapie prion proteins. Proc Natl Acad Sci USA 1986; 83:2310–2314.
25. McKinley MP, Bolton DC, Prusiner SB. A protease-resistant protein is a structural component of the scrapie prion. Cell 1983; 35:57–62.
26. Kretzschmar HA. Neuropathology of human prion diseases (spongiform encephalopathies). Dev Biol Standardization 1993; 80:71–90.
27. Marsh RF, Dees C, Castle BE, Wade WF, German TL. Purification of the scrapie agent by density gradient centrifugation. J Gen Virol 1984; 65:415–421.
28. Ceroni M, Piccardo P, Safar J, D.C. G, Gibbs CJ Jr. Scrapie infectivity and prion protein are distributed in the same pH range in agaraose isoelectric focusing. Neurology 1990; 40:508–513.
29. Safar J, Ceroni M, Piccardo P, et al. Subcellular distribution and physiochemical properties of scrapie-associated precursor protein and relationship with scrapie agent. Neurology 1990; 40:503–508.
30. Prusiner SB. Novel proteinaceous infectious particles cause scrapie. Science 1982; 216:136–144.
31. Serban D, Taraboulos A, DeArmond SJ, Prusiner S. Rapid detection of Creutzfeldt-Jakob disease and scrapie prion protein. Neurology 1990; 40: 110–117.
32. Grathwohl K-U, Horiuchi M, Ishiguro N, Shinagawa M. Sensitive enzyme-linked immunoabsorbent assay for detection of PrP^{Sc} in crude extracts from scrapie-affected mice. J Virol Methods 1997; 64:205–216.
33. Nowak T, Niedrig M, Bernhardt D. Inactivation of HIV-, HBV-, HCV-related viruses and other viruses in human plasma derivatives by pasteurisation. In: Brown F, ed. Virological Safety Aspects of Plasma Derivatives. Basel: Karger, 1993; 169–176.
34. Hart HF, Hart WG, Crossley J, et al. Effect of terminal (dry) heat treatment on non-enveloped viruses in coagulation factor concentrates. Vox Sang 1994; 67:345–50.
35. Chin S, Jin R, Wang XL, et al. Virucidal treatment of blood protein products with UVC radiation. Photochem Photobiol 1997; 65:432–5.
36. Bos OJ, Sunyé DG, Nieuweboer CE, van Engelenburg FA, Schuitemaker H, Over J. Virus validation of pH 4-treated human immunoglobulin products produced by the Cohn fractionation process. Biologicals 1998; 26:267–76.
37. Reid KG, Cuthbertson B, Jones AD, McIntosh RV. Potential contribution of mild pepsin treatment at pH4 to the viral safety of human immunoglobulin products. Vox Sang 1998; 55:75–80.
38. Horowitz B, Wiebe ME, Lippin A, Stryker MH. Inactivation of viruses in labile blood derivatives. 1. Disruption of lipid-enveloped viruses by tri(n-butyl)phosphate detergent combinations. Transfusion 1985; 25:516–22.
39. Lee D, Lebing W, Stenland C, Petteway S. Methods of inactivation of transmissible spongiform encephalopathies (TSEs) as applied to production chromatography processes. Downstream 1998; 27:18–22.

40. Taylor DM. Inactivation of transmissible degenerative encephalopathy agents: a review. Vet J 2000; 159:10–17.
41. Sofer G, Hagel L. Handbook of Process Chromatography: A Guide to Optimization, Scale-up, and Validation. London: Academic Press, 1997.
42. Prusiner S. Bioassays of Prions. In: Novel Infectious Pathogens Causing Scrapie and Creutzfeldt-Jakob Disease. New York: Academic Press, Inc., 1987:65–81.
43. Prusiner SB, Tremblay P, Safar J, Torchia M, DeArmond SJ. Bioassays of prions. In: Prusiner SB, ed. Prion Biology and Diseases. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1999:113–145.
44. Reed LJ, Muench H. A simple method of estimating fifty percent end points. Am J Hyg 1938; 27:493–497.
45. Dougherty RM. Animal virus titration techniques. In: Harris RJC, ed. Techniques in Experimental Virology. New York: Academic Press, 1964: 169–223.
46. Bolton DC, McKinley MP, Prusiner SB. Molecular characteristics of the major scrapie prion protein. Biochemistry 1984; 23:5898–5906.
47. Bueler H, Aguzzi A, Sailer A, et al. Mice devoid of PrP are resistant to scrapie. Cell 1993; 73:1339–1347.
48. Marsh RF, Castle BE, Dees C, W.F. W. Equilibrium density gradient centrifugation of the scrapie agent in Nycodenz. J Gen Virol 1984; 65:1963–1968.
49. Prusiner SB, Bolton DC, Groth DF, Bowman KA, Cocheram SP, McKinley MP. Further purification and characterization of scrapie prions. Biochemistry 1982; 21:6942–6950.
50. Bueler H, Raeber A, Sailer A, Fischer M, Aguzzi A, Weissmann C. High prion and PrP^{Sc} levels but delayed onset of disease in scrapie-inoculated mice heterozygous for a disrupted PrP gene. Mol Med 1994; 1:19–30.
51. Lee DC, Stenland CJ, Hartwell RC, et al. Monitoring plasma processing steps with a sensitive Western blot assay for the detection of the prion protein. J Virol Meth 2000; 84:77–89.
52. Lee DC, Stenland CJ, Miller JLC, et al. A direct relationship between the partitioning of the pathogenic prion protein and transmissible spongiform encephalopathy infectivity during the purification of plasma proteins. Transfusion 2001; 41:449–455.
53. Brown P, Cervenakova L, Diringer H. Blood infectivity and the prospects for a diagnostic screening test in Creutzfeldt-Jacob disease. J Lab Clin Med 2001; 137:5–13.
54. Safar J, Wille H, Itri V, et al. Eight prion strains have PrP^{Sc} molecules with different conformations. Nat Med 1998; 4:1157–1165.
55. MacGregor I, Hope J, Barnard G, et al. Application of a time-resolved fluoroimmunoassay for the analysis of normal prion protein in human blood and its components. Vox Sang 1999; 77:88–96.
56. Schmerr MJ, Jenny A. A diagnostic test for scrapie-infected sheep using a capillary electrophoresis immunoassay with fluorescent-labeled peptides. Electrophoresis 1998; 19:409–414.

57. Schmerr MJ, Jenny AL, Bulgin MS, et al. Use of capillary electrophoresis and fluorescent labeled peptides to detect the abnormal prion protein in the blood of animals that are infected with a transmissible spongiform encephalopathy. J Chromatogr 1999; 853:207–214.
58. Rubenstein R, Gray PC, Whelburg CM, Wagner JS, Tisone GC. Detection and descrimination of PrP^{Sc} by multi-spectral ultraviolet fluorescence. Biochem Biophys Res Commun 1998; 246:100–108.
59. Bieschke J, Giese A, Schulz-Schaeffer W, et al. Ultrasensitive detection of pathological prion protein aggregates by dual-color scanning for intensely fluorescent targets. Proc Natl Acad Sci USA 2000; 97:5468–5473.
60. Manuelidis L, Fritch W. Infectivity and host responses in Creutzfeldt-Jakob disease. Virology 1996; 216:46–59.
61. Lasmezas CI, Deslys J-P, Robain O, et al. Transmission of the BSE agent to mice in the absence of detectable abnormal prion protein. Science 1997; 275:402–405.
62. Grathwohl K-UD, Horiuchi M, Ishiguro N, Shinagawa M. Improvement of PrP^{Sc} detection in mouse spleen early at the preclinical stage of scrapie with collagenase-completed tissue homogenization and sarkosyl- NaCl extraction of PrP^{Sc}. Arch Virol 1996; 141:1863–1874.
63. Bruce ME, Will RG, Ironside JW, et al. Transmissions to mice indicate that "new variant" CJD is caused by the BSE agent. Nature 1997; 389: 498–501.
64. Pattison IH. Experiments with scrapie with special reference to the nature of the agent and the pathology of the disease. In: Gajdusek Dea, ed. Slow, Latent and Temperate Virus Infections. Washington, DC: US Government Printing Office, 1965:249–257.
65. McKinley M, Meyer R, Kenaga L, et al. Scrapie prion rod formation in vitro requires both detergent extraction and limited proteolysis. J Virol Meth 1991; 65:1340–1351.
66. Foster PR, Welch AG, McLean C, et al. Studies on the removal of abnormal prion protein by processes used in the manufacture of human plasma products. Vox Sang 2000; 78:86–95.
67. Diringer H, Hilmert H, Simon D, Werner E, B. E. Towards purification of the scrapie agent. Eur J Biochem 1983; 134:555–560.
68. Vey M, Pilkuhn S, Wille H, et al. Subcellular colocalization of the cellular and scrapie prion proteins in caveolae-like membranes. Proc Natl Acad Sci USA 1996; 93:14945–14949.
69. Baron H. Variant Creutzfeldt-Jakob disease: predicting the future? Blood Coagul Fibrinolysis 2001; 12:s29–36.
70. Cal K, Miller JLC, Stenland CJ, Gilligan KJ, Hartwell RC, Terry JC, Evans-Storms RB, Rubenstein R, Petteway SR Jr, Lee DC. Solvent-dependent precipitation of prion protein. Biochim Biophys Acta 2002; 1597:28–35.
71. Golker CF, Whiteman MD, Gugel KH, et al. Reduction of the infectivity of scrapie agent as a model for BSE in the manufacturing process of trasylol. Biologicals 1996; 24:103–111.

72. Tateishi J, Kitamoto T, Mohrl S, et al. Scrapie removal using Planova virus removal filters. Biologicals 2001; 29:17–25.
73. Foster P, McLean C, Welch A, et al. Removal of abnormal prion protein by plasma fractionation. Transfus Sci 2000; 22:53–6.
74. Prusiner SB. An introduction to prion biology and disease. In: Prusiner SB, ed. Prion Biology and Diseases. Cold Spring Harbor, NY: Cold Spring Harbor Press, 1999:1–66.
75. Liao Y-CJ, Lebo RV, Clawson GA, Smuckler EA. Human prion protein cDNA: molecular cloning, chromosomal mapping, and biological implications. Science 1986; 233:364–367.
76. Stenland CJ, Lee DC, Brown P, Petteway SR Jr, Rubenstein R. Partitioning of human and sheep forms of the pathogenic prion protein during purification of therapeutic proteins from human plasma. Transfusion 2002; 42:1497–1500.
77. Foster PR. Assessment of the potential of plasma fractionation processes to remove causative agents of transmissible spongiform encephalopathy. Transfus Med 1999; 9:3–14.
78. Pocchiari M, Peano S, Conz A, et al. Combination ultrafiltration and 6M urea treatment of human growth hormone effectively minimizes risk from potential Creutzfeld-Jacobs disease virus contamination. Horm Res 1991; 35:161–166.

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about the book . . .

The alarm sounded by Canada's recently confirmed case of bovine spongiform encephalopathy (BSE) has reaffirmed the exigency of establishing improved safeguards and more aggressive surveillance protocols in North America and around the world. Research converging on the probable causative agent—prion proteins—calls for intensive assessment of the headway gained in tracing prions, testing for transmissible neurodegenerative diseases, and developing methods for cornering the epidemic.

This timely book marshals techniques for prion protein assay and diagnosis of transmissible spongiform encephalopathies (TSEs).

Administered by an illustrious panel of 36 international contributors, ***Prions and Mad Cow Disease*** manifests evidence of infectious agents believed responsible for TSEs...explores prion protein detection via immunoassay, chemiluminescent ELISA, bioassay, and fluorescence...relates differential extraction, DELFIA, multispectral techniques, and in vivo diagnostics...consults comparable sheep-carried infection scrapie for BSE leads...examines chronic wasting disease, a TSE striking deer and elk populations...specifies measures for active BSE surveillance and safety monitoring of biological products...presents models for estimating probabilities of animal–animal BSE spread...and scientifically gauges the possibility of human contraction of BSE variants.

about the editors . . .

BRIAN K. NUNNALLY is a Research Scientist at Eli Lilly and Company, Indianapolis, Indiana. Dr. Nunnally has published several articles and lectured on a variety of analytical disciplines. He is an assistant editor of the journal *Analytical Letters* (Marcel Dekker, Inc.) and a member of the Analytical Chemistry Division of the American Chemical Society, the Society for Applied Spectroscopy, and the Coblentz Society. Dr. Nunnally received two B.S. degrees (1994) from South Carolina Honors College, University of South Carolina, Columbia, and the Ph.D. degree (1998) in chemistry from Duke University, Durham, North Carolina.

IRA S. KRULL is Associate Professor of Chemistry at Northeastern University, Boston, Massachusetts. The coauthor of *Analytical Method Development and Validation* (Marcel Dekker, Inc.), Dr. Krull is credited with over 250 published papers and several book chapters. His memberships include the American Chemical Society, the Central New England Chromatography Council, and Sigma Xi. Dr. Krull received the B.S. degree (1962) in chemistry from the City College of New York, New York, and the M.S. (1966) and Ph.D. (1968) degrees in organic chemistry from New York University, New York.

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