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Editors

# Veterinary Vaccines

# Progress in Vaccinology

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# Veterinary Vaccines

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# Series Preface

Vaccines have historically been considered to be the most cost-effective method for preventing communicable diseases. It was a vaccine that enabled global eradication of the dreaded disease smallpox. Mass immunization of children forms the anchor of the strategy of the World Health Organization (WHO) to attain "health for all" status by the year 2000.

Vaccinology is undergoing a dimensional change with the advances that have taken place in immunology and genetic engineering. Vaccines that confer short or inadequate immunity or that have side effects are being replaced by better vaccines. New vaccines are being developed for a variety of maladies. Monoclonal antibodies and T cell clones have been employed to delineate the immunodeterminants on microbes, an approach elegantly complemented by computer graphics and molecular imaging techniques. Possibilities have opened for obtaining hitherto scarce antigens of parasites by the DNA recombinant route. Better appreciation of the idiotypic network has aroused research on anti-idiotypic vaccines. Solid-phase synthesis of peptides is leading to an array of synthetic vaccines, an approach that is expected to attain its full potential once the sequences activating suppressor cells are discovered and the rules for presentation of antigens to T and B cells are better worked out.

A new breed of vaccines is on the horizon that seeks to control fertility. Originally conceived to intercept a step in the reproductive process, they are conceptual models for developing approaches to regulate the body's internal processes. The importance of lymphokines and monokines in the induction of the immune response and in killing parasites is realized, and specific or nonspecific routes are employed to elicit their formation. Interleukins and interferons have been produced by DNA recombinant methods and experimental approaches initiated to coexpress the genes for such regulators with microbial antigens. The old smallpox vaccine, vaccinia, is appearing in a new garb with genetically engineered foreign genes. The technology for manufacture of vaccines, especially for cell

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culture-based organisms, is undergoing changes, with new cell lines, promoters for better expression, and automation.

Contemporary vaccinology is a multidisciplinary science (and technology) that is developing rapidly. Findings are reported in disparate journals. Periodical reviews by experts assimilating relevant progress in a given field would be of immense value to investigators, funding agencies, manufacturers, and users of the vaccines, the public health authorities. This series aims to provide comprehensive reviews on topics relating to various aspects of vaccinology by leading investigators.

G.P. Talwar

# Introductory Remarks on Vaccine Development

Vaccines have been one of the major achievements in both human and veterinary medicine; however, in spite of nearly 200 years of vaccine history, vaccines still are basically the same. In general vaccines consist of whole microorganisms — either live and attenuated but replicating or inactivated killed and not replicating. What are the prospects for new concepts in vaccine development and vaccine technology? And is there a need to develop new techniques?

There is a need for new concepts to develop vaccines. First, there are diseases caused by microorganisms against which effective vaccines do not exist. With few exceptions there are no vaccines against diseases caused by chronic and persistent infections, including infections caused by retroviruses. There are a few vaccines against parasites, and an effective vaccine giving a long lasting response is desired against *Mycoplasma mycoides*, which causes contagious bovine pleuropneumonia or the caprine variant contagious caprine pleuropneumonia. Second, economically there are difficulties in producing antigens from several microorganisms because they are prohibitively expensive for vaccine use. For example, parasite antigens are not easily produced. Also some viruses, such as respiratory syncytial virus, do not give a high antigen production in established culture systems. Such production problems may be overcome by the aid of gene technology or perhaps even by chemical synthesis of antigens, e.g., oligopeptides. The production of antigen by gene technology also offers a potential economic advantage by permitting the use of conventional vaccine facilities whereas the risks of contagion attendant with whole microorganism use would require employing high-risk laboratories, representing microorganisms that are hazardous to humans or because of the risk of spread must be kept in high risk laboratories.

Coming back to the problem of developing vaccines against microorganisms causing chronic or persistent infection, in general the whole microorganism attenuated or killed will not induce protective immunity. The strategy of such microorganisms is to persist in the host under pressure of an immune response. It is, therefore, likely that the microorganism

tricks the host by modulating the immune response that facilitates its persistence. Therefore, a vaccine strategy would be to identify and select proteins (antigens) essential for the infection, i.e., the concept for subunit vaccines; further, the antigens have to be present in an immunogenic form in particles such as micelles or liposomes supplemented with a suitable immunomodulator (adjuvant). An even more effective way to modulate the immune response is to build the adjuvant component into the particle carrying the antigen, i.e., the immunostimulating complex. When we know the mechanisms of infection of a microorganism and when we have mapped what type of immune response is protective, we will be able to tailor-make the vaccine. To do that we need to know what type or isotype of antibody is required for protection, what type of cell-mediated immune response is required including type of T-helper cell, cytokines produced, or the need of cytotoxic T cells, characterized as $CD8^+$ and induced under restriction of MHC class I. In a well-constructed vaccine the antigen should have the capacity to interact with antigen-presenting cells to allow the antigen to be processed and allow the antigen fragments to be associated with MHC class II molecules and, if necessary, also with MHC class I molecules. All this is now readily possible. What is lacking at present is a more precise knowledge of the infection process and the type of immune response required to prevent the infection. With type development of adjuvant or immunomodulators we are dealing with substances that stimulate a number of cytokines, which have powerful effects and even cause side effects on the exposed individuals, as is the case with tumor necrosis factor, interleukin-1, and interleukin-6. Therefore, the evaluation of adjuvants has to be accompanied with toxicological studies.

However, the use of vaccines is in general dependent on the costs for their production. Particularly in regard to animal vaccines the cost–benefit ratio will determine whether a vaccine will be used, and, as this book will indicate, the conventional or first generation of vaccines is still in majority.

<div align="right">S. Höglund<br>B. Morein</div>

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CHAPTER 1

# Adjuvants for New Veterinary Vaccines

Iain J. East, Roy L. Kerlin, Klaus Altmann, and
Dennis L. Watson

## 1. Introduction

Many important diseases of domestic animals have been rendered impotent by the development of safe and effective vaccines to prevent or ameliorate infection. Up to now, veterinary vaccines consisted mainly of killed or attenuated whole organisms. Vaccines of this type that are registered for use in the state of Queensland, Australia are listed in Table 1.1 (63,161). While not including many of the vaccines found in other countries, the list is indicative of current vaccine technologies. Vaccines such as these, which mostly contain whole pathogens, are usually highly immunogenic and utilize either no adjuvant or a simple adjuvant, such as alum or oil, to prolong the effect of the vaccine. The term adjuvant was first defined by Ramon (117) in 1925 as a substance that, when used in combination with antigen, enhanced levels of immunity beyond those developed with the antigen alone. A synopsis of the adjuvants currently available for veterinary vaccines was recently published by Vanselow (152).
   The selection of adjuvants for veterinary use is constrained by ethical considerations. Many adjuvants manifest significant side effects such as generation of granulomas, pyrogenicity, induction of arthritis, and anterior uveitis. Some adjuvants are also cytolytic or have other undesirable effects (1). The only adjuvant approved for human use is aluminium hydroxide (152). However, aluminium hydroxide induces little cell-mediated immunity (17) and is therefore likely to be ineffective in vaccines against viruses, fungi, mycobacteria, and intracellular protozoans that are eliminated by cellular, not humoral, effector mechanisms. Both $Al(OH)_3$ and Freund's incomplete adjuvant (FIA) are approved for veterinary use, but with increasing emphasis on animal care and ethics, the future of an adjuvant such as FIA that may induce granulomas should not be taken for granted. In addition, the recent suggested links between aluminium and Alzheimer's disease could threaten the use of aluminium-based adjuvants.

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**Table 1.1.** Vaccines registered for veterinary use in Queensland.

| Organism | Vaccine type | Adjuvant |
|---|---|---|
| Bacteria | | |
| *Bacillus anthracis* | Live avirulent | Nil |
| *Brucella abortus* | Attenuated live | Nil |
| *Erysipelothrix rhusiopathiae* | Formalin killed | Aluminium |
| *Fusiformis necrophorus* | Formalin killed | Aluminium |
| *Bacteroides nodosus* | Formalin killed | Alum/oil |
| *Leptospira interrogans* | Formalin killed | Aluminium |
| *Campylobacter fetus* | Formalin killed | Oil |
| *Clostridium* spp. | Purified toxoid | Aluminium |
| *Corynebaccterium pseudotuberculosis* | Formalin treated culture products | Aluminium |
| Viruses | | |
| Wart vaccine | Formalin treated | Unknown |
| Bovine ephemeral fever | Attenuated live | Quil A |
| Scabby mouth | Attenuated live | Nil |
| Protozoa | | |
| *Babesia bovis* | Attenuated live | Nil |
| *Babesia bigemina* | Attenuated live | Nil |
| Rickettsia | | |
| *Anaplasma centrale* | Attenuated live | Nil |

Many of the shortcomings encountered with "first generation" whole-organism vaccines have been addressed in recent times by researchers working on a new generation of human and veterinary vaccines (38,119). This "second generation" of vaccines usually comprises one or few isolated proteins made by recombinant DNA technology. A problem with this approach, however, is that the immune response of an animal to a single soluble protein is often quite different to the immune response to attenuated or killed viruses, bacteria, or protozoa. Soluble proteins are rarely sufficiently immunogenic to promote a strong immune response when injected alone. Furthermore, it is important to ensure that an immune response is stimulated that can invoke appropriate immune effector mechanisms and provide immunity at various anatomical sites. It is imperative, therefore, that these second generation vaccine antigens are designed along with appropriate adjuvants to ensure maximal protection of vaccinated animals from disease.

## 2. Mechanisms of Adjuvant Action

### A. Introduction

The mechanisms by which adjuvants promote the immune response are poorly understood, although much ad hoc work has shown an influence of the type of adjuvant, the nature of the antigen, and the experimental

species vaccinated. It is important, therefore, to attempt to identify common features of adjuvant action in order to be able to predict the type of adjuvant to use in a given circumstance.

There appear to be at least four general ways in which adjuvants promote the immune response. First, some adjuvants maintain a depot of antigen at the injection site (12,83,124). Second, adjuvants are capable of promoting accumulation of immunoreactive cells at the site of injection and in the draining lymph nodes (18,37,145). Third, adjuvants modify the activities of cells that are concerned with generating, promoting, and maintaining the immune response (1). Fourth, adjuvants can modify the presentation of antigen to the immune system (100,128,137).

## B.  Maintaining a Depot of Antigen

The dogma entrenched in the vaccine literature maintains that the presence of an antigen depot in some manner promotes the immune response (51). The most common way to achieve this effect is to suspend the antigen in an oil-in-water emulsion. Historically this was first shown when Le Moignac and Pinoy (86) demonstrated that they could elicit a greater antibody response to *Salmonella typhimurium* when the bacteria were suspended in mineral oil. The best evidence that an antigen depot helps promote the immune response is the correlation between persistence of antigen at the site of injection and maintenance of serum antibody levels (12,40,67). Correlative studies have shown that a variety of materials that possess adjuvant activity such as bentonite and tapioca (155) assist with maintaining a depot of antigen at the injection site. Furthermore, polymerisation of antigen to form "beads" (85,88), precipitation of antigen with aluminium salts (156), entrapping antigen in dextran matrices (124), or coating antigen onto inert plastic supports (15) all cause a repository of antigen to be preserved at the injection site, and promote and maintain antibody levels for much longer periods than antigen given alone.

The question arises, however, of how the antigen depot actually contributes to the immune response. The first possibility is that antigen and adjuvant in the injection site together stimulate the development of a granuloma that contributes a significant proportion of the total antibody response. It is well-documented that the vaccine granuloma is a site of considerable immunological activity (130) and contains numerous antibody-containing cells (74,77). It was shown, however, that surgical excision of the primary injection site 30 min after inoculation did not markedly diminish subsequent antibody production, although titers were reduced (45). Furthermore, removal of the injection site 2 to 3 months after injection did not significantly alter antibody titers (83).

Another possibility is that slow release of antigen from the injection site is responsible for the immune stimulatory effects of depot-type

adjuvants. Soluble antigen in water is probably catabolized rapidly and effectively removed from detection by the immune system (72). In contrast, antigen in FIA is released slowly providing from an injection site (12). Two weeks after an injection of antigen in FIA, however, little antigen was still issuing from the vaccination granuloma (12,72). Indeed, 20 weeks after injection, approximately 30% of the original antigen was still present in an FIA-induced granuloma (83). The results suggested that the rate of antigen release from an oil adjuvant granuloma was insufficient to stimulate and maintain maximal antibody responses for more than a short period after vaccination (83). In another example, antigen mixed with the adjuvant saponin was shown to be retained at the site of injection and released slowly providing prolonged stimulation to the immune system (125). However, digitonin, a plant glycoside that has similar hemolytic and cholesterol-binding activities to saponin, also causes antigen retention at the site of inoculation, but is not a good adjuvant (125). These studies together suggest that the current perception of the way in which depot-type adjuvants promote the immune response should be reevaluated. It is possible that protracted release of antigen from a depot may be of some advantage to maintain the immune response. Indeed, it appears that persistence of B cell memory is contingent on the continual presence of antigen *in vivo* (53). However, the principal action of depot-type adjuvants may not relate directly to the antigen depot. Rather, it is more likely that antigen persistence in the draining lymph node, perhaps on the cell membrane of dendritic cells (148), is responsible for sustaining the immune response.

## C. Accumulation of Immunoreactive Cells

An effective immune response is the cumulative result of a tightly orchestrated series of cellular interactions that require the presence of appropriate cell types. It has long been known that injection of materials such as aluminium salts, that possess adjuvant activity, induce hyper-cellularity and paracortical enlargement of draining lymph nodes (145). Studies using $^{51}$Cr-labeled lymphocytes showed that this precedes the blastogenic response in a stimulated node, and is due, at least in part, to an increased influx of lymphocytes (37,169). This process, called "lymphocyte trapping," is probably an important component of the mechanism of adjuvant action. Both macrophages and T cells have been shown to mediate in the onset of lymphocyte trapping (46,168). Further-more, interleukin-1 (IL-1) has been implicated in affecting the retention of lymphocytes in lymph nodes (1), probably through a capacity to promote binding of lymphocytes to endothelial cells (21). Other studies have linked complement activation and $PGE_2$ release with the onset of "cell shutdown" (65,95). This phenomenon, where cell output from a stimulated lymph node is markedly diminished, is probably related to

lymphocyte trapping (96). Preliminary studies of lymphocyte trapping suggested that it was caused by plugging of lymphoid sinuses, thus causing nonspecific retardation of cell passage through the lymph node (169). This was subsequently shown to be unlikely as lymphocytes in transit through an antigenically stimulated lymph node do not have an altered transit time (20). Furthermore, the hypercellularity of stimulated nodes was shown to be a function of both an increase in number of lymphocytes passing into the node from blood, and to a decrease in the number of resident lymphocytes passing into the efferent lymph (20). It is important to note that antigen-specific lymphocytes are selectively recruited into an antigen-stimulated lymph node from the recirculating lymphocyte pool (64). Thus, adjuvants that stimulate lymphocyte trapping help to ensure optimal exposure of specifically responsive cells to the appropriate antigens in an environment replete with the necessary cells and growth factors to support an immune response.

## D. Modification of Cellular Responses

The published effects of adjuvants on cells involved in the immune response are many and varied (1,156). The difficulty is in establishing whether such effects are important for adjuvanticity or whether they are an unrelated by-product of adjuvant action. An example of this is the influence of adjuvants to induce inflammation and granuloma formation. Inflammation, hypercellularity, and granuloma formation at the site of injection have all been proposed as central to the capacity of an adjuvant to promote the immune response (72,156). Indeed, it was shown that only 48 hrs after injection of staphylococcal vaccines into the skin of sheep, the cellular immune response within the injection lesion reflected the type of response observed later in the serum (75,77). This suggests that processes going on in the site of granuloma development may influence the subsequent immune response. The adjuvant action of some materials, however, does not correlate with inflammation or granuloma formation. For example, modification of the distribution and content of hydrophilic and hydrophobic copolymers in oil emulsions led to the conclusion that induction of antibody synthesis was not associated with development of inflammation and subsequent generation of a granuloma (68). Similarly, the adjuvant action of saponin, including retention and splenic localization of antigen, was attributed to an ability to promote an inflammatory response (125). Abolishing the inflammatory properties of saponin by competitive inhibition with cholesterol, however, did not affect the adjuvant action of this compound (125).

Generation of an antigen-specific immune response implies cellular collaboration between lymphocytes and antigen-presenting cells (APC), and it would be expected that one of the actions of adjuvants may be the capacity to promote this union. Antigen-specific interaction between APC

and lymphocytes is mediated through cell membrane-bound class II MHC gene products (class II) complexed with processed antigen (82). It is not surprising then that at least two adjuvants, lipopolysaccharide (LPS) and Freund's complete adjuvant (FCA), directly stimulate class II expression by macrophages (13,170). Interestingly, muramyl dipeptide (MDP), the adjuvant-active moiety of FCA (13), does not affect class II expression by macrophages, although there may be some promotion of class II expression on splenocytes. This may be important, as B cells are efficient antigen-presenting cells (24).

Although cellular interactions are essential, the other important components of optimal immune responses are the soluble stimulatory factors (113). Proliferation of B and T cells and generation of antibody responses are dependent on appropriate cytokines (113). There is little doubt that the effect of adjuvants on cytokine production is the most confusing aspect of the poorly understood mechanisms of adjuvant action. The reason for this is that adjuvants may act at a variety of points in a cascade of events involving multiple cell types and many soluble factors. As with many other effects of adjuvants, it is important to distinguish between those effects that promote the immune system, and those that are merely by-products of an ongoing response. An example in this regard is the influence of adjuvants on IL-1 production and the effects of IL-1 on the immune response. IL-1 costimulates T cell proliferation and has a variety of other stimulatory effects on the immune response (35). This factor is also responsible for fever and IL-1 was first known as "endogenous pyrogen." Adjuvants such as LPS, MDP, and silica particles stimulate macrophages directly and induce IL-1 production (49,50,131). In the case of LPS, however, the lipid A moiety, which is responsible for most of the biological activity of LPS, has been chemically modified to reduce its toxicity and pyrogenicity without affecting adjuvanticity (120,121). Similarly, MDP is an adjuvant with multiple effects, a principal one of which is to activate macrophages and stimulate IL-1 production (112). In this case also, MDP was chemically modified to obtain forms, such as murabutide (22), that are nonpyrogenic, but still adjuvant active. This evidence together suggests strongly that pyrogenic IL-1 is not primarily responsible for adjuvanticity. Furthermore, although partially purified IL-1 derived from stimulated macrophages has been shown to act as an adjuvant in mice with bovine serum albumin as antigen (133), it is likely that there are lymphocyte activating factors other than IL-1 in supernatants from cultures of activated macrophages (134).

With regard to other interleukins and adjuvanticity, the best studied examples are interleukin-2 (IL-2) and γ-interferon (IFN). The T cell growth factor IL-2 has been tested as an adjuvant with viruses and other antigens and showed some activity, although multiple doses of IL-2 were necessary for an effect, and IL-2 is toxic at high doses (159). More success was obtained with IFN. Early studies with compounds that induced IFN

synthesis suggested that this factor was important for adjuvanticity (70). More recently it was shown in sheep that Quil A, dextran sulfate, and mineral oil adjuvants promoted IFN production (41). Interestingly, no IFN synthesis was detected in sheep given the adjuvant alhydrogel indicating that a high level of IFN was not a prerequisite for adjuvanticity (41). Since aluminium-precipitated adjuvants do not induce strong cell-mediated immunity (CMI) (156), this may point to an association between IFN and the induction of CMI. Certainly IFN is known to promote class II expression by macrophages and thereby may facilitate interaction of APC and T cells (134).

Adjuvants are known to have other, direct effects on cells involved with the immune response (156). For example, LPS activates macrophages (170), causes lymphokine release (50), stimulates class II expression (170), is a B cell mitogen (50), and promotes specific and nonspecific antibody synthesis (127). Another interesting example is the adjuvant effect of *Bordatella pertussis*. The major adjuvant-active components of this bacteria are LPS and pertussis toxin (PT). The activities of LPS have been described, but PT has a number of unusual and noteworthy effects. This toxin affects T cells both directly and indirectly. PT promotes $IgG_1$ and IgE antibody responses in mice (105), which indicates that T cell-derived lymphokines such as IL-4 are being secreted as a result of PT action (113). The indirect effects of PT relate to a capacity to selectively alter T cell recirculation through lymph nodes and may reflect an altered balance of CD4 and CD8 T cells in the affected node (156).

## E.  Effects on Antigen Presentation

It is clear that both soluble and particulate material, when injected subcutaneously into nonimmune animals, pass into draining lymph nodes in a cell-free form and accumulate in lymphatic sinuses (12). From this point antigen is taken up into macrophages and dendritic cells in the parenchyma of the node (60). It is likely that interdigitating, parafollicular dendritic cells in the T cell areas of the node and follicular dendritic cells in the follicles are crucial to the generation of T cell and B cell responses, respectively (8,69,148). Evidence that antigen is maintained on dendritic cells for long periods, and that follicular dendritic cells are intimately associated with the generation of germinal centers in stimulated lymph nodes (148) suggests that study of the effects of adjuvants on dendritic cells would be profitable. The data available in this area are rather sketchy. For example, injection of soluble bovine serum albumin (BSA) into guinea pigs generates an antibody response, but no CMI (25). Conjugation of BSA to dodecanoic acid, however, caused concentration of the antigen in the paracortical (T cell) region of the lymph node, and a consequent induction of CMI. In another experiment, human serum albumin (HSA) coupled to fatty acids activated complement and

localized on dendritic cells (which bear $C_3b$ receptors) in the spleen of rats, whereas unconjugated HSA did not (132). The conjugated HSA promoted greater antibody responses than native HSA (132).

Antigen is internalized by antigen-presenting cells and then processed and reexpressed on the cell surface with class II where it is capable of interaction with antigen receptors on T cells (82). The association of antigen with class II is contingent on the structure of the antigenic epitope against which the response is to be directed and is unlikely to be easily modified (80). There are a number of adjuvants, however, that promote interaction of antigen and APC; on the one hand to increase the efficiency of antigen uptake by APC, and on the other hand to effectively by-pass the need for APC and present antigen directly to T cells. For example, liposomes, which will be discussed in detail below, are artificial lipid bilayers that mimic the nature of a cell membrane (2) and can, under some conditions, substitute for APC (151). It is possible that other means of presenting large aggregates of antigen, either alone or on the surface of inert matrices, may have a similar effect (15,81,85,88,124).

It is possible that many adjuvant effects are due to enhanced antigen contact with APC. For example, antigen aggregates are usually more immunogenic than soluble antigen, perhaps by virtue of the greater amount of antigen phagocytosed by APC (85,88). Covalently bound polymeric antigen structures that enhance immunity may act in a similar fashion (71). This may also explain the greater immunogenicity of antigen–antibody complexes (57). Antibody bound to antigen can interact with Fc receptors on APC, activate macrophages, and facilitate ingestion of the complex (84). Antigens complexed with Fab portions of antibody that cannot bind to Fc receptors on APC are much less immunogenic (9).

If antigen is to be presented more efficiently to APC, then cognizance must be paid to the nature of the cell membrane. For example, increasing the hydrophobicity of antigen in oil emulsions may increase the immune response to injected antigen (28). Furthermore, addition of lipid to protein antigens promotes the response to the protein. In both of these cases it could be that antigen interacts more efficiently with the hydrophobic lipid bilayer of the macrophage cell membrane. Thus, there would be increased antigen uptake by the APC leading to more effective stimulation of T cells. This effect could be predicted to play a role in the adjuvant action of oil emulsions generally, as antigen is spread across the surface of the oil–water interface and would thereby be in a position to interact more favorably with cell membranes than aqueous soluble antigen. Detergents may have a similar effect by forming micelles that aggregate antigen (147). The detergent in the micelles may interact with the cell membrane and allow more efficient antigen processing. In this regard, antigen that is phagocytosed by macrophages is processed in phagolysosomes and associates only with class II MHC molecules to be reexpressed on the cell surface (82). It is only molecules that pass through

the cell membrane or the phagolysosomal membrane into the cytoplasm that can interact with class I MHC molecules and stimulate cytotoxic T cell responses, that are important in viral immunity. Live viruses pass into the cytoplasm as a function of their reproduction in the cell, and as a consequence invoke class I restricted immune responses (80). Most killed antigen vaccines, however, do not induce class I restricted immunity (80,100). It is possible that judicious use of detergents in adjuvants may help to give killed antigens access to the cytoplasmic milieu that allows association of antigen with class I antigens and thereby invoke class I restricted immunity to the antigen. It is possible to elicit class I restricted cytotoxic T cells using small protein antigens if antigens are presented as immune stimulatory complexes (ISCOMs) (100), although the reasons for this are not clear.

## 3.  Examples of Adjuvants in Use

### A.  Introduction

To date, the development of adjuvants has been largely empirical and their effects as described above are diverse and complex. This has complicated attempts to discover unifying mechanisms of adjuvant action (156). It is difficult, therefore, to predict which adjuvants will be effective with a particular antigen or vaccine. Little work has been published on the modulation of immune responses to vaccines for cattle or sheep by different adjuvants and even less on the immune response to vaccines containing a single protein antigen.

### B.  Experimental Vaccines

With vaccines against protozoans, FCA has proved effective but there are few alternatives described. Taylor et al. (146) were able to protect cattle from *Babesia divergens* using a combination of FIA and MDP. Timms et al. (149) were successful with killed *B. bovis* presented in saponin and Goodger et al. (52) with a soluble antigen polymerized with glutaraldehyde.

Wells et al. (160) conducted a study comparing different adjuvants to vaccinate against *Trypanosoma brucei* using a soluble glycoprotein. Oil-based adjuvants were superior to both aluminium hydroxide and bacteria-based adjuvants in stimulating the immune response and conferring protection from challenge infection. Saponin was less effective than FCA in promoting an immune response. However, both FCA and FIA produced skin reactions, which, in the authors' estimation, "made their use unacceptable in meat producing animals." Saponin did not cause a reation at the site of injection.

A number of workers have had considerable success in the search for efficacious and less toxic adjuvants. Vanselow et al. (153) compared the efficacy of Quil A (a saponin derivative), aluminium hydroxide gel, and dextran sulfate as adjuvants for a live, attenuated bovine ephemeral fever vaccine. Quil A was the most effective adjuvant and produced only transient skin reactions. Quil A was also effective in improving the performance of a foot-and-mouth disease vaccine in both guinea pigs and cattle (29). Only transient inflammatory reactions were observed at the site of injection in these experiments.

Willadsen et al. (162,163) purified a protective antigen from the midgut of the cattle tick *Boophilus microplus*. During these studies the progressive stages of protein fractionation were assessed by vaccine trials using FCA. In contrast, Wong and Opdebeeck (165) have conducted an essentially similar purification of *B. microplus* antigens and found that Quil A is equally effective as an adjuvant.

Effective vaccination of sheep against footrot (*Bacteroides nodosus*) has been complicated by the existence of several serotypes. Killed vaccines in a range of adjuvants induce immunity, however, absorption onto alum first improves the effect whether the absorbed material is subsequently combined with Quil A (39) or oil (135). Purified pili have also been used as a vaccine and with these, Stewart et al. (136) found first that FIA was as effective as FCA and subsequently (135) that absorbing the pili onto alum first improved the efficacy of the vaccines when presented in oil. However, the purified pili were not as effective as whole killed bacteria when vaccinating against heterologous serotypes. A recombinant pilus vaccine against ovine footrot has been produced (38) which when absorbed onto alum and emulsified in FIA reduced the incidence and severity of footrot.

Foot-and-mouth disease (FMD) has been successfully controlled for many years with an inactivated virus vaccine. The current vaccine, however, suffers from problems with instability and incomplete activation (36,106). Much work has been done to develop alternative vaccines and the work has centered on the highly immunogenic region comprising amino acids 141–160 of the capsid protein VP1. Both native VP1 purified from the virus and a recombinant form produced as a fusion protein with the *Escherichia coli* protein LE1413 are effective as vaccines for cattle when combined with FIA (79). Other recombinant forms have been produced (164) but have not been tested in cattle. Variants of the 20-mer peptide sequence are highly immunogenic and as a synthetic vaccine, coupling to a carrier protein is unnecessary and may even have adverse effects (19). Dimarchi et al. (34) successfully protected cattle from FMD using a peptide containing two regions of VP1 viz. 141–158 and 200–213 juxtaposed together. This vaccine, however, was presented in FCA. A homopolymer of peptide 141–160 was created by adding a cysteine residue at each end and oxidizing in air. This homopolymer was tested

with several adjuvants but was more effective in FIA than when a combination of Al(OH)$_3$ and saponin was used. The addition of MDP to the FIA emulsion improved the immune response further (16). Finally a copolymer of MDP and 141–160 produced a high level of neutralizing antibodies in guinea pigs without additional carriers or adjuvants. These animals, however, were not challenged with live virus and neither has this copolymer vaccine been tested in cattle.

Recent studies with an experimental staphylococcal mastitis vaccine have identified dextran sulfate as the most efficacious adjuvant (157). This is a killed cell-toxoid vaccine comprising pseudocapsule-enshrouded *Staplylococcus aureus* organisms. Dextran sulphate selectively stimulates synthesis of IgG$_2$ anti-pseudocapsule antibody. IgG$_2$ is cytophilic for ruminant neutrophils and it is known that IgG$_2$ anti-pseudocapsule antibody is a powerful opsonin and hence an important mediator of host defence (158). Interestingly, the IgG$_2$-stimulatory property of dextran sulfate does not apply with all antigens. When the hapten DNP was coupled to *S. aureus* the addition of dextran sulfate did not influence the ratio of IgG$_2$:IgG$_1$ anti-DNP antibody in sheep (76).

A more extensive range of literature is available if one considers the studies in laboratory rodents and excellent reviews have been written by Warren et al. (156) and Allison and Byars (1). The applicability of this work to cattle and sheep, however, is not clear. Several studies have reported that findings with adjuvants and vaccines in laboratory models do not extrapolate to large animals (11,19,23,103,106).

## C. Subunit Vaccines

It would be enlightening to consider the development of successful subunit vaccines and their choice of adjuvants. However, many of these vaccines are commercially valuable and experimental detail is neither published nor readily available. In some cases, synthetic antigens have proven to be effective vaccines but suitable adjuvants have not been identified. Rand et al. (119) described a recombinant antigen that protects cattle from the cattle tick, *Boophilus microplus*, however, as yet it has only been tested with FCA. Another example is the heat-stable toxin of *E. coli*. An effective synthetic vaccine has been made from an 18 residue synthetic peptide. Various carriers and adjuvants have been assessed but only coupling to ovalbumin and emulsifying in FCA produced an effective vaccine (44).

Adjuvants have varied effects and the required immune response must be matched to the adjuvant. This was exemplified in the vaccine against *Staphylococcal mastitis* described earlier. The commercial vaccine "Fecundin," designed to increase the incidence of twinning in sheep flocks is an estrone-6 albumin conjugate. The vaccine is injected combined with DEAE-dextran. DEAE-dextran is an adjuvant that stimulates

a weak and transient immune response resulting in multiple ovulations without causing persistent anestrus (26). Other polyelectrolytes such as alginic acid, dextran sulfate, and polyethyleneimine were found to be unsatisfactory (66). The weak immune response required for hormone vaccines, however, is in direct contrast to the strong response necessary for vaccines against infectious diseases.

Vaccination against bacterial toxins using new technologies will almost certainly be successful because this type of vaccine will differ little from existing toxoid vaccines. An effective immune response will require only a high circulating antibody titer to neutralize the toxin. However, other vaccines that comprise soluble recombinant proteins will have to address not only the problem of poor immunogenicity but the need to stimulate cellular immunity.

Recent studies have identified a wide range of potential adjuvants including dextran derivatives (124), pluronic polymers (1), liposomes (137), synthetic carriers (6), and immunostimulating complexes (ISCOMs) (100). Many of these, however, have only been tested in model systems or with whole organism vaccines. The most promising of these are described below.


## 4. New Generation Adjuvants


### A. Immunostimulating Complexes

In general, antigens incorporated into ISCOMs are significantly more immunogenic than antigens presented in the form of micelles (101,102), liposomes (78,104), or inactivated viral complex (6). The humoral immune response of mice to vaccination with ISCOMs containing influenza virus hemagglutinin and neuraminidase glycoproteins has been shown to follow a classic serum antibody response, viz. early appearance of IgM followed by an even distribution of all IgG isotypes (89). An experimental ISCOM vaccine against equine influenza virus induced 10-fold higher serum antibody titres in mice and guinea pigs than conventional killed whole virus or virus micelles (98,138) and this vaccine is now produced commercially. In another case, an experimental ISCOM vaccine composed of 3 $\mu$g of feline leukemia virus antigens (FeLV) was significantly more effective than a commercially available, conventional adjuvanted inactivated whole FeLV vaccine (Leukocell) possessing the same protein dose. Not only did the ISCOM vaccine promote neutralizing antibody levels in 97% of seronegative cats, it also caused an increase in neutralization antibody levels in a majority of seropositve animals (109,110). Further, animals vaccinated with the ISCOM vaccine were protected against infection after oronasal challenge with the virus (109,111).

Recent studies have demonstrated that whereas vaccination of neonates with a live attenuated virus such as measles virus (MV) failed to protect as a consequence of passively transferred circulating maternal antibody interfering with virus replication, ISCOMs containing MV specific F protein stimulated active immunity (33). ISCOMs are also capable of stimulating cell-mediated immunity as measured by delayed type hypersensitivity responses. Analysis of measles virus-specific T-cell clones derived from mice vaccinated with F-protein containing ISCOMs were phenotypically Thy-1$^+$, L3T4$^+$, LyT-2$^-$, a pattern characteristic of murine helper T cells (107). CMI responses as measured by virus-specific DTH or by lymphocyte proliferation assays have been efficiently induced in different animal models by immunization with ISCOMs containing glycoproteins from cytomegalovirus (154), rabies virus (108), influenza virus (73), and measles virus. Recently it has been demonstrated that ISCOMs containing gp160 of HIV-1 were able to stimulate the proliferation of MHC class 1 restricted CD8$^+$ HIV-specific cytotoxic T-lymphocytes (139). This contrasts with the situation observed with most other adjuvants and antigen presentation systems, in that the presentation of soluble antigens to antigen presenting cells leads to the induction of MHC class II restricted T-lymphocytes.

Whereas the incorporation into ISCOMs of amphipathic molecules such as viral glycoproteins is a relatively straightforward process (62, 91,102), the incorporation of nonamphipathic proteins is more difficult due to the absence of exposed hydrophobic domains. Generally non-amphipathic proteins must be modified to some degree in order to achieve efficient incorporation into ISCOMs. At least three techniques have been employed for exposing internalized hydrophobic regions of proteins to allow more efficient incorporation into ISCOMs. These methods include subjecting the protein to pH 2.5 (99,17,116), heating to 70°C (62), or exposing the protein to chaotropic reagents such as 6 M urea (43). Bovine serum albumin has been incorporated into ISCOMs by denaturing the protein using either acidic treatment or by heating, however, ovalbumin can only be incorporated using the latter technique since it is rapidly denatured at pH 2.5. Although these harsh techniques may expose internalized hydrophobic domains and thereby enable incorporation of the antigen into ISCOMs, epitopes required for neutralizing activity may be denatured. This problem has been encountered with the amphiphathic glycoprotein gp51 of bovine leukemia virus (97). Using a panel of monoclonal antibodies it was demonstrated that the detergents, Mega-10 and Triton X-100, usually employed in the generation of ISCOMs denatured the epitopes on gp51 responsible for generating neutralizing antibody. An alternative approach to exposing internalized hydrophobic regions was employed to achieve the incoorporation of small, peptides into ISCOMs (89). The conjugation of hydrophobic fatty acids to the amino-terminal end of synthetic peptides may facilitate the

incorporation of hydrophilic peptides into ISCOMs. Furthermore, the use of conventional heterobifunctional linkers may be considered when attempting to link nonantigenic haptens to preformed ISCOMs containing an amphiphathic protein (90).

## B. Liposomes

Although conventional nonadjuvanted phosphatidylcholine-cholesterol liposomes appear to produce significantly lower humoral immune responses compared to ISCOMs, a considerable amount of work has been directed to enhancing the adjuvanticity of liposomes (54,55). The principal advantage liposomes have over ISCOMs appears to be the relative ease of incorporation of nonamphipathic proteins. Whereas hydrophilic antigens can be incorporated inside the lipid bilayer, hydrophobic proteins are embedded in the lipid bilayer (54). Antigen incorporation in liposomes of up to 80% has been reported, especially in the generation of dehydration–rehydration vesicles (DRV) (56,104). Studies on increasing the adjuvanticity of liposomes have concentrated on increasing structural stability, incorporation of lipophilic adjuvants into the liposome bilayer, addition of cytokines, and targeting of liposomes to immunocompetent cells. Reducing the fluidity of the liposome bilayer by substituting phosphatidylcholine (PC) with phospholipids of higher liquid-crystalline phase transition temperatures or the introduction of cholesterol and sphingomyelin, which can participate in intermolecular hydrogen bonding, significantly increased the half life of liposomes *in vivo* (126). The stability of liposomes (144) and the magnitude of the resultant immune response may be increased (10) by the addition of charged phospholipids into the bilayer. The route of immunization also appears to affect the half-life of liposomes *in vivo* since high-density lipoproteins rapidly sequester the phospholipids from liposomes possessing fluid bilayers (55). Subcutaneous or intramuscular immunization is preferred with these vehicles since up to 80% of liposomes injected by these routes are retained at the side of injection where they are engulfed by macrophages or sequestered in lymph nodes draining the injection site (150).

The lipid bilayer composition not only influences the half life of the liposome *in vivo* but also appears to influence the immune response to particular antigens (30,31,56,143,166). Differences in the immune response to antigens in solid and fluid liposomes have been explained in terms of differences in the processing of soluble tetanus toxoid (TT) and insoluble membrane antigens (polio virus protein 3vp2) by APC. Membrane antigens in solid liposomes are transferred into the plasma membranes of APCs and associate with the MHC without first being processed. Soluble antigens on the other hand must be processed by APC and antigen expressed subsequently with MHC on the cell surface. Solid liposomes may therefore interfere with one of the stages of antigen

processing, between liposome internalization and reexpression of the processed peptide in conjunction with MHC molecules (55).

Recently nontoxic monophosphoryl lipid A (120,140) and lipophilic derivatives of muramyl dipeptide (3,129) have been incorporated into liposomes. Monkeys immunized with liposomes containing the adjuvant 6-O-stearoyl-N-acetylmuramyl-L-alanyl-D-isoglutamine together with *Plasmodium falciparum* merozoites were protected against challenge with a homologous strain of human malaria (129). Encouraging results have also been obtained from the incorporation of intact lipid A into liposomes containing a synthetic malaria sporozoite antigen. Whereas the fusion protein alone was nonimmunogenic in rabbits, strong primary and secondary immune responses were obtained when it was encapsulated in liposomes containing lipid A (122,123).

The incorporation of intact *Salmonella* (78,101,109) lipopolysaccharide into liposomes has been found to induce a marked CMI response that protected mice against lethal challenge with a virulent strain of *Salmonella* possessing the same O-antigen (32). Liposomes containing hepatitis B surface antigens have also been shown to induce strong DTH reactions (93).

Immunomodulators especially IL-2 have been incorporated into liposomes. Liposomes containing IL-2 coincorporated with TT were found to stimulate greater antibody titers in BALB/c mice than IL-2 and TT encapsulated in separate liposomes (142). Increased antibody titers were observed in all the IgG isotypes examined. Aged mice vaccinated with liposomes possessing coincorporated IL-2 and influenza virus antigen had a significantly reduced mortality following challenge with influenza virus compared to mice vaccinated with liposomes containing only virus antigen (94). It is believed that the incorporation of IL-2 inside the liposome increases the half-life of IL-2 *in vitro* and *in vivo* (4,94).

The surfaces of liposomes can be modified with lectins or Fab fragments to specifically target them to the appropriate B or T cell phenotype. This approach would utilise monoclonal antibodies defining T-cell subset-specific epitopes. The covalent coupling of mannosylated albumin to TT containing DRV was found to increase antitoxoid $IgG_1$ and $IgG_{2b}$ titers in BALB/c mice in comparison to conventional TT DRV (47). The enhanced adjuvanticity of mannosylated liposomes is due to enhanced uptake by macrophages, which express mannose receptors on their surfaces.

## C. Vaccinia

A recombinant vaccinia virus may not primarily be regarded as an adjuvant. When Ramon's (117) definition is considered, however, the presentation of an antigen by Vaccinia certainly results in an immune response greater than when the antigen is presented alone.

Three recombinant vaccinia viruses have been developed as veterinary vaccines for cattle. The most promising of these are constructs that express the hemagglutinin or the fusion genes of Rinderpest (167). Both protected cattle from a challenge infection with 1000 times the lethal dose of virus. The vaccine will also protect sheep and goats from the disease, peste-des-petits-ruminants. Mackett et al. (92) have described a construct containing the G protein of vesicular stomatitis virus that partially protects cattle from experimental challenge. In addition, a vaccinia virus expressing rabies antigens is effective in cattle (5,42).

The advantages of vaccinia as a vector for vaccines include induction of high levels of virus neutralizing antibody and generation of specific cytotoxic T-lymphocytes. Such effects are achieved through the capacity of vaccinia to reproduce within host cells and markedly increase levels of antigen available to stimulate the immune system. In addition, vaccinia constructs gain access to the cell cytoplasm and allow presentation to T cells of both virus antigen and the recombinant antigen in the context of class I MHC molecules. This is important for the generation of cytotoxic T cells that are necessary for recovery from intracellular pathogens (14). In addition, initial evidence suggests that incorporation of the gene for IL-2 in a vaccine recombinant will have the double benefit of further enhancing immunogenicity and attenuating the virus (118).

A potential problem with vaccinia is the immune response to the Vaccinia virus itself. After each injection, the immune response will be greater and the boosting effect less as the vaccinia is rapidly eliminated. This would be of importance if several different vaccinia-based vaccines were administered sequentially. One alternative would be the development of a vaccinia recombinant carrying antigens from several different pathogens simultaneously (114).

## D.  Cytokines

In an earlier section, we described the adjuvant effects of individual lymphokines. In practical terms, there has been little real success in using isolated lymphokines as adjuvants. This is not surprising given the large number of stimulatory factors and cellular interactions necessary to generate an immune response. Perhaps as we gain greater knowledge of the precise action of lymphokines on cells of the immune system, we will be able to concoct a "cytokine recipe" to promote the particular response required in a given circumstance. An example of some interest is the use of IFN as an adjuvant. When used as an adjuvant, IFN promotes antibody and T cell-mediated responses (58,115) and has been shown to enhance protection invoked by a vaccine against murine malaria (115). Interestingly, conjugation of IFN and antigen promoted cell-mediated immunity, but had little effect on antibody responses (59). A note of caution should be made about the use of cytokines from other species in

vaccines, because of the possibility of developing an immune response to the foreign cytokine. This would depend, of course, on the degree of similarity between the proteins in the different species. The possibility of coexpressing genes for interleukins and genes for antigens in the same vaccinia constructs is an exciting area with important consequences for viral vaccine development (118).

## E. Covalent Complexes

Small proteins and peptides such as those generated using molecular biological techniques are, in general, poorly immunogenic. The immunogenicity of antigens usually increases with increasing molecular weight (27). This probably relates to the capacity to engage T cell help in the immune response. Larger molecules contain more potential epitopes, giving an APC that has taken up the antigen greater chance to interact with specific T cells. This has been known for many years and has been dealt with by coupling small antigens to carrier molecules such as BSA or ovalbumin (48), diphtheria toxoid (87), or synthetic polymers (6) to increase the molecular weight of the antigen. Other means to promote immunogenicity are to couple peptides to themselves, thus creating polymeric antigens (71). An extension of this concept is to couple a number of antigenic peptides together to create a larger molecule that invokes a greater immune response to each separate peptide (7). Thus each peptide could be viewed as being coupled to a carrier molecule constituted by the other peptide molecules. An even more interesting concept is to couple the protein or peptide to an adjuvant active molecule such as MDP (6). A way to improve the immune response to proteins, particularly CMI, is to couple them to lipids (28). As discussed earlier, this may enhance antigen uptake by APC through better contact with cell membranes.

Finally, a problem with the use of carriers is that there is a phenomenon where there is suppression of the immune response to a new epitope on a carrier to which the host is already immune (61). This may be circumvented, however, by using *Bordatella pertussis* as an adjuvant. Studies have shown that pertussis toxin can break suppression of DTH responses caused by high zone tolerance in mice (141) perhaps as a result of modifying T cell recirculation.

## 5. Conclusion

In recent years, major developments have occurred in the understanding of mechanisms of action of adjuvants. Unfortunately these studies still do not allow us to predict with confidence which adjuvant will work, particularly with recombinant vaccines. In spite of this, several sub-

unit vaccines have either been developed or reached field trial stage with potential for release in the immediate future. The adjuvants for these, however, have been chosen on an ad hoc basis. Development of recombinant DNA technologies to create subunit antigens has outstripped our current level of understanding of adjuvant action. Clearly, much more work needs to be done on the nature of immunopotentiation and adjuvant action before we can, with confidence combine new generation antigens with appropriate adjuvants to make successful vaccines.

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CHAPTER 2

# Genetically Engineered Bluetongue Virus-Like Particles and Their Potential for Use as Vaccine in Sheep

P. Roy

## 1. Introduction

Protection against a viral disease can be accomplished by using a live attenuated virus vaccine, an inactivated virus, or virus subunits either derived (extracted) from infectious material or produced by genetic engineering involving specific gene expression in a vector. Such vectors may be based on bacterial, yeast, or other cellular systems into which the gene is introduced. Certain viruses can also be used as vectors for gene expression. In recent years the baculoviruses, which are pathogenic to insects, have received considerable attention because of their potential for use as viral insecticides and for their potential use as vectors for the introduction and expression of foreign genes into insects and insect cell lines.

Bluetongue disease in sheep and cattle was first described in the late eighteenth century. In a typical case description in sheep the onset was marked by high fever lasting about 5–7 days. By 7–10 days distinctive lesions appeared in the mouth, and the tongue became severely affected and turned dark blue; hence the name "bluetongue" was suggested (31). For many decades the disease was believed to be confined to Africa. The first confirmed outbreak elsewhere occurred in the island of Cyprus (8); subsequently outbreaks have been reported in different parts of the world (3). According to Gambles (8) there had been a number of outbreaks in Cyprus beginning in 1924, but in 1943–1944 it was responsible for about 2500 deaths in sheep. The mortality rate in flocks reached 70%. The same disease in a less severe form had been seen in Palestine in 1943 and in Turkey in 1944, 1946 and 1947 (see review article, 10). An apparently new disease entity of sheep known as "soremuzzle" with close resemblance to bluetongue was first recognized in the United States in 1948. By 1951 bluetongue had been reported in Israel, Pakistan, and the Indian subcontinent (18). In 1956 a major epizootic of bluetongue began in Portugal and extended into Spain (19). Within the first 4 months 179,000 sheep, approximately 75% of the affected animals, were killed. The

outbreaks of the disease in the Middle East, Asia, Southern Europe, and the United States in the early 1940s and 1950s led to the description of bluetongue as an "emerging disease" (11).

The disease has been recognized not only in sheep, goats, and cattle but also other domestic animals (e.g., water-buffaloes and camels) and wild ruminants (Blesbuck, white-tailed deer, elk, pronghorn antelope, etc.). In sheep, the disease is acute and mortality may be high, whereas in cattle and goats the disease is usually milder. However, in contrast to sheep, infected bovines (e.g., cattle) experience prolonged viremia. As early as 1905, Robertson and Theiler (10) showed that the etiological agent of the disease was a filterable virus (31). To date, isolates of the virus have been made in tropical, semitropical and temperate zones of the world including North and South America, Australia, Southern Europe, Israel, Africa, and southeast Asia. An important factor in the distribution of bluetongue virus (BTV) worldwide is the availability of suitable vectors, usually biting midges (gnats) of the genus *Culicoides*. Whenever the required vectors are present BTV can be endemic and infected vectors can be transported by prevailing winds to areas where, if they come into contact with susceptible animals, they may infect them thereby instituting epizootics.

Since early 1900, an attenuated live virus vaccine had been used for more than 40 years despite the evidence that the vaccine was not safe and the resultant immunity was not adequate. Neitz (25) was the first to recognize antigenically different types of bluetongue virus (BTV) and to provide evidence for strain variation in virulence. He found that each strain produced solid immunity against reinfection, but variable protection against challenges with heterologous strains. To date, some 24 different serotypes (BTV-1, -2, etc.) have been identified from different parts of the world (3,27). Modified live virus vaccines have been developed in South Africa and in the United States. In South Africa, sheep are presently vaccinated with three pentavalent live attenuated virus vaccines at 3-week intervals. In the United States, although five BTV serotypes have been identified (BTV-2, -10, -11, -13, and -17), a modified live virus vaccine is available only for BTV-10.

Bluetongue virus (BTV) is the prototype of the genus *Orbivirus* (within the family Reoviridae). The virion contains an icosahedral core which consists of 10 segments of double-stranded RNA as well as two major (VP3 and VP7) and 3 minor protein species (VP1, VP4, and VP6). This core is surrounded by an outer capsid consisting of 2 major proteins, VP2 and VP5 (13,21,36,37). It has been demonstrated both *in vivo* (using intertypic reassortant viruses) (17) and *in vitro* (by translation of each RNA segment) (23) that RNA segment 2 codes for VP2. Using immunoprecipitation techniques, Huismans and Erasmus (14) have shown that VP2 is a major serotype-specific antigen. This was confirmed by analyzing the intertypic reassortant viruses (17). Subsequently, it has been dem-

onstrated that solubilized VP2 polypeptide induces neutralizing anti-
bodies that protect sheep against viral infection (15).

It had previously been demonstrated that BTV has high capabilities for
reassorting the RNA segments between different serotypes. Thus, the
whole virus "vaccines" might play a significant role in the generation of
endemic strains (1,27,32,33). Since genetic engineering techniques allow
safe, large-scale production of subunit vaccines, we have applied these
techniques to the development of vaccines for bluetongue disease using
baculovirus expression vectors.

## 2. Expression of Bluetongue Virus Serotype 10 Outer Capsid Proteins VP2 and VP5

### A. Baculovirus Expression System

This expression system utilizes the major late promoter of the polyhedrin
gene in *Autographa californica* nuclear polyhedrosis virus (AcNPV) (24).
The life cycle of this virus is characterized by the production of two forms
of progeny; extracellar virus particles (ECV) and occluded virus particles
(OV). ECVs are produced relatively early in infection (from 12 hrs
onward) and are released by budding from the cell surface. They mediate
the systemic infection of the insect and also account for the mode of
infection in cell culture. Later in the infection cycle (from 18 hrs onward)
viral progeny are occluded into a paracrystalline matrix composed pri-
marily of a 29-kDa protein called polyhedrin. These occlusions, called
polyhedra, protect the progeny virus during horizontal transmission and
effect their release in a new host by dissolving in the alkaline environment
of the insect gut. Polyhedra accumulate in infected cells for 4–5 days until
cell lysis, by which time polyhedrin may constitute up to 50% of the total
cell protein. Because cell-to-cell infection is propagated by ECVs, the
synthesis of polyhedrin protein is a nonessential function for the replica-
tion of AcNPV in cell culture. The use of AcNPV as an expression system
therefore involves replacement of the polyhedrin gene with a foreign
gene, which, due to the control of the polyhedrin promoter, has the
potential to be expressed to a high level.

A number of transfer vectors are available to construct such recom-
binant baculoviruses. One that has found particular favour is pAcYM1
(22). This, in common with most other baculovirus expression vectors,
consists of a restriction enzyme fragment of the AcNPV genome encom-
passing the polyhedrin gene, cloned into a high copy-number bacterial
plasmid. The polyhedrin gene sequence has been deleted in pAcYM1 and
replaced by a *Bam*HI linker to allow for the insertion of a foreign gene
immediately downstream of the polyhedrin promoter. The unchanged
wild-type AcNPV DNA sequences that flank the inserted gene mediate

homologous recombination when a cell is transfected with the plasmid DNA and wild-type AcNPV DNA (Fig. 2.1).

Recombinant baculoviruses are sought by screening for plaques (under agarose) that lack visible polyhedra (i.e., virus clones that may have an inserted foreign gene in lieu of the initial sequences of the polyhedrin protein). The screening is either performed directly, or indirectly following liquid culture of progeny viruses derived from all the transfected cells. Polyhedrin-minus virus clones are then analyzed for (a) the presence of viral DNA containing the foreign gene (Southern analyses), (b) its mRNA species (Northern analyses), and (c) the foreign gene protein (using ELISA).

## B.  Construction of AcNPV-BTV Recombinant Transfer Vectors

In order to analyze structure–function relationships of the 10 RNA segments and their gene-products, we have synthesized the DNA copies of all 10 RNA segments of BTV-10. The availability of complete cDNA clones representing the 10 discrete double-stranded RNA (dsRNA) segments and the availability of their sequence database (28, 29) allowed us to manipulate each DNA to be expressed to high level in insect cells using recombinant baculoviruses.

The strategy employed for construction of the baculovirus transfer vector containing each BTV-10 gene is represented in Figure 2.2. Each of the cDNA clones has been manipulated and inserted into the *Bam*HI site of the transfer vector. In order to transfer each gene into the AcNPV genome, *S. frugiperda* cells were cotransfected with recombinant DNA containing only one of the 10 BTV genes and the infectious AcNPV DNA. Putative recombinant viruses were isolated from the infected cells by selecting progeny viruses exhibiting no evidence of occlusion bodies and after 3 successive cycles of plaque purification, stocks of 2 recombinant viruses were obtained. Each polyhedrin-free recombinant virus was then screened for BTV DNA and mRNA by standard "Southern" and "Northern" hybridization procedures. Ten such recombinant viruses were selected each containing one BTV-10 gene (29). The recombinants were designated as follows: AcBTV-10.1 (VP1), AcBTV-10.2 (VP2), AcBTV-10.3 (VP3), AcBTV-10.4 (VP4), AcBTV-10.5 (VP5), AcBTV-10.9 (VP6), AcBTV-10.7 (VP7), AcBTV-10.6 (NS1), AcBTV-10.8 (NS2), and AcBTV-10.10 (NS3).

## C.  Expression and Characterization of BTV Antigens

In order to demonstrate that BTV proteins were synthesized in recombinant baculovirus infected cells, *S. frugiperda* cells were infected with one of the 10 recombinant viruses and infected cell lysates were analyzed by gel electrophoresis. As shown in Figure 2.3, each recombinant virus syn-
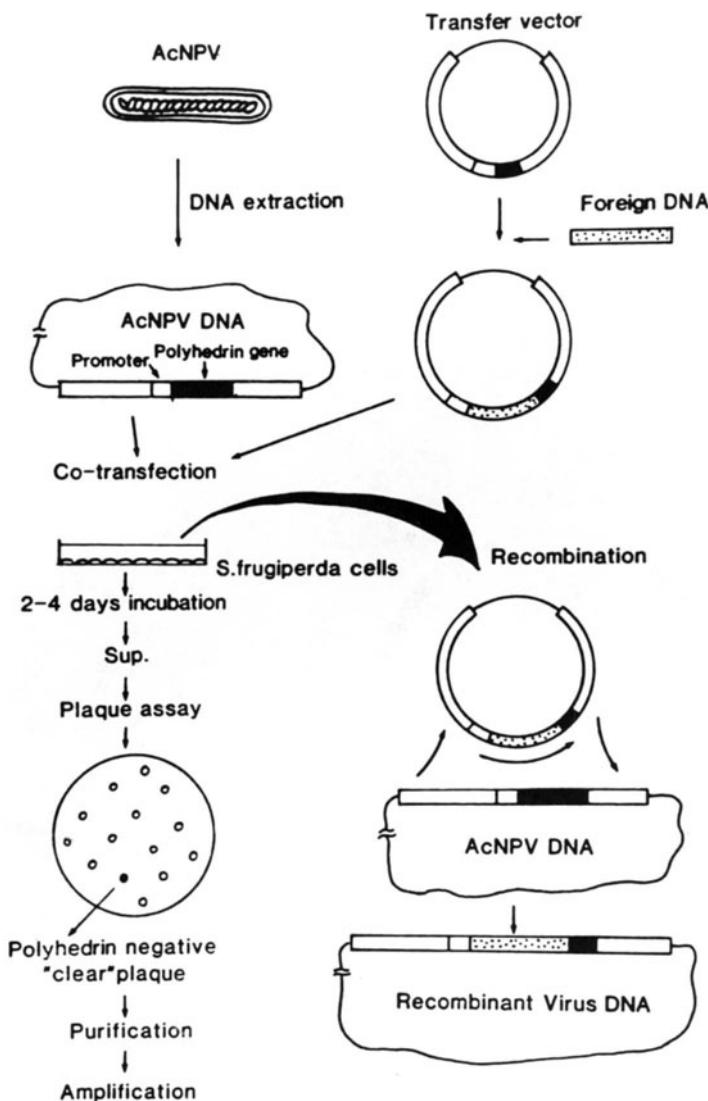
**Fig. 2.1.** General protocol for the insertion of foreign DNA into infectious *Autographa californica* nuclear polyhedrosis virus. Foreign DNA inserted at a locus of a cloned AcNPV subgenomic fragment is introduced into *Spodoptera frugiperda* culture together with infectious AcNPV DNA by transfection procedures. *In vivo* recombination occurs between the AcNPV DNA sequences flanking the foreign insert and homologous DNA sequences on the replicating AcNPV genome forming a novel recombinant DNA molecule, which can in turn be packaged into infectious recombinant AcNPV virus within 48–72 hrs.
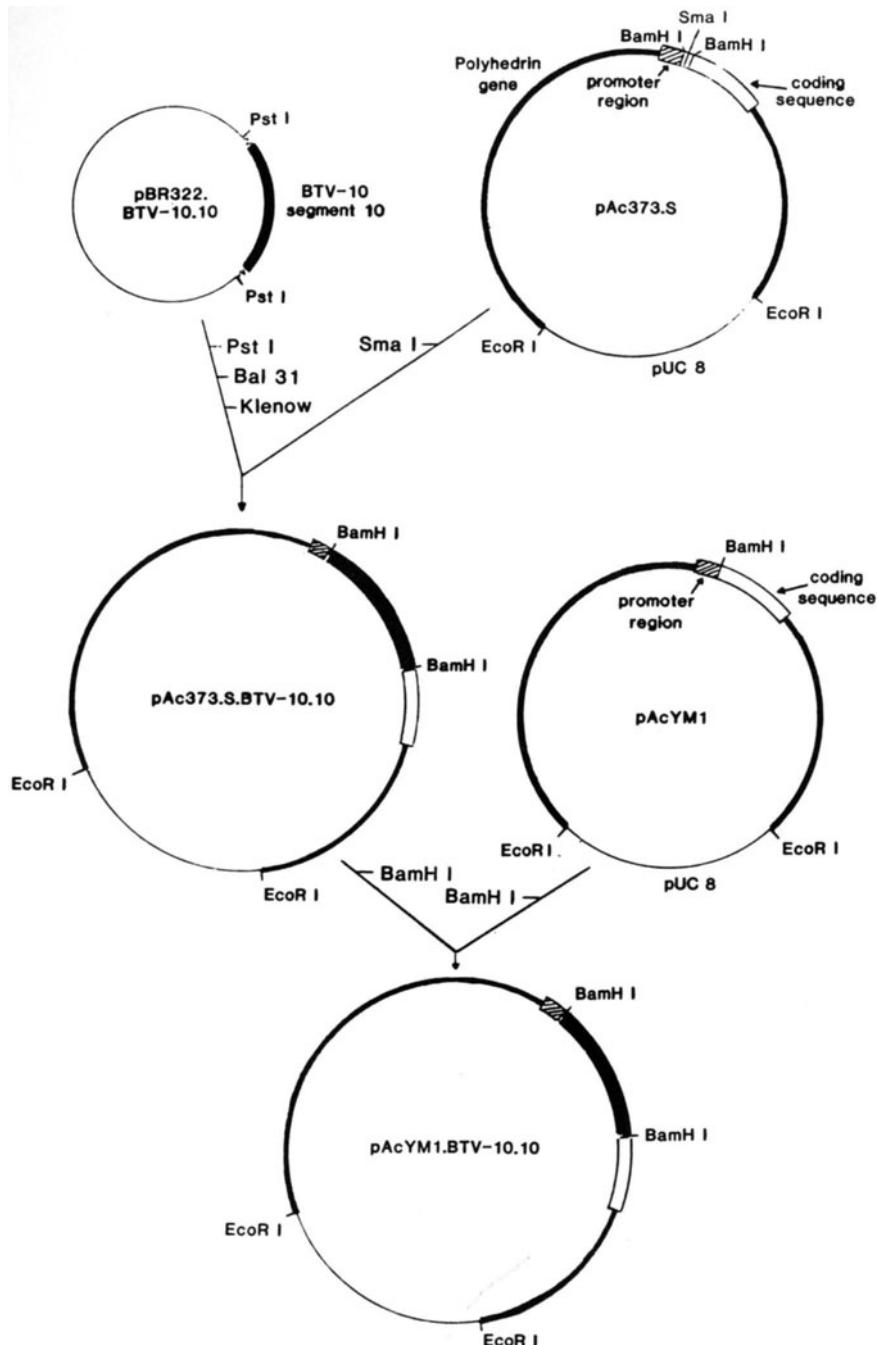
**Fig. 2.2.** Construction of recombinant baculovirus transfer vector containing BTV-10 gene (e.g., RNA segment 10 of BTV-10).
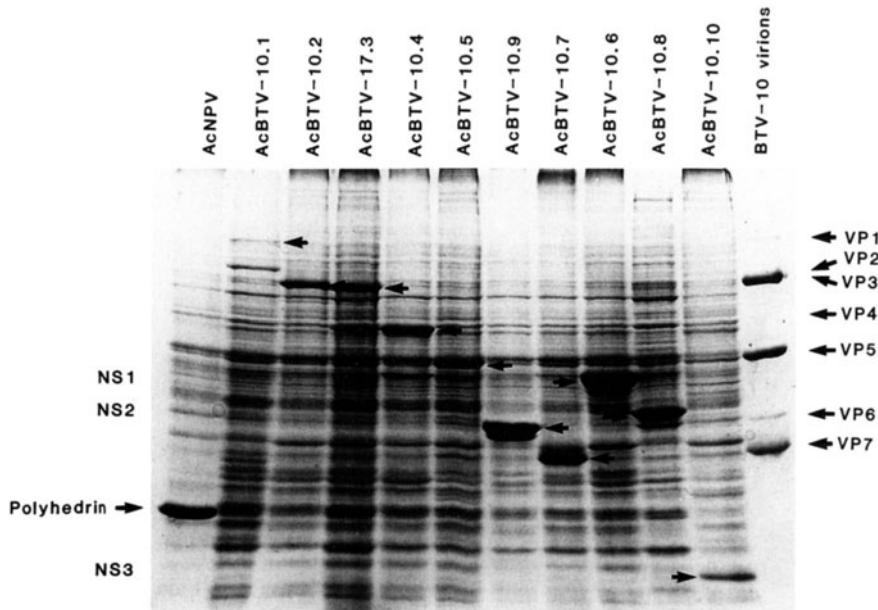
**Fig. 2.3.** SDS-PAGE analyses of recombinant baculoviruses (AcBTV) that express the 10 gene products of BTV-10, compared to marker proteins (kD), BTV virion proteins, AcNPV, and mock infected insect cells (Sf). In addition to the BTV proteins, the AcNPV polyhedrin protein is identified. The resolved proteins are stained with Coomassie Brilliant Blue.

thesized a protein with a molecular size equivalent to the estimated sizes (e.g., 25 to 150 kD) of BTV proteins. It was clear that the level of expression of each protein was significantly higher than the authentic BTV proteins, some proteins (e.g., VP7 and NS1) were expressed in the order of 40 to 50 mg/liter of culture.

In order to confirm that the expressed proteins were indeed BTV derived proteins, they were subjected to Western analyses using BTV-10 antisera. Strong positive signals obtained for each expressed protein have confirmed their origin.

## D. Neutralization of Bluetongue Virus Infection by Antisera Raised to Expressed VP2 and VP5 Antigens

To assess the neutralization ability of the two outer capsid proteins VP2 and VP5, monospecific antisera were raised to the expressed VP2 and VP5 proteins. Each recombinant protein was analyzed by polyacrylamide gel electrophoresis (10% SDS-PAGE) and visualized by soaking the gel in 0.25 M KCl at 4°C. The protein bands were excised, macerated, and

**Table 2.1.** Plaque reduction neutralization titers of antisera raised to expressed VP2 and VP5.

| Antisera | BTV serotypes | | | |
| --- | --- | --- | --- | --- |
| | 10 | 11 | 13 | 17 |
| Rabbit VP2 antisera | >640 | >160 | 0 | >160 |
| Preimmune rabbit sera | 0 | 0 | 0 | 0 |
| Mouse VP5 ascitic fluid | 0 | 0 | 0 | 0 |
| Control ascitic fluid | 0 | 0 | 0 | 0 |
| Mouse antisera | | | | |
|   AcBTV-10-2 infected *S. frugiperda* cells | 205 ± 74[a] (n = 4) | ND[b] | ND | ND |
|   AcBTV-10-5 infected *S. frugiperda* cells | 51 ± 23[b] (n = 4) | ND | ND | ND |
|   AcNPV infected *S. frugiperda* cells | 55 ± 40 (n = 4) | ND | ND | ND |

[a] Significantly different from AcNPV infected *S. frugiperda* cells at the $p = 0.05$ level.
[b] ND, not done.
[c] Not significantly different from AcNPV infected *S. frugiperda* cells at the $p = 0.05$ level.

used to immunize either rabbits, to produce antiserum, or mice, to produce ascitic fluids, using Freund's incomplete adjuvant. The resulting rabbit antisera raised to the expressed VP2 and mouse ascitic fluid raised to the expressed VP5 recognized the corresponding authentic proteins in BTV-10, confirming that both proteins were immunologically equivalent to those of the authentic BTV-10 proteins (16,20).

These sera, were subsequently tested for their ability to neutralize BTV-10 *in vitro* using plaque reduction assay and the plaque reduction neutralization titers were expressed as the reciprocal of the antiserum dilution that gave a 50% reduction in plaque number (Table 2.1). VP2 antisera neutralized the virus at a titer of greater than 640 whereas VP5 ascitic fluid, control ascitic fluid, and preimmune rabbit sera did not. The VP2 antiserum was also tested for its ability to neutralize the heterologous BTV serotypes 11, 13, and 17. Serotypes 11 and 17 were neutralized to a titer greater than 160 whereas serotype 13 was not. This pattern of cross-serotype neutralization reflects the pattern of homologies between the VP2 proteins of the serotypes (7,9,38). In order to check that the failure of the expressed VP5 protein to induce neutralizing antisera was not due to denaturation of the protein during SDS-PAGE, whole infected *S. frugiperda* cells were also used to raise mouse antisera. Groups of four mice each received two inoculations of $3 \times 10^6$ infected cells, intraperitoneally, and the resulting sera were tested for their ability to neutralize. Titers were 205, 51, and 55 for cells infected with AcBTV-10-2, AcBTV-10-5, and AcNPV, respectively. Thus, even in the absence of denaturation by SDS-PAGE, VP5 was unable to elicit neutralizing antibodies even though VP2 could.

The data presented support the conclusion that the outer capsid protein VP2 plays a direct role in neutralization of BTV while VP5 does not, although a synergistic effect when VP5 used with VP2 cannot be ruled out.

## 3. Vaccination of Sheep with Expressed Antigens and Protection against Virulent Bluetongue Virus

### A. Vaccination with VP2 and VP5 Proteins

To determine the protective capabilities of these recombinant proteins against virus infection in sheep, experiments were performed using BTV-free sheep in collaboration with the scientists in The Veterinary Research Institute, Onderstepoort, South Africa (30). Groups of Merino sheep were used for the vaccination trials as follows. Three groups of four sheep each (groups I, II, and II) were injected subcutaneously with various doses of the recombinant BTV-10 VP2 protein (derived from AcBTV-10.2). Booster doses were administered on day 21 (and also on day 42 for group I) (Tables 2.2 and 2.3). The sheep in group I received approximately 50 $\mu$g of VP2 antigen per inoculation, while groups II and III received approximately 100 and 200 $\mu$g, respectively, per inoculation. To investigate if VP5 (the second outer capsid protein) plays a role in the induction of neutralizing antibodies, four sheep (group IV) were injected with a mixture of VP2 (ca. 50 $\mu$g) and VP5 (ca. 20 $\mu$g) and were similarly boosted on days 21 and 42. A control group of four sheep (group V) received only saline. To evaluate if adjuvant enhanced the immunity and induction of neutralizing antibodies in sheep, two animals in each group received vaccine without adjuvant, while the other two were given vaccine emulsified in incomplete Freund's adjuvant.

Serum samples were collected from each sheep at regular intervals between days 7 and 75 postimmunization and were tested for the presence of neutralizing antibodies against BTV-10 by the plaque reduction neutralization test. All the sheep immunized with or without adjuvant elicited BTV-10-neutralizing antibodies, albeit to various levels (Table 2.2). Higher antibody titers were obtained when adjuvant was included for sheep in groups III and IV (Table 2.2). In contrast, adjuvant did not seem to have any effect when low doses (50 to 100 $\mu$g) of VP2 antigen were used (groups I and II in Table 2.2). When a small amount of VP5 (ca. 20 $\mu$g) was combined with ca. 50 $\mu$g of VP2 antigen, higher titers of neutralizing antibodies were elicited (Table 2.2). In all cases, the plaque reduction titers decreased with time. No neutralizing antibodies were detected in the sera of sheep inoculated with saline alone (group V).

**Table 2.2.** Serum plaque reduction titers of sheep inoculated with recombinant BTV antigens.[a]

| Group No. | Antigen | Sheep No. | Adjuvant | Inoculation (day) | | | Serum neutralization titers against BTV-10[b] (days) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 0 | 21 | 42 | 25 | 42 | 48 | 50 | 52 | 60 | 67 | 74 |
| I | VP2: ~50 µg | 1 | − | √ | √ | √ | 32 | 32 | 64 | 64 | 32 | 64 | 16 | 8 |
| | | 2 | − | √ | √ | √ | 32 | 32 | 32 | 32 | 16 | 8 | 8 | 4 |
| | | 3 | + | √ | √ | √ | 16 | 16 | 32 | 32 | 32 | 16 | 12 | 8 |
| | | 4 | + | √ | √ | √ | >4 | 4 | 16 | 16 | 16 | 8 | 8 | 8 |
| II | VP2: ~100 µg | 5 | − | √ | √ | − | >32 | 64 | 16 | 16 | 16 | 16 | 8 | 8 |
| | | 6 | − | √ | √ | − | >32 | 64 | 32 | 32 | 16 | 16 | 12 | 8 |
| | | 7 | + | √ | √ | − | 32 | 32 | 16 | 16 | 16 | 8 | 6 | 4 |
| | | 8 | + | √ | √ | − | 16 | 8 | >4 | >4 | >4 | >4 | >4 | 4 |
| III | VP2: ~200 µg | 9 | − | √ | √ | − | >32 | 128 | 32 | 32 | 32 | 16 | 16 | 8 |
| | | 10 | − | √ | √ | − | >32 | 64 | 16 | 16 | 16 | 16 | 8 | 8 |
| | | 11 | + | √ | √ | − | >32 | 128 | 64 | 64 | 32 | 32 | 32 | 16 |
| | | 12 | + | √ | √ | √ | >32 | 512 | 128 | 128 | 128 | 64 | 64 | 32 |
| IV | VP2: ~50 µg | 13 | − | √ | √ | √ | >4 | >4 | 16 | 8 | 8 | 8 | 4 | 4 |
| | | 14 | − | √ | √ | √ | >4 | 4 | 16 | 8 | 8 | 8 | 4 | 4 |
| | VP5: ~20 µg | 15 | + | √ | √ | √ | >32 | 128 | 512 | 256 | 128 | 128 | 128 | 96 |
| | | 16 | + | √ | √ | √ | 32 | 64 | 128 | 128 | 64 | 32 | 32 | 24 |
| V | Saline | 17 | − | √ | √ | √ | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 |
| | | 18 | − | √ | √ | √ | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 |
| | | 19 | + | √ | √ | √ | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 |
| | | 20 | + | √ | √ | √ | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 |

[a] Pairs of animals were inoculated with (+) or without (−) incomplete Freund's adjuvant on the days indicated (√).

[b] Reciprocal of the dilution that caused a 50% plaque reduction.

**Fig. 2.4.** Immune precipitation of ^{35}S-labeled BTV-10 protein with sera from sheep injected with 100 μg of VP2 alone (lane B) or with 50 μg of VP2 and 20 μg of VP5 (lane C). Lane A shows the immunoprecipitation of ^{35}S-labeled BTV proteins with anti-BTV-10 antiserum.
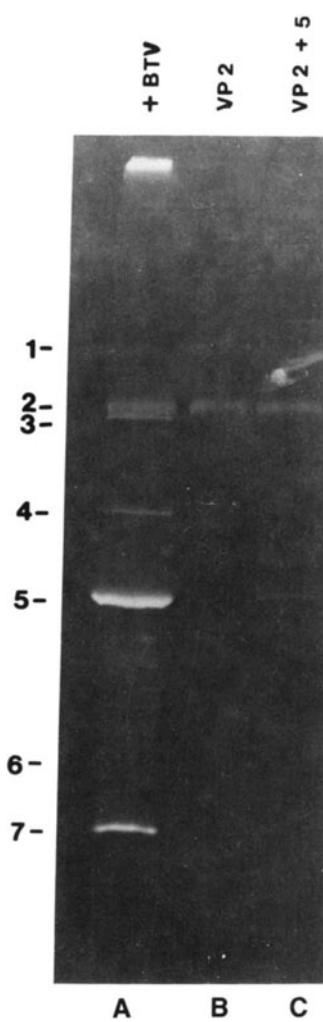
## B. Immunoprecipitation of BTV-10 Proteins by Immunized Sheep Sera

Immunoprecipitation analyses were used to analyze the specificities of the immune responses to the various combinations of expressed BTV antigens. From each group of sheep, only sera with high neutralizing-antibody titers were analyzed. The assays were performed by incubating samples of ^{35}S-labeled soluble protein fraction (S100) obtained from BTV-infected BHK-21 cell cultures with a sample of the respective serum. The 48-day serum of a sheep that received 200 μg of VP2, precipitated VP2. The 51-day serum of a sheep that received both the VP2 (50 μg) and VP5 (20 μg), precipitated both proteins (Fig. 2.4) (30).

**Table 2.3.** Immune status of vaccinated sheep after virulent virus challenge.

| Group No. | Inoculum | Sheep No. | Serum neutralization titers against BTV-10 (21 days postchallenge) | Clinical reaction index[a] | Viremia[b] (days postchallenge) |
|---|---|---|---|---|---|
| I | VP2: ~50 μg | 1 | 160 | 0.0 | — |
| | | 2 | 640 | 1.4 | 4–6 |
| | | 3 | 40 | 0.0 | — |
| | | 4 | 320 | 3.1 | — |
| II | VP2: ~100 μg | 5 | 40 | 0.0 | — |
| | | 6 | >20 | 0.0 | — |
| | | 7 | >20 | 0.0 | — |
| | | 8 | 80 | 0.0 | — |
| III | VP2: ~200 μg | 9 | 80 | 0.0 | — |
| | | 10 | 40 | 0.0 | — |
| | | 11 | 80 | 0.0 | — |
| | | 12 | >20 | 0.0 | — |
| IV | VP2: ~50 μg | 13 | 40 | 0.0 | — |
| | VP5: ~20 μg | 14 | 40 | 0.0 | — |
| | | 15 | 120 | 0.0 | — |
| | | 16 | 60 | 0.0 | — |
| V | Saline | 17 | >640 | 7.4 | 4–9 |
| | | 18 | 640 | 5.0 | 4–10 |
| | | 19 | 640 | 4.6 | 4–9 |
| | | 20 | >640 | 5.1 | 4–10 |

[a] Clinical reaction index: $(a + b + c)$: (a) the fever score—the cumulative total of fever readings above 40°C on days 3–14 after challenge (maximum score 12); (b) the lesion score—lesions of the mouth, nose and feet were each scored on a scale of 0–4 and added together (maximum score 12); (c) the death score—4 points if death occurred within 14 days postchallenge.

[b] Viremia assayed in eggs; —, not detected; numbers refer to days sheep blood tested positive for viremia.

## C. Protection Capabilities of VP2 and VP5 Antigens against Virulent Virus Challenge

To assess the ability of the recombinant viral antigens to induce a protective immunity, on day 75 (33 days after the second booster injection) all sheep were challenged by subcutaneous injection with infective sheep blood of a South African strain of virulent BTV-10. From day 1 postchallenge, rectal temperatures were recorded twice daily and the sheep were carefully examined for clinical manifestations of bluetongue disease. The clinical reaction index was expressed numerically (Table 2.3, footnote a). Whole-blood samples were collected daily after the virus challenge for the first 15 days and were screened for viremia by passage in 10- and 12-day-old embryonated chicken eggs (Table 2.3). The recovered virus was identified as BTV-10. Plaque reduction titers were determined for sera taken at 21 days postchallenge (Table 2.3).

Apart from two sheep of group I that received a low dose (ca. $50\,\mu$g) of VP2 (Table 2.3, sheep 2 and 4), all of the sheep injected with recombinant BTV antigens were immune to virulent virus challenge. None of these sheep developed any clinical symptoms of bluetongue disease or demonstrable viremia, although they did show mild anamnestic antibody responses indicative of virus replication. Surprisingly, however, virus was recovered from the blood of only one sheep (no. 2) and not from that of the other (no. 4). All of the control sheep, on the other hand, developed typical bluetongue disease with a relatively high clinical reaction index. In addition, the postchallenge blood of the control sheep was viremic, and their sera contained high neutralizing titers, which is characteristic of a primary infection.

The results confirmed that the outer capsid protein VP2 is the main determinant of the neutralization-specific immune response and that it induces protection. Our data indicated that $50\,\mu$g of the expressed VP2 alone was insufficient to confer total protection. Two successive injections of $100\,\mu$g of VP2 provided full protection, a finding that closely correlates with that of Huismans and associates (15). However, $50\,\mu$g of VP2 together with $20\,\mu$g of VP5 protected the sheep. Other amounts of the two antigens have yet to be assessed. Why the VP5 antigen enhances the neutralization (and protective) response is not known. No neutralizing monoclonal or monospecific antibody that reacts specifically with VP5 protein has yet been obtained. However, our data indicate that a combination of VP2 and VP5 antigens elicited significantly higher titers of BTV-neutralizing antibodies. It is possible that VP5 enhances the immune responses indirectly by interaction with VP2 and by affecting the conformation of VP2 and, consequently, its serological properties.

## 4. Synthesis of Virus-Like Particles Lacking Genetic Materials

Formation of complex structures and evaluation of the interactions of their protein components can be attempted *in vitro*, but this may introduce artifacts, depending on the experimental conditions used. Synthesis of proteins in eukaryotic cells by an expression vector provides an opportunity to investigate macromolecular interactions under more natural, intracellular conditions.

However, until recently, a limitation of the baculovirus expression system has been that simultaneous expression of several proteins within a single cell requires coinfection with two recombinant viruses, each containing a single foreign gene. To overcome various technical problems of coinfection, baculovirus multiple-expression vectors, such as pAcVC3, have been constructed (3) and used to express more than one foreign gene (34). This vector has duplicated copies of the polyhedrin promoter
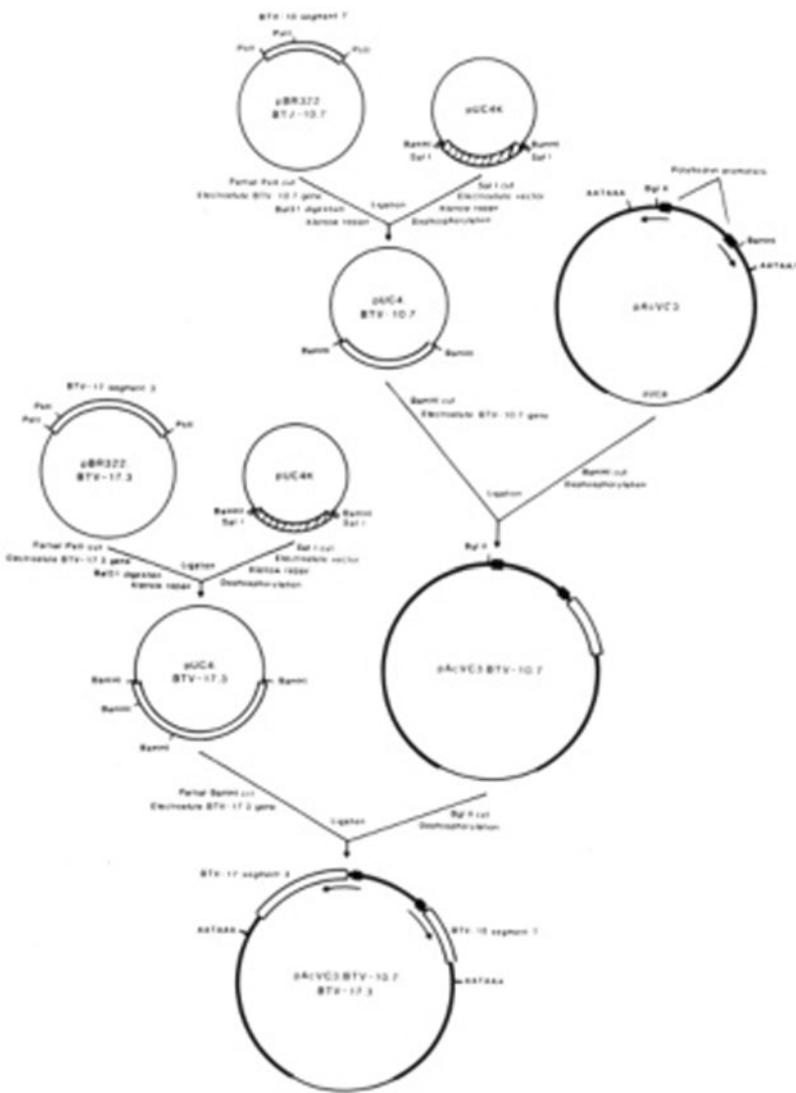
**Fig. 2.5.** Construction of a dual-expression transfer vector showing the appropriate manipulations for insertion of the BTV L3 and M7 genes.

and transcription termination sequences. In plasmid transfer vector pAcVC3 unique enzyme restrictions sites are located downstream of each copy of the (AcNPV) polyhedrin promoter. The sites allow two foreign genes to be placed under the control of their own copy of the polyhedrin
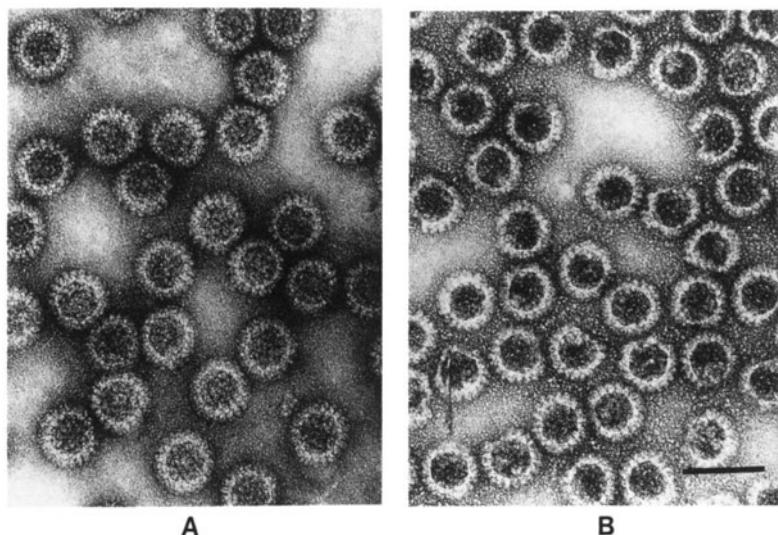
**Fig. 2.6.** Electron micrographs of authentic BTV core particles (A) and the empty core particles (B) synthesized in insect cells by a dual recombinant baculovirus.

transcriptional machinery. The promoters are arranged in opposite orientations to minimize the possibility of homologous sequence recombination and excision of either of the foreign genes (Fig. 2.5). To synthesize BTV core-like and virus-like particles, dual expression vectors have been used.

## A. Expression of Major Core Proteins VP3 and VP7 Using Dual Expression Vectors and Synthesis of Core-Like Particles

In order to synthesize core-like particles (CLP), cDNA copies representing RNA segments L3 of BTV-17 and M7 of BTV-10 were cloned into the dual transfer vector pAcVC3 as shown in Figure 2.5 and dual recombinant baculovirus was isolated (5). When *S. frugiperda* cells were infected with the recombinant baculovirus they synthesized two unique protein species that comigrated with VP3 and VP7 derived from purified BTV cores or virus particles (Fig. 2.6A). The sizes of the expressed proteins agreed with those expected for VP3 and VP7 on the basis of their amino acid compositions [i.e., 103,416 (26) and 38,548 (39) Da, respectively] (28).

Confirmation that the expressed proteins represented authentic BTV polypeptides was provided by Western immunoblot analyses with antiserum raised to BTV-10 virus particles (5). This antiserum reacted strongly with the VP3 and VP7 species in a cell lysate of *S. frugiperda*

cells infected with the dual recombinant baculovirus, while no reaction was detected with mock-infected or wild-type AcNPV-infected cells. Virion-derived BTV-10 core particles were included as a control, which showed that the BTV-10 antiserum also contained antibodies to minor core protein VP6 (but not to VP1 or VP4).

The electron micrographs of *S. frugiperda* cells infected with the recombinant baculovirus showed large aggregates of foreign material in the cytoplasm that, under higher magnification, appeared to consist of spherical particles (5). This material was isolated by lysing cells with NP40 and purification of the structures by discontinuous sucrose gradient centrifugation. When examined under the electron microscope, the material was found to consist of empty core-like particles whose size and appearance were indistinguishable from authentic BTV cores prepared from BTV-infected BHK cells (6).

## B.  Simultaneous Expression of Four Bluetongue Viral Proteins and Isolation of Virus-Like Particles (VLPs)

To determine if double capsid particles can be synthesized a second dual transfer vector was constructed by inserting cDNA copies respresenting the complete coding sequences of the BTV L2 and M5 genes in pAcVC3 vector (6). The recombinant virus was isolated by similar procedures as described above and expression of VP2 and VP5 protein were confirmed; although no morphological structures could be detected.

To assess the interaction of VP2 and VP5 with the BTV CLPs, insect cells were coinfected with both dual recombinant baculoviruses (to coexpress VP2, VP3, VP5, and VP7). The cells were harvested at 48 hrs postinfection and lysed with the nonionic detergent Nonidet P-40, and the released particles were purified to homogeneity by centrifugation on discontinuous sucrose gradients. Expression of four BTV proteins was confirmed by analyzing the particles in gel electrophoresis (Fig. 2.7). When examined under the electron microscope, the particles observed consisted of core surrounded by a thick outer capsid (Fig. 2.8, "P," large arrows). The diameters of the largest particles were estimated to be of the order of 85 nm, i.e., comparable to those of BTV. Some simple CLPs were observed in the preparation (Fig. 2.8, "C" thin arrows). Their diameters were estimated to be of the order of 65 nm. A range of inter-mediate structures was also observed, apparently with various amounts of the outer capsid proteins attached. These may reflect different stages in particle assembly. Interestingly, the centers of all types of particles (CLPs, VLPs, intermediate VLPs) exhibited an icosahedral configuration. The smaller size of the central area of the VLPs is presumably due to the density of the outer capsid proteins. The icosahedral configuration of the center was also apparent in several authentic BTV particles where stain had penetrated the particles.
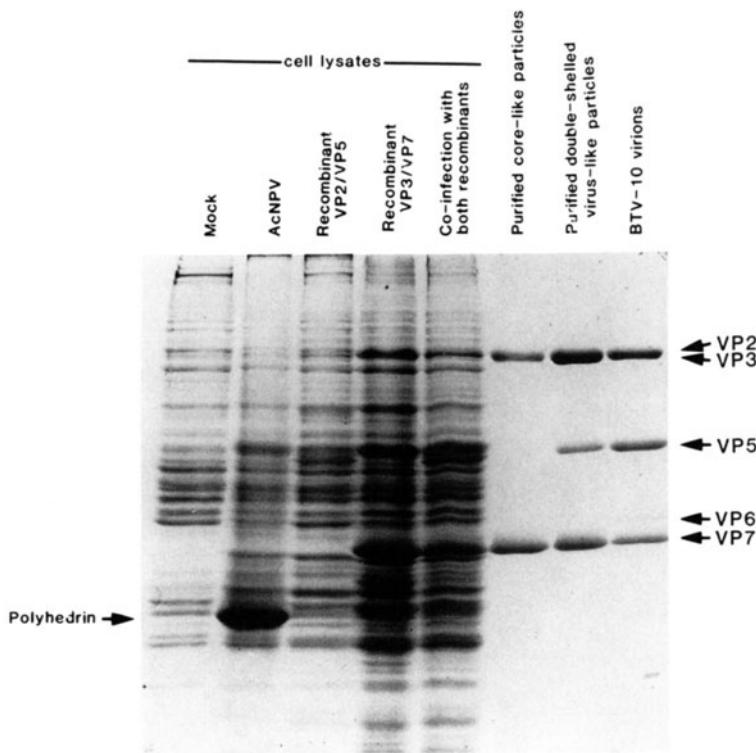
**Fig. 2.7.** SDS-PAGE analyses of BTV cores derived by coexpression of VP3 and VP7, and particles made by coexpression of VP2, VP3, VP5, and VP7.

## C. Biochemical and Immunological Characterization of VLPs

In order to determine if all four proteins were indeed present in the VLPs, the expressed particles were analyzed by SDS-PAGE and Western immunoblot analyses and shown to contain large amounts of VP2 and VP5 (Fig. 2.7) in addition to VP3 and VP7. However, due to the various amounts of VP2 and VP5 proteins attached to the cores, the stoichiometries of the proteins were not determined. Phenol extraction of purified expressed particles and examination by optical density measurements or agarose gel electrophoresis failed to demonstrate the presence of nucleic acid.

The immunogenicity and hemagglutinating activity of the VLPs was investigated. Guinea pig sera raised against purified CLPs and the double-shelled VLPs were tested for their ability to neutralize the infectivity of BTV-10. As expected, serum raised to the cores exhibited no neutralizing activity, while in a 50% plaque reduction test, substantial neutralization was demonstrated by the serum raised to the double-shelled VLPs at a
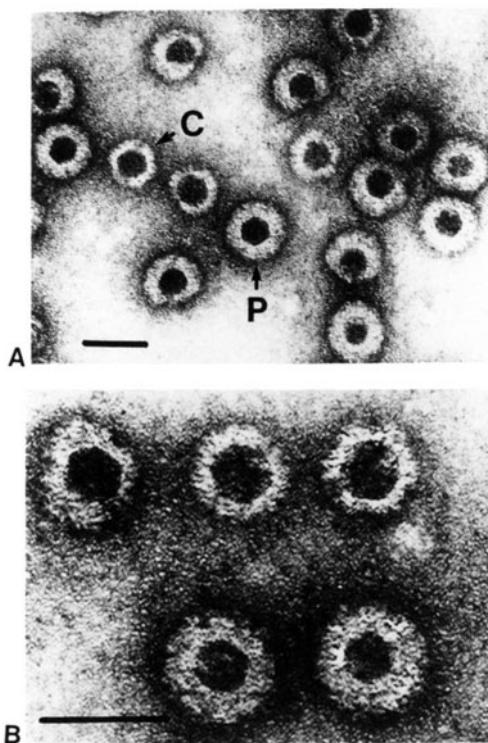
**Fig. 2.8.** Coexpression of VP2, VP3, VP5, and VP7 by recombinant baculoviruses forms virus-like particles (A). C, core formed by VP3 and VP7; P, double capsids formed by VP2, VP3, VP5, and VP7. The high-magnification micrograph (×30,000) shows the appearance of expressed VLPs (B).

dilution of 1:10,000. Monospecific serum raised in guinea pigs to VP2 gave titers of <640. As expected, none of the other three monospecific sera (to VP3, VP5, or VP7) raised in rabbits or mice neutralized BTV. Purified VLPs exhibited hemagglutination (Table 2.4) as is observed with authentic virus (35). Purified CLPs did not hemagglutinate. These data agree with the demonstration that VP2 is the hemagglutinating protein of BTV (18). Monospecific antiserum raised to VP2 inhibited hemagglutination by the VLPs. Monospecific antisera raised to the other component proteins (VP3, VP5, or VP7) had essentially no effect (Table 2.4). Unlike authentic BTV, the VLPs were noninfectious when assayed in mammalian cells.

From the morphological appearances, biochemical constituents, and immunological properties, the assembled empty particles appeared to be similar to the double capsid structures of BTV, lacking the genetic materials and the three minor proteins.

**Table 2.4.** Hemagglutination analysis of BTV double-shelled VLPs.[a]

| Substrate or serum tested | HA or HI titer | Plaque reduction neutralization titer |
|---|---|---|
| Substrate (20 μg) | HA titer | |
|   Single-shelled CLPs | <2 | |
|   Double-shelled VLPs | 2048 | |
| Serum | HI titer | |
|   Preimmune GP | 4 | 0 |
|   GP anti-VLP | >2048 | 10,000 |
|   GP anti-CLP | <2 | 0 |
|   GP anti-VP2 | Not done | <640 |
|   Preimmune rabbit | 16 | 0 |
|   Rabbit anti-VP2 | >1024 | <640 |
|   Rabbit anti-VP7 | 2 | 0 |
|   Preimmune mouse | 4 | 0 |
|   Mouse anti-VP5 | 8 | 0 |
|   Mouse anti-VP3 | 32 | 0 |

[a] HA titers are expressed as the reciprocal of the highest serial dilution that gave complete hemagglutination. Antisera raised to the particles and baculovirus-expressed BTV proteins were used in hemagglutination inhibition (HI) and plaque reduction neutralization tests. The inhibition titers are expressed as the reciprocal of the highest serial dilution of the serum that gave complete inhibition of hemagglutination. The plaque reduction neutralization titers are expressed as the reciprocal of the serum dilution that gave a 50% reduction in plaque numbers. Guinea pig (GP), rabbit, and mouse sera were used as indicated.

# 5. Efficacy of VLPs as Vaccine

The assembly of the BTV like particles lacking genetic material is indicative of the potential of this technology for the production of a new generation of viral vaccines. Since baculovirus expressed VP2 proteins together with expressed VP5 had conferred protection against virulent virus challenge in sheep, it could be anticipated that these particles with correct structural conformation should have even better protective capabilities for the disease. The efficacy of their protective capabilities was therefore assessed in sheep.

## A. Vaccination of Sheep with VLPs

Twenty-four Merino sheep were divided into three groups and each group was inoculated with different amounts of purified particles (10–200 μg/dose) in the presence of either alumunium hydroxide [Al(OH)$_3$], incomplete Freund's adjuvant (IFA), or Montamide ISA-50 (a generous gift from Seppic, Paris) as indicated in Table 2.5. For each concentration of proteins, two sheep were used. In addition, four sheep received only saline and another four, a live attenuated BTV-10 vaccine developed in Onderstepoort, South Africa. Each animal was boosted with equivalent

**Table 2.5.** Vaccination of sheep.

| Inoculum | Adjuvants | Sheep No. | Serum neutralization titers against BTV-10 on day: | | | | | | | | | | | | | | | | | | | CRI[a] | Viremia |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 21 | 26 | 28 | 33 | 40 | 42 | 49 | 56 | 63 | 70 | 77 | 85 | 91 | 98 | 105 | 112 | 117* | 125 | 132 | 138 | | |
| 200 μg VLP | Al(OH)₃ | 1 | <4 | 16 | 16 | 12 | 12 | 12 | 8 | 4 | 4 | 4 | 4 | 4 | 4 | <4 | <4 | <4 | <4 | 160 | 1280 | 1280 | 2.3 | D5 |
| 200 μg VLP | | 2 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | 320 | 640 | 5.4 | D4–11 |
| 100 μg VLP | | 3 | <4 | <4 | <4 | <4 | <8 | <8 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | 40 | 640 | 640 | 3.4 | D4–6 |
| 100 μg VLP | | 4 | <4 | 32 | 32 | 32 | 32 | 48 | 64 | 48 | 32 | 48 | 48 | 16 | 8 | 8 | 4 | 4 | 4 | 40 | 40 | 40 | 1.7 | — |
| 50 μg VLP | | 5 | <4 | <4 | <4 | <4 | 8 | 32 | 16 | 8 | 8 | 4 | 4 | <4 | <4 | <4 | 4 | <4 | <4 | 60 | 1280 | 2560 | 0.1 | — |
| 50 μg VLP | | 6 | <4 | <4 | <4 | <4 | 8 | 8 | 12 | 12 | 12 | 12 | 8 | 6 | 8 | 4 | 4 | <4 | <4 | 160 | 1280 | 1280 | 0.0 | — |
| 10 μg VLP | | 7 | <4 | 64 | 64 | 64 | 64 | 64 | 48 | 48 | 32 | 32 | 16 | 6 | 8 | 4 | 4 | <4 | <4 | 160 | 160 | 240 | 0.0 | — |
| 10 μg VLP | | 8 | <4 | <4 | <4 | <4 | 12 | 12 | 8 | 8 | 8 | 8 | 8 | 8 | 4 | 4 | 4 | <4 | <4 | 160 | 320 | 320 | 0.0 | — |
| 200 μg VLP | ISA-50 | 9 | 8 | 128 | 256 | 512 | 384 | 256 | 192 | 192 | 128 | 128 | 128 | 64 | 64 | 32 | 32 | 32 | 32 | 320 | 320 | 240 | 0.0 | — |
| 200 μg VLP | | 10 | 4 | 128 | 128 | 128 | 96 | 96 | 64 | 192 | 64 | 64 | 128 | 32 | 16 | 12 | 8 | 8 | 8 | 60 | 160 | 160 | 0.0 | — |
| 100 μg VLP | | 11 | <4 | 128 | 256 | 256 | 256 | 256 | 192 | 128 | 128 | 96 | 64 | 32 | 16 | 16 | 16 | 16 | 16 | 120 | 240 | 240 | 0.0 | — |
| 100 μg VLP | | 12 | <4 | 64 | 64 | 64 | 64 | 64 | 96 | 96 | 96 | 96 | 96 | 64 | 32 | 32 | 16 | 16 | 16 | 320 | 320 | 160 | 0.0 | — |
| 50 μg VLP | | 13 | <4 | 128 | 256 | 256 | 256 | 256 | 256 | 128 | 128 | 128 | 64 | 32 | 16 | 16 | 8 | 8 | 8 | 80 | 80 | 80 | 0.0 | — |
| 50 μg VLP | | 14 | 16 | 64 | 128 | 512 | 512 | 256 | 256 | 128 | 128 | 128 | 128 | 32 | 32 | 16 | 8 | 8 | 8 | 80 | 80 | 80 | 0.0 | — |
| 10 μg VLP | | 15 | <4 | <4 | <4 | <4 | 32 | 32 | 24 | 24 | 24 | 24 | 16 | 4 | 4 | 4 | 4 | 4 | 4 | 80 | 320 | 160 | 0.0 | — |
| 10 μg VLP | | 16 | <4 | 64 | 64 | 64 | 128 | 128 | 128 | 64 | 96 | 64 | 64 | 16 | 16 | 16 | 16 | 16 | 16 | 80 | 260 | 120 | 0.0 | — |
| 200 μg VLP | IFA | 17 | <4 | 8 | 16 | 16 | 48 | 32 | 32 | 24 | 24 | 24 | 16 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 640 | 640 | 1.4 | — |
| 200 μg VLP | | 18 | <4 | 64 | 64 | 64 | 128 | 64 | 128 | 128 | 128 | 128 | 128 | 64 | 64 | 32 | 16 | 16 | 16 | 640 | 1280 | 1280 | 0.7 | — |
| 100 μg VLP | | 19 | <4 | >4 | >4 | >4 | 4 | 4 | 4 | 4 | 12 | 24 | 12 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | 480 | 320 | 0.8 | — |
| 100 μg VLP | | 20 | 16 | 16 | 16 | 16 | 32 | 64 | 64 | 64 | 64 | 64 | 48 | 16 | 16 | 8 | 4 | 4 | 4 | 4 | 640 | 320 | 0.0 | — |
| 50 μg VLP | | 21 | <4 | 8 | 8 | 8 | 24 | 24 | 24 | 24 | 24 | 24 | 16 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 640 | 640 | 0.0 | — |
| 50 μg VLP | | 22 | <4 | 32 | 64 | 64 | 48 | 32 | 32 | 64 | 48 | 32 | 32 | 16 | 16 | 8 | 4 | 4 | 4 | 8 | 480 | 160 | 0.0 | — |
| 10 μg VLP | | 23 | <4 | 32 | 64 | 64 | 48 | 32 | 48 | 32 | 32 | 32 | 24 | 8 | 8 | 8 | 4 | 4 | 4 | 4 | 480 | 160 | 0.0 | — |
| 10 μg VLP | | 24 | <4 | <4 | 4 | 16 | 16 | <4 | 16 | 12 | 12 | 12 | 12 | 4 | <4 | <4 | <4 | <4 | <4 | <4 | 120 | 120 | 0.0 | — |
| Saline Control | | 25 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | 640 | 480 | 3.5 | D4-14 |
| | | 26 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | 2560 | 2560 | 7.7 | D4-14 |
| | | 27 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | 480 | 2560 | 6.9 | D6-14 |
| | | 28 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | 320 | 1280 | 9.7 | D4-13 |
| BTV-10 ATT Vaccine | | 29 | 512 | — | — | — | — | 640 | 320 | 320 | 320 | 320 | 320 | 320 | 320 | 320 | 320 | 320 | 320 | 320 | 1280 | 1280 | 0.7 | — |
| | | 30 | 512 | — | — | — | — | 320 | 320 | 320 | 320 | 320 | 320 | 320 | 320 | 320 | 320 | 320 | 320 | 320 | 320 | 320 | 0.0 | — |
| | | 31 | 512 | — | — | — | — | 640 | 640 | 640 | 640 | 640 | 640 | 640 | 320 | 320 | 320 | 320 | 320 | 640 | 640 | 640 | 0.0 | — |
| | | 32 | 512 | — | — | — | — | 480 | 480 | 480 | 480 | 480 | 480 | 320 | 320 | 320 | 320 | 320 | 320 | 320 | 640 | 640 | 0.0 | — |

[a]Challenge, CRI, clinical reaction index.

quantities of protein on day 21. Serum samples were collected from each sheep at regular intervals between days 21 and 4 months after the first inoculation.

## B. Neutralizing Antibodies Induced in Sheep by VLPs in the Presence of Various Adjuvants

From day 21 after the primary inoculation, serum samples from each animal were collected at regular intervals, diluted as required with phosphate-buffered saline, and tested for the presence of neutralizing antibodies against BTV-10 by the plaque reduction neutralization assay. All the immunized sheep elicited BTV-10 neutralizing antibodies, albeit to various levels (Table 2.5). However, the efficacy of the adjuvants differed. For example, VLPs induced higher NA titers when used in conjunction with IFA than with $Al(OH)_3$. When ISA-50 was used, as little as $10 \mu g$ VLPs was able to induce NA titers in vaccinated sheep. ISA-50 appeared better adjuvant than either IFA or $Al(OH)_3$. As expected all animals given live virus had a high level of neutralizing antibodies, and the control sheep had no BTV antibody. Surprisingly, vaccination with high amounts of VLPs (e.g., $200 \mu g$) showed some negative effect with very little or no antibodies being produced (e.g., sheep numbers 1, 2, 3 and 12). In contrast it appeared that as little as $10 \mu g$ of VLPs was sufficient to elicit neutralizing antibodies.

## C. Protection Conferred by VLPs against Bluetongue Disease in Sheep

To determine if VLPs could confer protection against virulent virus challenge, all sheep were challenged by subcutaneous injection with 1 ml of infected sheep blood containing virulent South African serotype 10 at day 117 (i.e., 4 months) after the primary immunization of the sheep. The clinical reactions were monitored from 3 to 14 days postchallenge. The severity of clinical manifestations of bluetongue disease was expressed in numerical form, as described earlier. Viremia tests of whole blood samples and neutralization tests of the postchallenge sera were performed. Sheep given higher levels of VLPs ($50 \mu g$ to $200 \mu g$) and IFA showed mild clinical reactions. However, no clinical symptoms were detected in the sheep immunized in the presence of ISA-50. The blood samples of all challenged sheep that had received either IFA or ISA-50 showed no sign of viremia. Only three sheep (nos. 1, 2, 3) had viremic blood, all of which received the $Al(OH)_3$ adjuvant. All the postchallenge blood samples of all control sheep, on the other hand, were viremic and their sera contained high neutralizing antibody titers indicating a primary infection.

## Conclusion

For nearly a century, bluetongue has been associated with disease and mortality in sheep and cattle. Despite the fact that this can have serious economic repercussions for some countries in terms of import and export regulations for sheep and cattle, live attenuated vaccines have been developed only for use in South Africa and in the United States. In South Africa, sheep are vaccinated with pentavalent live attenuated virus vaccine at 3-week intervals. In the United States, although five BTV serotypes have been identified (BTV-2, -10, -11, -13, and -17), a modified live virus vaccine is available only for BTV-10. Conventional live attenuated virus vaccines have certain inherent disadvantages. In the case of bluetongue, such virus vaccines can cause fetal infection with teratological consequences. When used as a polyvalent vaccine, interference occurs between the component BTV serotypes, resulting in the development of incomplete immunity. Moreover, live attenuated vaccine strains may be neutralized passively by the antibody in maternal colostrum, hence vaccination of newborns results in lower protection than that observed in newborns given inactivated or subunit vaccines.

Recent developments in biotechnology have made it possible to synthesize double-shelled virus like particles, mimicking authentic virions but lacking the harmful genetic material and viral replicating machinery. Therefore, these particles are as safe as subunit vaccines, and potentially as effective as "whole" virus vaccine. Indeed, when these particles were given as *second generation vaccines*, the initial trials indicated that a very small amount (10 μg) of VLPs in the presence of appropriate adjuvant protected the sheep against the disease.

We have yet to determine the minimum amount of VLP needed for complete protection and the duration of the immunity conferred by these particles. It is also essential to perform similar vaccination trials in cattle, since they are a major reservoir host of BTV. Another important aspect of vaccine development is the role of adjuvants. Our data demonstrate that Montanide ISA-50 enhanced the neutralizing-antibody responses in sheep more effectively than incomplete Freund's adjuvant. The value of the baculovirus-expressed BTV-like structures as vaccines therefore needs to be investigated further.

Our previous studies involving cDNA–RNA hybridization experiments, as well as complete sequence analysis of cDNA clones of viral RNA species, have demonstrated that both outer capsid proteins VP2 and VP5 are among the most variable proteins of different BTV serotypes. Depending on the serotype, they exhibit sequence relationships to other BTV serotypes (29). Data indicating that antigens of one BTV serotype elicit antibody responses that neutralize infection by other BTV serotypes (13) have been obtained. There is every reason to believe that it should be possible to make vaccine chimaeras representing different

BTV serotypes (e.g., involving the expression of several BTV VP2 genes), as well as chimaeras containing protein sequences representing other pathogens (e.g., chimaeric genes involving VP2, and/or VP5, and/or VP3, and/or VP7 sequences and selected sequences of viral bacterial, fungal, or protozoan pathogens). This is an exciting prospect for the future.

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CHAPTER 3

# Progress towards Peptide Vaccines for Foot-and-Mouth Disease

D.J. Rowlands

## 1. Introduction

Despite the impressive advances that have been made in recent years in our understanding of the mechanisms of immune responses to pathogenic organisms and the antigenic and chemical structures of the organisms themselves, these have yet to result in the widescale introduction of novel vaccines. In both veterinary and human fields the vaccines in use today are basically similar to those developed at the dawn of the era of vaccination and fall into two groups: killed vaccines, in which the virulent pathogen is rendered innocuous by chemical or physical inactivation, and attenuated vaccines, in which the virulence of the pathogen is reduced by laboratory culture in a largely empirical fashion. One exception is the surface antigen vaccine against hepatitis B virus, which is expressed in yeast and is the only practical vaccine to have emerged so far from the application of recombinant DNA techniques (57).

Although conventional vaccines have been enormously beneficial in the control of many major infectious diseases of both man and animals, there is still room for significant improvement and there remain those diseases for which no vaccine is currently available, e.g., AIDS. One attractive route for the development of new vaccines consists of identifying the most important antigenic determinants on the surfaces of pathogenic organisms and mimicking their features in the form of synthetic peptides, thus producing vaccines by chemical rather than biological processes. Despite the formidable problems associated with this approach, the potential advantages that would be attendant on its success justify the investigation. This chapter will review progress in this area as related to vaccines against foot-and-mouth disease (FMDV) and discuss the reasons why the results obtained in the laboratory with peptide vaccines against this disease have been more encouraging than with many other systems.

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## 2. FMD

FMD has long been recognized as a major scourge of the most important domestic livestock species kept by man. The earliest historical reference that clearly describes this disease is by Fracastorius in 1546 (32). Cattle, goats, sheep, and pigs are all susceptible to the disease, as are many other species under natural or laboratory conditions. The virus produces a febrile illness with often massive vesicular lesions on the oropharynx and feet and systemic infection involving many organs of the body. The severity of disease symptoms varies according to the strain of virus and the species infected. Domestic cattle are usually the most severely affected species whereas in goats and sheep the infection can be almost inapparent. Mortality due to infection with the virus is not usually very high, except in young animals, but morbidity typically approaches 100% in unprotected herds. This is due both to the uniform susceptibility of individuals to infection and the speed and efficiency with which the virus is spread. Virus is transmitted by the consumption of contaminated food-stuff and experimentally by aerosol. There is also good circumstantial evidence that under appropriate meteorological conditions virus can be naturally transmitted by aerosol over large distances (29,44). Although mortality may not be particularly high, infected animals are severely debilitated for a considerable time and there is a permanent loss of productivity, estimated to be ~25% (14). Apart from the primary economic losses associated with the disease, there are secondary con-sequences related to marketing and export restrictions that can be even more financially damaging. For these reasons FMD is still probably the most feared infection of livestock throughout the world.

   Although strict control measures such as the slaughtering of infected herds, quarantining of imported stock, and the use of vaccines have eradicated or prevented the introduction of the disease from some regions (e.g., North America and Australia) it is still endemic in large parts of the world and poses a constant threat.

## 3. History of FMD Vaccines

The first vaccines successfully used to protect against FMD consisted of lymph drawn from the lesions of infected cattle tongues and inactivated with formaldehyde (90). Although this procedure was highly effective in inducing protective immunity, the source of virus was clearly inappro-priate to produce the vast quantities of vaccine required to make a serious impact on the control of the disease.

   The situation improved with the introduction of the Frenkel vaccine (41). This is produced from formaldehyde-inactivated virus that is cul-tured *in vitro* on strips of epithelium taken from cattle tongues collected

after slaughter in abattoirs. The Frenkel vaccine is highly efficacious and is still used today in some areas, e.g., the Netherlands. However, the major breakthrough that enabled the production of the huge quantities of vaccine required to protect the global herd was the development of large-scale tissue culture techniques using the baby hamster kidney cell line, BHK21 (22). This methodology allows the virus to be grown in deep cell suspension cultures of up to 10,000 liters and produces most of the $\sim 2 \times 10^9$ doses of vaccine that are used annually today.

During the early days of vaccine development it became apparent that vaccination did not always protect animals from developing the disease. Investigation of this problem led to the recognition of important antigenic variation by the virus. It is now established that the virus occurs as seven distinct serotypes (14), the definition of a serotype in this case being that an animal recovered from infection by one serotype is still fully susceptible to infection by any of the other six. The seven serotypes of the virus are not all distributed uniformly throughout the areas endemic for the disease. The most important serotypes, O, A, and C, are found in all endemic areas of the world but three serotypes, SAT1, SAT2, and SAT3, are confined to the African continent and Asia 1 is found only in Asia.

In addition to serotype differences there is considerable antigenic variation among virus isolates from within each serotype and these differences can be so great that vaccination with virus of one strain may provide little protection against other strains within the serotype. This is especially true as immunity wanes and the cross neutralizing activity in serum falls below the protective level. This problem clearly has complicating consequences on the choice of vaccine strains; an ideal strain being one that raises high levels of broadly reactive neutralizing antibodies. Protection against FMD is correlated with the serum titer of virus-neutralizing antibodies at the time of exposure, there apparently being little benefit derived from the recall of immunological memory. For this reason it is usually necessary to revaccinate stock two or three times a year to maintain good levels of immunity.

It is clear that properly controlled vaccination programs can have a dramatic effect on the incidence of FMD. This is exemplified by the situation in Western Europe in which the number of outbreaks per year have been reduced from many tens of thousands to a handful in the 20–30 years since widescale vaccination has been practised (Fig. 3.1). Control has not been so effective in other regions due to a combination of local logistical problems in distribution and control of vaccination and the techniques of animal husbandry employed.

The great majority of FMD vaccine in use today consists of chemically inactivated tissue culture grown virus that is adjuvented with aluminium hydroxide gel or mineral oil emulsion; the latter is essential for the induction of solid immunity in pigs. Earlier vaccines, and some still in use today, were inactivated with formaldehyde. However, the virus
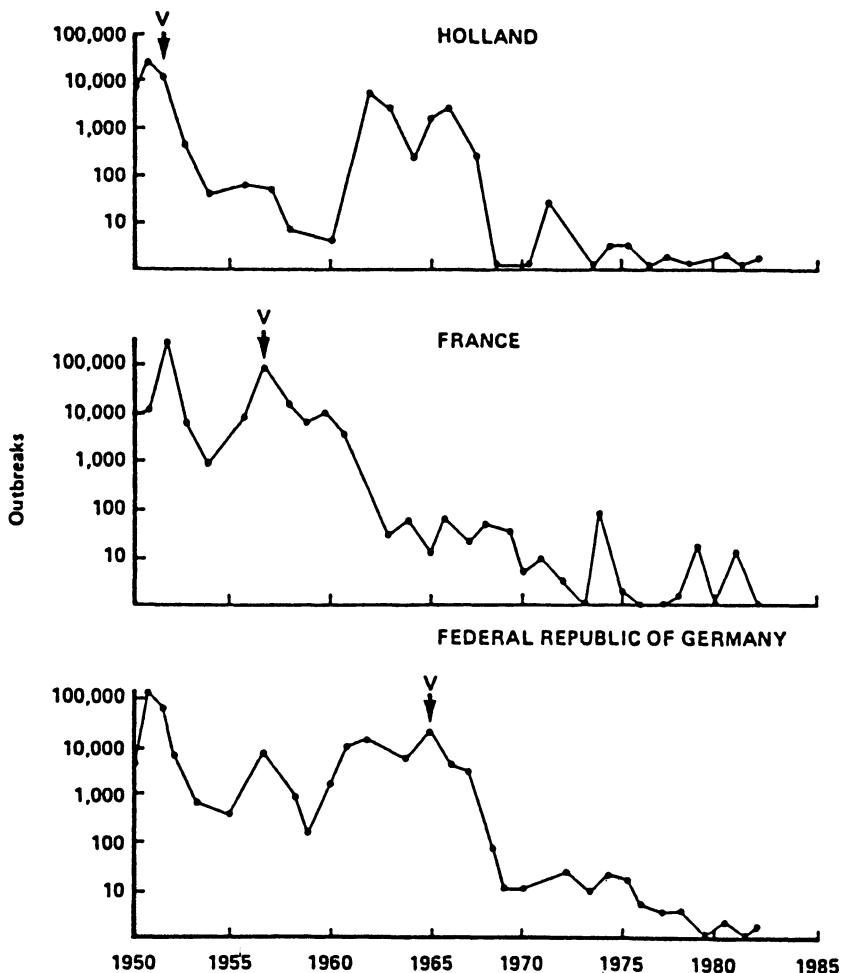
Fig. 3.1. The influence of the introduction of vaccination on the frequency of outbreaks of FMD in Holland, France, and the Federal Republic of Germany (16).

inactivation kinetics obtained using formaldehyde are nonlinear and there is compelling evidence that residual live virus in such vaccines has on occasions caused outbreaks of disease (8,50). Aziridine compounds do produce linear kinetics of virus inactivation and binary ethyleneimine is most commonly used to neutralize virus infectivity in current vaccines.

Attempts have been made to derive live attenuated vaccine strains of FMDV but these have generally proved unreliable and little is used in practice. Two problems have prevented the development of effective live virus vaccines, both related to the speed and flexibility with which the

virus genome can change. First, a range of attenuated strains would be needed to cope with the antigenic diversity displayed by the virus; second, reversion to a virulent phenotype is a continuous worry and problem with such a highly mutable virus.

## 4. Desirable Features of Novel FMD Vaccines

Despite the proven efficacy of current FMD vaccines there are a number of ways in which improvement would be desirable.

1. Current vaccines are produced from huge quantities of infectious, virulent virus. Consequently sophisticated methods must be adopted to prevent escape of virus and contamination of the environment and there is an ever present risk of failure of containment. A means of vaccine production that did not rely on the cultivation of virulent virus would clearly be advantageous.
2. A chemically synthesized vaccine would not only obviate the problems of handling live virus, but the scale and complexity of the production plant would be considerably reduced.
3. As with many biological products, current FMD vaccines are relatively unstable and need to be stored and distributed under refrigerated conditions. Even with suitable storage facilities the shelf life of the vaccine is limited. The inherent stability of chemically synthesized vaccines would reduce these problems considerably.
4. The anticipated stability of peptide vaccines would also allow for novel methods of delivery, which may improve the duration of the period during which protective immunity is achieved and reduced the frequency at which booster vaccinations are required. Both of these desirable objectives may be met by the incorporation of part of the vaccine payload within microcapsules designed to release boosting doses of antigen at defined intervals after injection.
5. Current vaccines are impure and occasionally cause anaphylactic responses following sensitization to contaminating material, such as serum proteins. A synthetic product would be pure and chemically defined.
6. Biological constraints and complications in vaccine virus culture are other problems that would be eliminated by the use of a snythetic product. Antigenic variation is a major feature of FMDV and virus isolates can vary quite markedly in the degree of protection they can confer against heterologous strains. Consequently, the choice of virus strains is extremely important for the production of broadly effective vaccines. However, there are poorly understood relationships between virus and host cell that can result in the concomitant selection of antigenic variants of viruses during the process of adaptation for

growth in the suspension culture cells used for vaccine production
(10). Such biological quirks would clearly not apply to a synthetic
product.

7. Finally, current production methods rely on the use of serum for cell
culture purposes and there is always the slight, but real, possibility that
adventitious disease causing agents may be introduced from this
source. The recent outbreak of bovine spongiform encephalopathy in
cattle in the UK is a salutary reminder of the damage that can arise
from unexpected sources.

It would appear that the perceived advantages that a synthetic peptide
vaccine would have over the current product are sufficient to justify the
pursuance of research in this area. Clearly many aspects of peptide
immunity need to be fully explored before conclusions can be reached on
the practical potential of synthetic FMD vaccines. However, at the very
least such studies will improve our fundamental understanding of the
antigenic character of the virus and the mechanisms of the induction of
protective immunity.


# 5. General Properties of FMDV

The FMDVs comprise the aphthovirus genus of the family Picornaviridae,
the other genera being the enteroviruses (e.g., poliovirus), the rhino-
viruses (e.g., human rhinovirus), and the cardioviruses (e.g., Mengovirus)
(70). The nonenveloped particles of viruses in this family have a diameter
of ~30 nm and comprise four proteins, VP1–4, which encapsidate a
single-stranded RNA genome of ~8000 nucleotides. There are 60 copies
of each protein per particle, of which VP1–3 form the icosohedral capsid
with VP4 lining the inner surface. The molecular weights of VP1–3
typically range form ~24K to 30K, with VP1 being the largest. In FMDV,
however, each of the proteins has a molecular weight of ~24K, VP1
consisting of 213, VP2 218, and VP3 230 amino acids. VP4, which typically
comprises ~70 amino acids, is somewhat larger in FMDV being com-
posed of 85 amino acids.

The infectivity of FMDV is easily destroyed by environmental con-
ditions. The virus is extremely labile in mildly acidic conditions; in low
ionic strength solutions it is disrupted at pH values as high as 6.8. The
pentameric units of the icosohedral shell disassociate to produce soluble
12 S subunits composed of five copies each of VP1, −2, and −3. VP4
separates as an insoluble aggregate and the RNA is released (85). An
extraordinary feature of the virus is that it includes an endonuclease
activity that cleaves the genomic RNA *in situ* within the virus particle
unless stored under refrigerated conditions (19). It has been suggested
that this "suicidal" ribonuclease activity many be used as an alternative to

chemical inactivation in the production of conventional vaccines (27). At higher temperatures (>50°C) virus is inactivated by disruption into products that are similar to those produced by acid degradation. Other properties that serve to differentiate FMDV particles from those of other picornavirus are their extremely high bouyant density in cesium chloride solutions (≥1.43 g/ml) and their permeability to rather large photo-inactivating compounds such as proflavine. As will be seen later, the recently determined molecular structure of the virus offers some explanation for these properties.

The RNA genome is of positive sense, that is to say it can act directly as a mRNA for the translation of virus proteins, and hence is infectious in the absence of any other virus components, provided precautions are taken to prevent its degradation by RNase. The RNA is translated into a single large polyprotein that is proteolytically processed into the functional virus proteins. The structural proteins, VP1–4, are encoded in the 5′ region of the virus RNA. To the 5′ side of the coding sequences there is an extraordinarily long untranslated region that, in common with the cardioviruses, includes a polycytidilic acid tract [poly(C)] varying in length from 100 to 200 residues. There is some evidence for FMDV that shortening of this poly(C) tract can attenuate the virulence of the virus (46) and there is more direct evidence that the same occurs with cardioviruses (30). Thus manipulation of this part of the genome via infectious cDNA clones (93) may be of value in rational attempts to develop stable and defined live vaccine viruses.

Many picornaviruses can replicate rapidly in tissue culture cells to produce cytopathic effect but FMDV is probably the fastest growing. Some strains can complete the replication cycle in 2–3 hrs *in vitro* and this rapid growth probably accounts for the speed with which infection can proceed to frank disease *in vivo* and why the maintenance of high neutralizing antibody titers is essential for protection, there being insufficient time to recall a memory response.


## 6. Antigenic Properties of FMDV

Protection against FMD is correlated with the level of circulating antibody with appropriate virus-neutralizing specificity (89). As antibody levels decline following immunization, so susceptibility increases and vaccination must be repeated at regular intervals in order to maintain protective levels of immunity. Although intact virus particles are highly immunogenic and as little as 1–10 μg can induce protective levels of immunity in cattle, degradation of the capsid into the pentamer or 12 S subunits results in a reduction of immunogenicity of about two orders of magnitude. Moreover, adsorbtion of antivirus particle antiserum with slight excess of pentamer subunits fails to remove the neutralizing activity
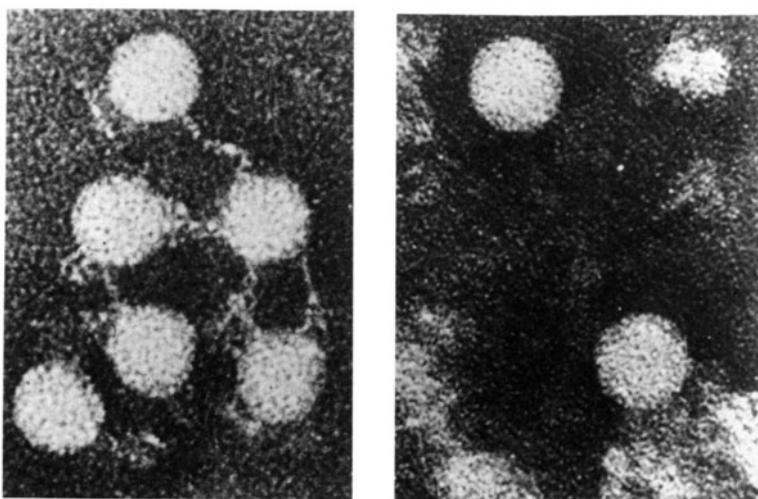
**Fig. 3.2.** Electron micrographs of FMDV native (left) and trypsin-treated particles reacted with IgM antibodies (17).

of the serum (75). Thus the immunogenic properties of virus particles and viral subunits are both quantitatively and qualitatively different. It is for these reasons that storage of vaccines under conditions that maintain the integrity of the particles is especially important.

The immunogenicity of some virus strains, especially those in the O serotype, is significantly reduced by treatment with proteolytic enzymes, such as trypsin. Serum adsorbtion experiments and immune electron microscopy have shown that an important antigenic site on the virus is destroyed by trypsin without affecting the overall morphology of the particle (17). This was further supported by the observation that trypsin-treated virus fails to react with neutralizing IgM antibody (Fig. 3.2). Trypsin treatment also prevents viruses of all serotypes from attaching to susceptible cells, with consequent reduction in infectivity (91). These results suggest that the enzyme destroys both the cell receptor binding site and a major antigenic determinant on the virus. Analysis of the proteins of trypsin-treated virus showed that only VP1 was affected, it being cleaved into two smaller fragments (21). It was concluded from experiments of this sort that the VP1 protein contains both a major antigenic determinant responsible for eliciting neutralizing antibodies and the cell receptor recognition domain. The importance of VP1 was further confirmed by the demonstration that the isolated protein, separated from the other capsid components chromatographically or by polyacrylamide gel electrophoresis, is capable of eliciting a virus-neutralizing and protective response in laboratory animals and pigs (5,53,59). In view of these properties of the VP1 protein attempts were made to produce a vaccine

using recombinant DNA techniques to express VP1 as a fusion protein in *Escherichia coli* (51). This was extremely successful in terms of the amount of protein that was produced and, although it was demonstrated that protective immunity could be induced in cattle, the immunogenicity of the material was too low for it to be of practical value.

# 7. Identification of the Immunodominant Site

As outlined above, several lines of evidence had suggested that an important antigenic determinant, in terms of eliciting virus-neutralizing and protective antibody, was located on VP1. The precise location of this determinant was elucidated in the early 1980s from a variety of evidence. As more sequence information was accumulated it became apparent that amino acid variation was not randomly distributed across the VP1 protein but tended to be concentrated in two regions, between residues ~40–60 and ~132–160 (amino acid numbers are based on the sequence of VP1 of serotype $O_1$ virus) (52). The former tract is highly variable between viruses of different serotypes but is considerably less so between viruses within a serotype whereas the 132–160 tract varied both within and between serotypes.

The 132–160 region is highly variable in size as well as sequence, the length varying from 24 amino acids in serotype $C_1$ virus to 34 amino acids in serotype $SAT_3$ virus (63). In striking contrast to the overall variation in this portion of the protein, the triplet Arg-Gly-Asp at position 145–147 is almost invariant (63). The hypervariability of the 132–160 tract suggested (a) that it did not play a significant role in determining the folding of the protein and that it therefore was oriented at the surface and (b) it was likely to be important in determining the antigenic characteristics of the virus.

The application of predictive algorithms to the sequence of VP1 suggested that the 132–160 sequence was hydrophilic in nature and that part of the tract (144–159) had the propensity to form an amphipathic $\alpha$-helix (68). The variable tract between amino acids 40 and 60 was predicted to be more hydrophobic. Although the accuracy of prediction of antigenic sites from such analyses has not been particularly striking, it is a general rule that the more hydrophilic regions of proteins are likely to be exposed to aqueous solvent at the surface of proteins and therefore potentially antigenic.

The most direct evidence as to the location of linear antigenic determinants on VP1 was produced by Strohmaier et al. (83). These investigators purified fragments of VP1, which had been produced by enzymatic or chemical cleavage, and determined their position on the protein by limited terminal sequencing, the amino acid sequence of the protein having been determined from the nucleotide sequence of molecular

clones (52). The fragments were used to vaccinate mice and the sera analyzed for the presence of virus-neutralizing antibodies. By comparing the sequences of the active and inactive fragments it was deduced that there were two linear antigenic regions on the protein involving residues 146–154 and 201–213 at the C' terminus (Fig. 3.3).

Virus-neutralizing monoclonal antibodies (MAbs) have also been used extensively to investigate the antigenic structure of FMDV by competition binding experiments and the analysis of neutralization escape mutant viruses selected in the presence of MAb. In general these studies have supported the concept that an immunodominant site is located within the 132–160 sequence of VP1, especially with viruses of serotypes A and C (11,56,69,81,87,92). In fact most MAbs to serotype A and C viruses behave like antipeptide antibodies in that they recognize peptides in direct ELISA tests (11,56). The antigenic structure of viruses of serotype O appears to be more complex. A high proportion of type O virus MAbs are conformation dependent and some have been shown to recognize regions within the 140–160 sequence in combination with residues from the C terminal portion of VP1 (6,64,92). The location of MAb resistance conferring amino acid substitutions within the structural proteins and the cross-resistance properties of mutant viruses (58) suggest that there are several important antigenic sites on the virus. However, recent X-ray crystallographic studies (65) suggest that some mutations may indirectly confer resistance to neutralization by affecting the conformation of the VP1 132–160 region (see below).

## 8. Synthetic Peptide Immunogens

The evidence outlined in the last section was used as the basis for the synthesis of synthetic peptide immunogens and the successful use of such materials to produce virus-neutralizing (9,68) and protective (9) responses in experimental animals was reported. Peptides representing the sequences of VP1 amino acids 141–160 and 200–213 from the C' terminal portion of the protein were both shown to elicit virus neutralizing antibodies but the specific activity of the internal tract was found to be highest and most subsequent work has concentrated on this region. The first synthetic peptide work used sequence from serotype $O_1$ virus but it has been shown subsequently that the equivalent sequences from the VP1 141–160 region of examples of viruses from all seven serotypes are also able to induce virus-neutralizing antibody (38).

In the earlier studies synthetic peptides were chemically coupled to carrier protein molecules, such as keyhole limpet hemocyanin (KLH), since it was generally perceived that peptides of 15–20 amino acids in length would not be sufficiently immunogenic alone to elicit a significant response. Coupling was achieved either with glutaraldehyde, which forms
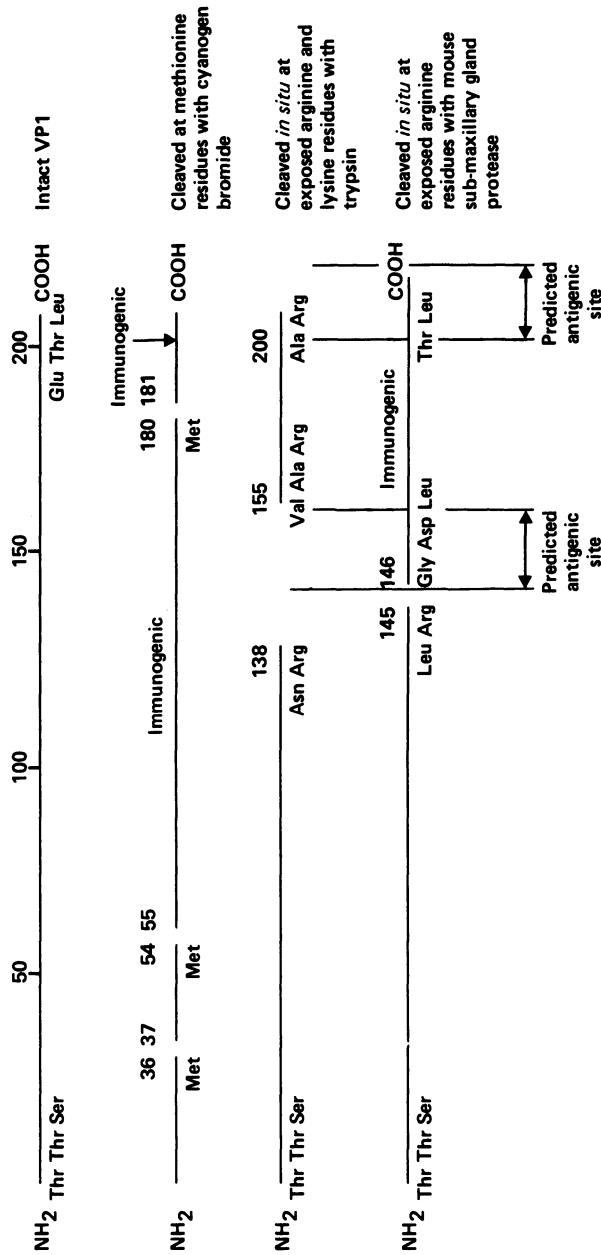
**Fig. 3.3.** Immunogenicity of defined cleavage fragments of VP1 of FMDV (83).

covalent linkages between amino groups on the peptide and carrier protein, or by including a nonnatural cysteine residue at the C' end and linking this residue to amino groups on the carrier protein using the bifunctional coupling reagent $N$-maleimidobenzoyl-$N$-hydroxysuccinimide ester (MBS). However, further studies showed that the coupled peptides did not perform as classical haptens since anamnestic responses induced by boosting injections of coupled peptide were not restricted by the protein to which the peptide was linked (35). In fact, following an initial priming with peptide coupled to KLH, good anamnestic responses were induced with free peptide alone. This suggests that carrier protein is not essential for the mounting of immune responses and it was subsequently shown that free peptide can both prime and boost the immune system, provided it is appropriately administered either within liposomes or as an aqueous emulsion in oil (incomplete Freund's adjuvant). Protective immunization of cattle with an uncoupled synthetic peptide delivered with complete Freund's adjuvant has also been reported (28). The immunizing peptide in this case consisted of residues 141–158 and 200–213 linked by a Pro-Pro-Ser spacer and including Cys residues at both N and C termini. Such a structure would allow extensive polymerization via disulfide bond formation.

Protection tests can be readily performed in the guinea pig, which has long been used as a laboratory animal model for studies on FMDV since the animal evidences clinical manifestations of disease reminiscent of those seen in natural hosts, with lesions developing on the feet and oropharynx. Compilation of protection data from animals that had been vaccinated with either inactivated virus particles or synthetic peptide 141–160 showed interesting qualitative differences in the responses to the two vaccines (Fig. 3.4) (34). As had been shown before, there was a general correlation in the inactivated virus-vaccinated animals between the level of serum-neutralizing antibody achieved at the time of challenge and the proportion of animals protected. However, this correlation was far from perfect and some animals with high antibody titers were susceptible to disease while, conversely, others with much lower titers were protected. With the peptide vaccinated animals, on the other hand, all animals which developed neutralizing titers above a rather low threshold level were protected. A possible explanation for this rather surprising result may be that some of the antibodies present in the spectrum induced by virus particles are more effective in neutralizing virus *in vitro* than they are *in vivo* and so the protected status of the animal is influenced by the relative proportion of antibodies of different specificities in the overall responses. Peptides necessarily induce a narrower range of antibody specificities that, at least in the case of protection of guinea pigs against FMD, are particularly effective *in vivo*.

Another surprising property of peptide vaccines is that they can be more effective in inducing cross-reactive neutralizing responses than
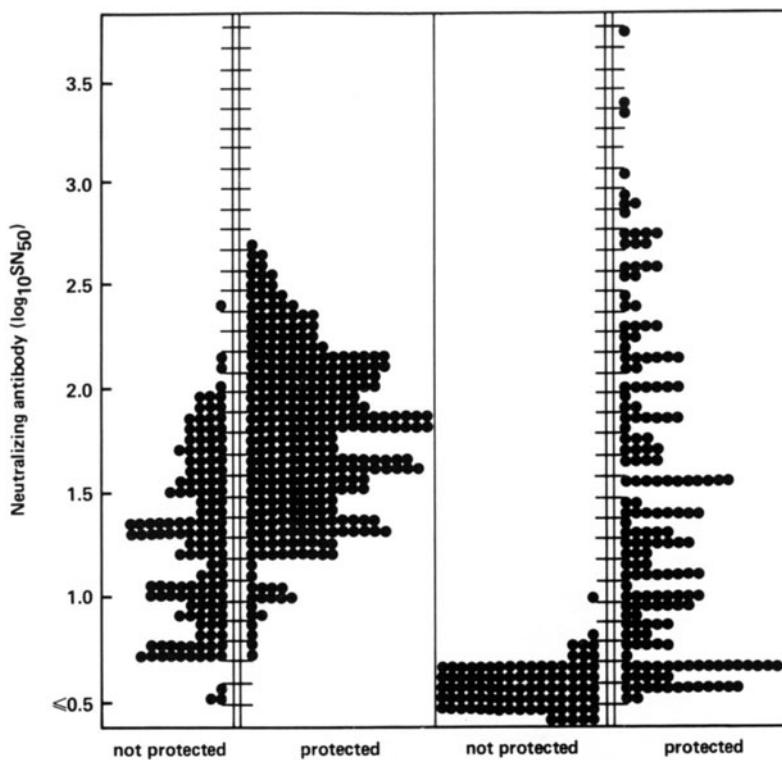
**Fig. 3.4.** Relationship between serum-neutralizing antibody titer and protection against FMDV challenge in guinea pigs vaccinated with inactivated virus (a) or synthetic peptide (b). Each point represents a single animal (34).

intact virus (Fig. 3.5) (66). As pointed out earlier, antigenic variation is a major problem in vaccination against FMD and candidate vaccine virus strains are tested for their abilities to induce antibody responses that will effectively neutralize as broad a range of field isolate strains of virus as possible. The results of such tests are expressed as *r* values, i.e., the ratio of the neutralization titre against a heterologous virus to that against the virus used to vaccinate (76). Using this method of analysis it was shown that peptides representing the VP1 141–160 region of examples of both serotype O and A viruses induced more broadly cross-reactive responses than did the viruses from which the sequences were derived (11,66).

An explanation of this finding may be related to the fact that a synthetic peptide antigen could not be expected to mimic the entire surface of a protein epitope, which, from X-ray crystallography studies of antigen antibody complexes, involves 20 or more amino acid side chains covering

| Virus Serum | BFS 1860 | BFS 1848 | KAUF B64 | KAUF B7 | AUS 1/81 | V1 | HKN | ISA 7/83 | TAI 1/80 |
|---|---|---|---|---|---|---|---|---|---|
| Virion (BFS 1860) | 1.00 | 0.20* | 0.21* | 0.23* | 0.18* | ≤0.03* | 0.48 | 0.20* | 0.10* |
| Peptide 141–160 | 0.68 | 1.00 | 0.58 | 0.41 | ≥1.00 | ≤0.08* | ≥1.00 | 0.59 | 0.11* |

\* indicates values of r < 1.0 at p= 0.05

**Fig. 3.5.** Cross-neutralizing activity of sera from guinea pigs vaccinated with inactivated virus or peptide VP1 141–160 against a range of serotype O viruses. The results are expressed as *r* values, i.e., the ratio of heterologous to homologous neutralizing titers.

an area of ~700 $\text{Å}^2$ (3). Despite this large area of epitope–paratope contact, a number of lines of evidence suggest that relatively few epitopic residues are crucial for antibody binding (42,43). However, the less critical portions of the epitope surface must, of course, be stearically compatible with antibody binding. Following this line of reasoning it would seem plausable that a peptide would induce a spectrum of antibody species that all recognize a cluster of residues critical for binding but that differ markedly in the stereocompatibility of the flanking regions, since there would be no selective pressure in the immune response to the peptide for structural restriction in these regions. Consequently different antibody species within the polyclonal antipeptide response may be able to recognize viruses that share the critical binding determinant but differ in the topographical context in which it appears at their surfaces.

In addition to the broadly cross-reactive neutralizing antibody responses elicited by peptides representing known naturally occurring sequences, artificial manipulation of the sequences have, in some cases, produced peptides that induce even more cross-reactive antibodies; even neutralizing and protecting across the serotype barrier (66).

In contrast to the high degree of cross-reactivity generally induced by peptide immunogens, some mutations within the virus can totally prevent the binding of antipeptide antibody. A dramatic example of this was provided by a study of naturally occurring variant viruses isolated from a single sample of serotype $A_{12}$ virus (73). Four such viruses differed from each other at only two positions, amino acids 148 and 153 of VP1. Antigenically the viruses fell into two groups, depending on whether or not the amino acid at position 153 was a proline. Antisera raised to peptides representing the sequences from one group did not neutralize viruses from the other group and vice versa. A similar pattern of cross-neutralizing activity was also seen with antisera raised against the intact viruses, again emphasizing the importance of this region of the virus in the overall immune response. Analysis of the solution structures of

peptides representing the four virus sequences by CD and NMR techniques showed that they fell into two conformation groups that mirrored their antigenic classification (79). The overiding importance of specific residues within the 141–160 region in determining antigenic properties of other strains and serotypes of FMDV has been reported (11,56,66).

A potential concern about the use of synthetic peptide vaccines is that the antiviral response elicited would be sufficiently narrow to facilitate the rapid selection of neutralization-resistant mutant viruses. However, rigorous attempts using antisera to peptide 141–160 of serotype $O_1$ virus failed to select such mutants under conditions which readily produced MAb resistant viruses (66). An antipeptide-resistant mutant virus was finally derived after multiple passage in the presence of the antipeptide antibody, using an antispecies antibody to enhance neutralization. This virus was resistant to neutralization only by the antipeptide serum from one animal, that used for the selection, and was susceptible to neutralization by other antisera raised to the same peptide. This encouraging result suggests that the selection of resistant viruses through the use of peptide vaccines would not be a major problem.

As mentioned earlier the results obtained with FMDV peptides are more encouraging than with most (perhaps any) other systems. A partial explanation for this was provided by an analysis of the proportion of antipeptide antibodies induced by 141–160 peptides that are cross-reactive with virus particles. Approximately 30–40% of the antipeptide antibodies fall into this category (67); a surprisingly high proportion and considerably greater than that observed with a human rhinovirus peptide which can also elicit virus neutralizing activity (39), albeit at a lower level than the FMDV peptide. To further understand the reasons for the relative success of FMDV peptides in inducing antiviral antibodies, the virus was crystallized and its structure determined by X-ray crystallography.

# 9. Structure of FMDV

The structure of FMDV, serotype O-1, has been resolved to a resolution of 2.9 Å (1). The basic structural properties of the virus resemble those of other members of the picornavirus family (48,55,72). VPs 1, 2, and 3 share a common structural motif, a wedge-shaped $\beta$-barrel composed of 8 strands of $\beta$-structure linked by loop regions of varying length (Figs. 3.6 and 3.7). It is the sequence and structure of these loop domains that largely dictate the surface properties of the virus, such as antigenic character. One copy each of VP1, 2, and 3 together form the basic structural unit or promoter from which the virus capsid is constructed. Five promoter units assemble to form the pentamer subunit with the VP1 proteins pointing to the axis of 5-fold symmetry (Fig. 3.7). The narrow
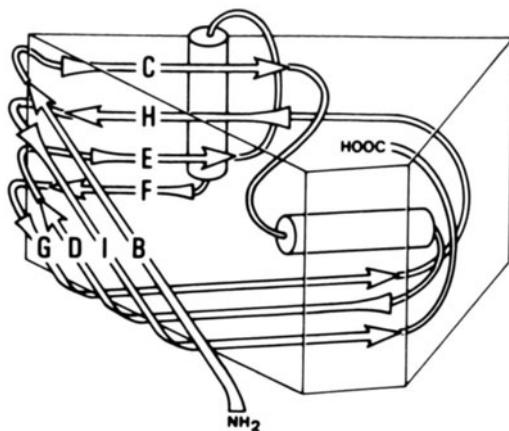
**Fig. 3.6.** Cartoon of the $\beta$-barrell structural motif common to the major structural proteins VP1, -2, and -3 of picornaviruses (48).

part of the VP1 wedge is aligned toward the 5-fold symmetry axis while those of VP2 and 3 are arranged in the opposite direction toward the 3-fold axis of the icosahedral capsid. The complete capsid is an assembly of 12 pentameric subunits. Acid-induced degradation of FMDV reverses this final assembly step to produce 12 S pentamer subunits. As with other picornaviruses, VP4 is located on the innermost surface of the virus particle and is the only structural protein that is not exposed at the outer surface. As indicated earlier, VP1–3 of FMDV are shorter than their equivalents in the other picornaviruses, particularly in the case of VP1. The truncations of the proteins have occurred principally in the loops
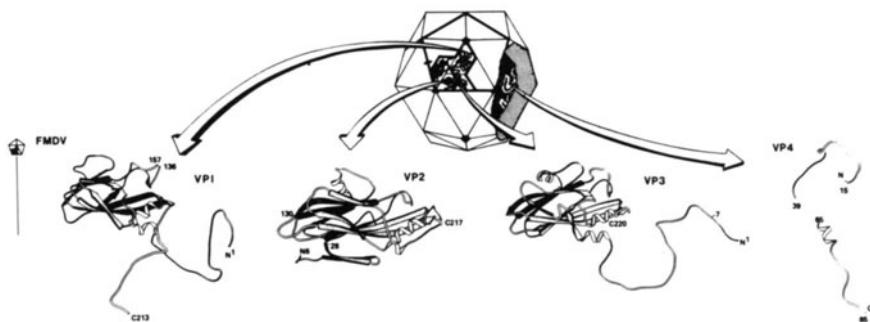


**Fig. 3.7.** Ribbon diagrams of the folding of the structural proteins of FMDV. Note that the region between VP1 136 and 157 is unresolved (1). Reprinted by permission from Nature, Vol. 327, p 711. Copyright © 1989 Macmillan Magazines Ltd.

adjoining the β-strands of the core β-barrel structures and the overall result of this is to reduce the average thickness of the protein shell and to produce a smoother outer surface. It has also resulted in the exposure of a pore at the 5-fold axis. The pore is formed by the intertwining of the N-terminal portions of the five VP3 proteins of the pentameric subunit, which are partially linked by disulfide bonds at this position. This interwined β-tube structure is common to all picornaviruses but, with the exception of FMDV, the aperture at the outer surface of the virus particle is covered by VP1 residues. The exposure of this pore probably accounts for some of the permeability-related properties of FMDV, such as its susceptibility to inactivation by drugs that can intercalate into the RNA (18) and the high buoyant density in cesium salts (74).

In both the enteroviruses, as typified by poliovirus (48), and the human rhinoviruses, as typified by HRV 14 (72) and 1A (49), there is a deep depression or canyon surrounding the 5-fold axis of the virus. This is produced by the upward tilting of the VP1 β-barrel toward the 5-fold axis and the VP2 and -3 β-barrels toward the 3-fold axis, thus leaving a depression in between. A similar structural arrangement exists in the cardioviruses, as typified by Mengo virus (55), except than in this case the canyon is partly filled in to produce a series of pits around the 5-fold axis. There is evidence for human rhinovirus that the cell receptor binding domain is located within the canyon and that this location protects the necessarily conserved features of the structure from immune surveillance (71). In FMDV, however, there is no evidence for the presence of a significant depression at the surface. This is due partly to the general reduction in size of the structural proteins, particularly VP1, and partly to the location of the C-terminal portion of VP1. This arises at the surface at about residue 200 and the remainder of the protein traverses the surface in the clockwise direction to terminate over the adjacent 5-fold related protomer. This portion of VP1 occupies a position equivalent to that of the canyon in the other picornaviruses.

One of the most striking and interesting features to emerge from the resolution of the FMDV structure is the location and nature of the portion of VP1 sequence termed the G–H loop (i.e., the looping linking strands G and H of the β-barrel). The amino acids forming this region are approximately residues 132–159 and include that part of the sequence that has been implicated as a major antigenic determinant and shown to be effective as a synthetic peptide immunogen. The G–H loop was found to be highly disordered so that no structure could be assigned to it between residues 136 and 158. The disorder started at VP1 residue 134, which is a cysteine linked by a disulfide bond to cysteine residue 130 of VP2. Two partially occupied positions for the disulfide bond could be seen in the electron-density maps. Residues 135 and 136 were progressively more disordered and between residue 137 and 157 the electron density was too weak to discern any structure.
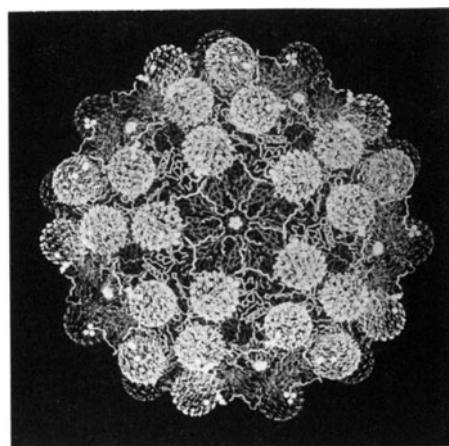
**Fig. 3.8.** Computer graphics representation of the $\alpha$ carbon tracings of FMDV. The view is centered on an axis of 5-fold symmetry. The spherical clusters of dots represent the volume potentially occupied by the G–H loop of VP1 which is too disordered to identify in electron density maps. Courtesy of Dr. D. Stuart et al., Department of Molecular Biophysics, University of Oxford.

It appears then that the portion of VP1 that is particularly effective as a synthetic peptide immunogen occurs as a large loop at the surface of a more or less featureless virus particle and, moreover, has minimal inter-action with the remainder of the capsid (Figs. 3.8 and 3.9). These features may help to explain the high proportion of virus-reactive antibodies elicited by peptides since, in the absence of strong interactions between the G–H loop of VP1 and other regions of the virus particle, it may adopt *in situ* conformation(s) similar to those of the synthetic peptide representation of the same sequence. Although this may be true to a first approximation, it is not the whole story for two reasons. First, antivirus MAbs have been described which appear to recognize sequences from both the G–H loop and the C-terminal portion of VP1 as parts of con-formational epitopes (64). This shows that the G–H loop does not necessarily function as an autonomous antigenic unit on the virus. Second, there is evidence that both the conserved Arg-Gly-Asp sequence at positions 145–147 within the G–H loop (7,84) and sequences from the C terminal region of VP1 are together important for cell receptor binding (31), again suggesting that there is some structural interaction between the two regions.

That adjacent sequences can influence the position/conformations of the G–H loop (Fig. 3.10) is further suggested from the properties of neutralization escape mutant viruses selected with MAbs. MAbs that have been shown by epitope mapping to recognize sequences within the G–H loop and the C-terminal region of VP1 selected neutralization
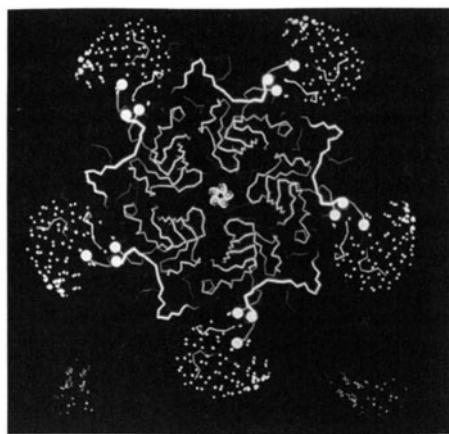
**Fig. 3.9.** $\alpha$ carbon tracing of the surface-oriented protein strands of a pentamer of FMDV. The view is centered on the axis of 5-fold symmetry. The heavy white lines transversing monomer units in a clockwise direction and terminating in spheres (representing disordered residues) are the C-terminal portions of VP1. The clusters of dots represent potential occupancy of the disordered G–H loop of VP1 which starts and finishes at the positions represented by the remaining spheres (1). Reprinted by permission from Nature, Vol. 327, p 713. Copyright © 1989 Macmillan Magazines Ltd.

resistant viruses with amino acid substitutions at positions 43, 48, and 59 of VP1. These residues are located in the B–C loop, which is adjacent to the G–H loop toward the 5-fold axis. That these substitutions can affect the orientation of the G–H loop was suggested by the fact that the mutant viruses could be distinguished from the parent virus in both neutralization and ELISA tests using anti peptide 141–160 antiserum. Furthermore, resolution of the structure of the mutant viruses showed that the orientation of the G–H loop was different from the parent virus, as judged by a reversal of the proportional occupancy of the two positions for the disulfide bond linking VP1 134 and VP2 130 (65). This also resulted in stronger electron-density maps from residue 136 to 143 so that this portion of the G–H loop could be modeled for the mutant viruses. The role of the disulfide bond between VP1 134 and VP2 130 in modulating the orientation of the G–H loop has recently been demonstrated by the resolution of the crystal structure of the virus in the presence of dithiothreitol (D. Stuart, personal communication). Under these reducing conditions the disulfide bond is broken and the structure of the entire G–H loop can be clearly seen overlying VP2 and 3 residues on the "southern" (i.e., distal from the 5-fold axis) side of the protomer.
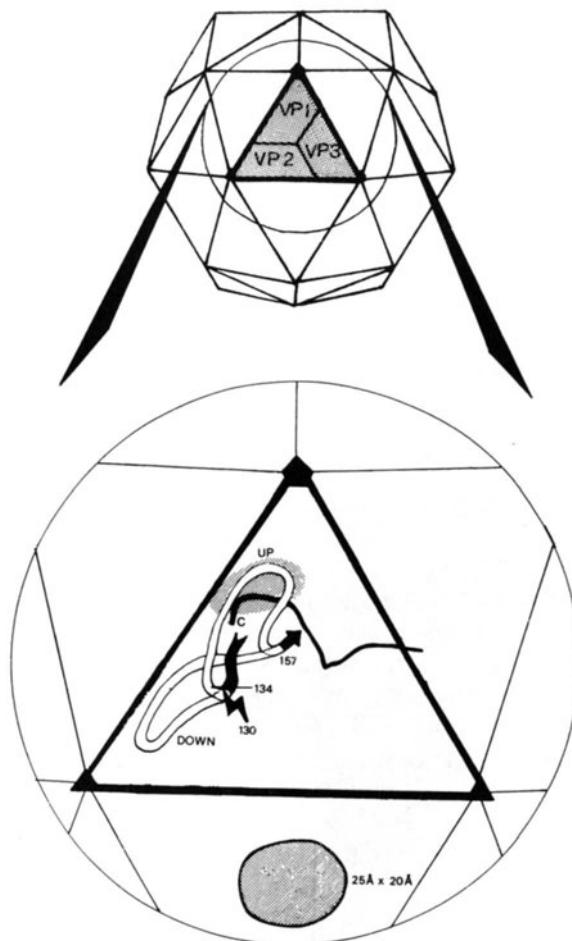
**Fig. 3.10.** Diagram representing the possible alternative conformations (the "up" and "down" position) of the G–H loop of FMDV VP1 (65). Reprinted by permission from Nature, Vol. 347, p 571. Copyright © 1990 Macmillan Magazines Ltd.

The presence of cysteine residues capable of linking the G–H loop of VP1 and VP2 is known to occur only in viruses of the $O_1$ serotype. However, there is circumstantial evidence from serotype $A_{22}$ viruses that mutations in VP2 at positions that underlie the G–H loop in the orientation seen with reduced $O_1$ virus can modulate antigenic and cell attachment properties of the virus, properties that can be directly attributed to the G–H loop (10). It is therefore possible that modulation of the orientation/configuration of the G–H loop by substitutions in regions

that are structurally adjacent on the virus particle is a common strategy adopted by the virus to permit antigenic variation. Such mechanisms are, of course, complimented by the selection of substitutions within the G–H loop itself (11,56,69,81,87,92).


## 10.  Genetic Restriction of Immune Response to Peptides

Although G–H loop peptide vaccines have been shown to routinely induce high levels of neutralizing and protective antibodies in guinea pigs results in target species, such as pigs and cattle, have been less impressive. Protective responses have been induced in both of these species but the percentage protection of pigs has been lower than for guinea pigs and even less so for cattle (M.J. Francis, personal communication). Although the specificity of the antipeptide antibodies induced in cattle and pigs appears to be indistinguishable from that induced in guinea pigs, the titers of neutralizing activity achieved are generally lower and more variable between individuals. A possible explanation is that the helper T cell epitope(s) present in the VP1 140–160 sequence is less effective in cattle and pigs than it is in guinea pigs.

T cell help is essential for the stimulation of antigen reactive B cells and provides the signals necessary for them to proliferate and mature into antibody secreting plasma cells (Fig. 3.11). Helper T cells recognize processed fragments (T cell epitopes) of the immunizing antigen in combination with class II molecules of the major histocompatibility complex (MHC) and presented at the cell surface. The T cell receptor proteins thus recognize T cell epitopes in conjunction with specific
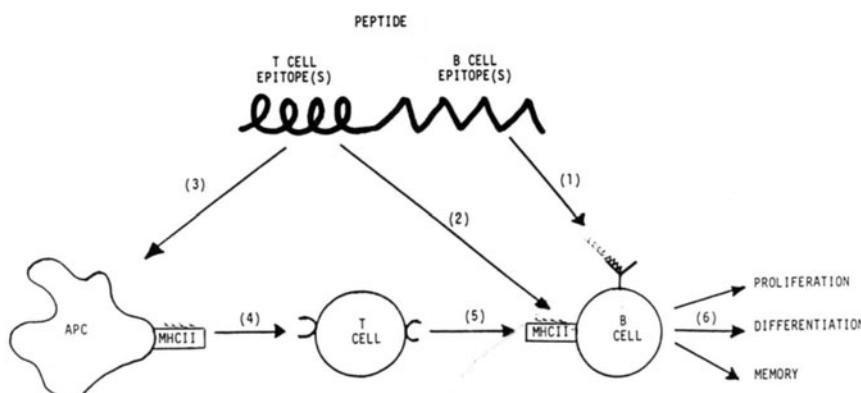


**Fig. 3.11.** Diagrammatic representation of the functions of helper T cell and B cell epitopes of a synthetic peptide immunogen in the induction of a full humoral antibody response (33).
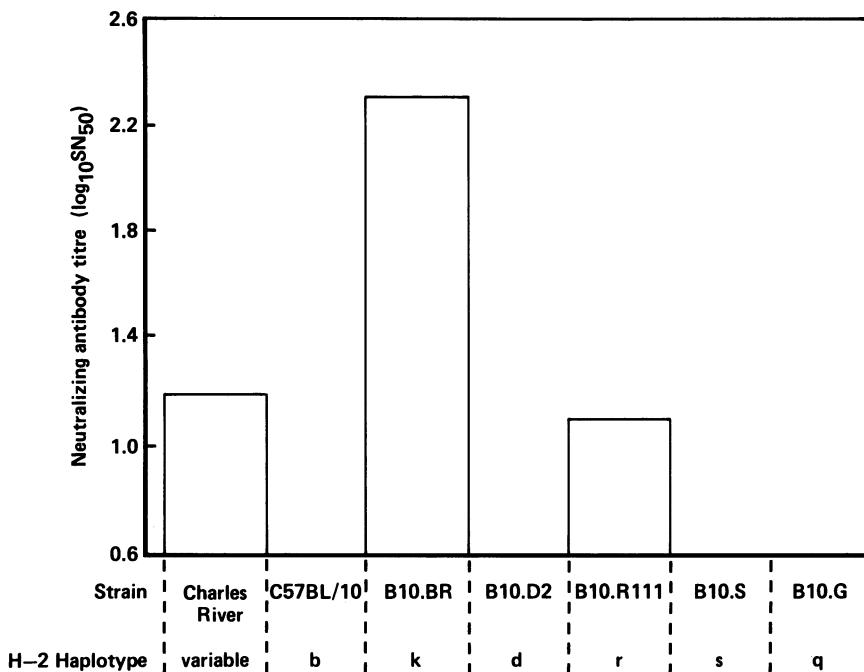
**Fig. 3.12.** FMDV neutralizing antibody responses of a range of outbred and congenic mouse strains to VP1 141–160 peptide (36).

portions of the MHC molecules. Since MHC molecules are restricted in number in an individual but are polymorphic within outbred populations there is a genetically defined variation in responsiveness to a given T cell epitope between individuals within a species. The T cell receptor, on the other hand, is analogous to immunoglobulin genes in that recombinatorial events during the development of T cells produce a vast array of specificities.

Congenic mice of defined MHC haplotypes were used to investigate genetic restriction of responsiveness to FMDV peptides and possible ways of overcoming such restrictions. Immunization of a number of such mouse strains with uncoupled VP1 141–160 peptides produced a range of responses dependent on the MHC haplotype (Fig. 3.12). Some strains produced no measurable antibody response and these were used to investigate methods of overcoming the restriction by incorporating specific T cell epitopes, known to be effective in such strains, into the peptide immunogen (40). These approaches showed that it is indeed possible to overcome MHC restriction in this way since colinearly synthesized peptides consisting of the FMDV sequence linked to foreign T cell epitopes (one from ovalbumin and two from sperm whale myo-
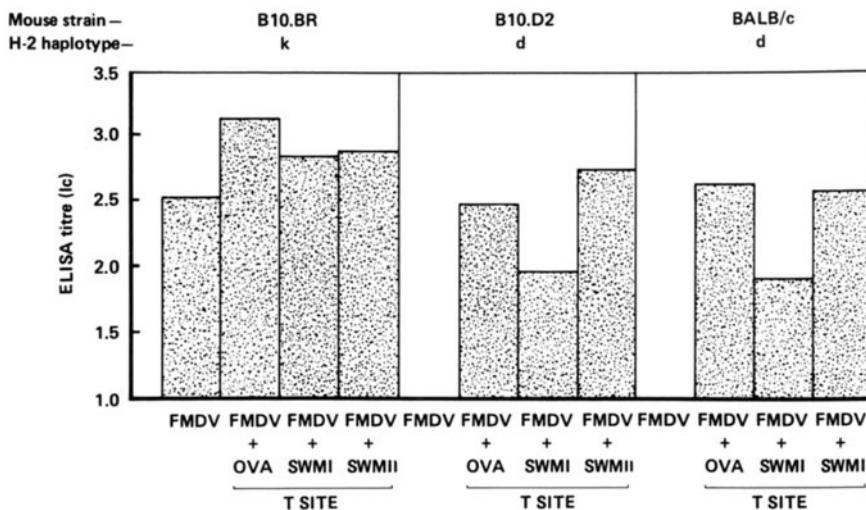
Fig. 3.13. Antibody titers elicited in FMDV VP1 141–160 peptide responder (B10.BR) or nonresponder (B10.D2 and BALB/c) strains of mice by 141–160 peptide colinearly synthesized with a 17 amino acid extension of the VP1 sequence or with heterologous helper T cell epitopes as assayed by ELISA (36).

globin), which are known to be functional in the non-FMDV peptide responsive mouse strains, induced antibody responses to the FMDV determinant as indicated by ELISA (Fig. 3.13). A control peptide consisting of VP1 141–160 extended with the natural VP1 sequence of equivalent length did not elicit a response in nonresponder mice and induced a response equivalent to that obtained with the 141–160 peptide alone in responder strains. Although each of FMDV-foreign T cell epitope hybrid constructs elicited the production of antibodies to the FMDV component, interesting qualitative differences came to light when the sera were tested for virus-neutralizing activity (Fig. 3.14). Two of the hybrid peptides induced neutralizing antibodies; the third did not. Examination of the specificities of the antibodies present in the different antisera by peptide mapping showed that the construct that did not induce neutralizing activity elicited antibodies with very narrow specificity. These recognized only the N-terminal portion of the FMDV peptide. Thus the principle of overcoming MHC restriction to FMDV peptide responses by the inclusion of defined T cell epitopes has been established, as it has with other peptide immunogen systems (12,45,54), but there are subtleties in the immunological interactions between B and T cell epitopes in synthetic constructions that require explanation. These are presumably related to the ways in which the peptides are processed and presented by B cells to recruit helper function from T cells.
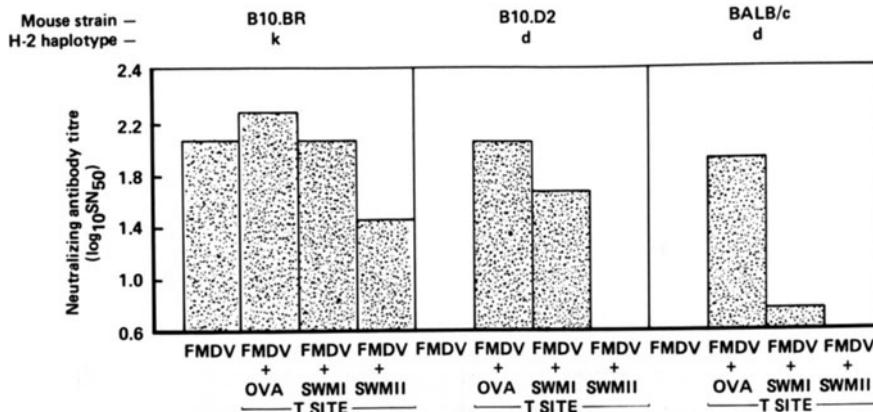
**Fig. 3.14.** Antibody titers elicited in FMDV VP1 141–160 peptide responder (B10.BR) or nonresponder (B10.D2 and BALB/c) strains of mice by 141–160 peptide colinearly synthesised with a 17 amino acid extension of the VP1 sequence or with heterologous helper T cell epitopes as assayed by virus neutralization (36).

Having demonstrated that combined T and B cell epitope peptides can overcome genetic restrictions of responsiveness in mice it is clearly important to investigate the potential for this approach in target species such as cattle. Recent studies have begun to define the region an FMDV structural proteins to which cattle helper T cells respond (25) and it will be interesting to determine the immunogenicity of peptides incorporating these sequences.

Antibody specificity and titer are not the only factors that govern the protective efficacy of anti-FMDV response since Di Marchi et al. (28) found a poor correlation between the level of virus-neutralizing antibody elicited in cattle by a synthetic peptide vaccine and the degree of protection afforded. This is in contrast to results obtained in cattle and pigs (13,61, M. Francis, personal communication) with biosynthetically produced peptide fusion proteins or chemically synthesized peptides coupled to carrier protein. Two properties of the antipeptide responses elicited by the peptide used in the De Marchi et al. (28) work may explain, at least partially, the lack of good correlation between antibody induction and protection. Mulcahy et al. (62) have shown that the ratio of virus reactive $IgG_1$ to $IgG_2$ antibodies induced in cattle by inactivated virus is generally higher than that elicited by the peptide. Thus isotype composition may be of importance in determining the effectiveness *in vivo* of the antibodies induced.

Antibody affinity has been shown to be of importance in determining the efficiency of virus neutralization in many systems and Steward et al. (82) have shown that the affinity of binding of antipeptide antibodies in

cattle sera to virus particles correlated with the protected status of the animals. It is, therefore, of importance to investigate factors such as peptide conformation, adjuvant formulation, and peptide presentation, which may influence the affinity and isotype distribution of antibodies induced with peptide vaccines.

## 11.  Peptide Antigen Presentation

Although synthetic FMDV peptides can raise protective levels of antibody, the immunogenicity of such material is low. For example, whereas $1\,\mu g$ of inactivated virus particles is sufficient to elicit a good neutralizing antibody response, approximately one hundred times this weight of 141–160 Cys peptide is required to induce comparable levels. Furthermore, peptides representing the natural FMDV VP1 sequence without an additional C-terminal cysteine residue elicit virtually no response. The improved immunogencity of the cysteine-containing peptides is probably due to dimerization of chains via disulfide linkages since peptide dimers produced by head-to-tail linkage or by a terminal coupling by methods other than cysteine – cysteine bridging also show enhanced immunogenicity (36).

An ingenious system for producing well-defined polymeric peptide constructs has been described by Tamm (86). In his system a core poly-lysine structure is produced by conventional peptide synthesis using lysine modified by $t$-Boc residues on both the $\alpha$ and $\varepsilon$ amino groups. Thus a branched structure is built up with a doubling of the number of amino groups available for further coupling at each cycle of synthesis. When the desired number of free amino groups per polymeric unit has been achieved then specific peptide sequences can be attached to each one by proceeding with conventional peptide synthesis or by chemically coupling preformed monomeric peptide chains. Peptides presented in this way have been shown to be considerably more immunogenic than monomeric preparations. A further advantage of this system is that it provides a convenient method for incorporating different peptides representing B or T cell epitopes into the same structural unit and so could be useful in producing synthetic immunogens that would be effective in the face of variations in MHC compositions between individuals within a population.

Another approach to improve the immunogenicity of peptide vaccines by presenting them as polymeric arrays has been to express them, by genetic engineering techniques, as fusion products in which they are linked to proteins that have the property of self-assembling into multmeric structures (2,20,24,26,88). The use of the core antigen protein of hepatitis B virus (HBc) has proved to be a particularly effective fusion partner for this purpose (24,78,80). HBc particles are extremely good immunogens and, moreover, they can be expressed to high levels in a number of
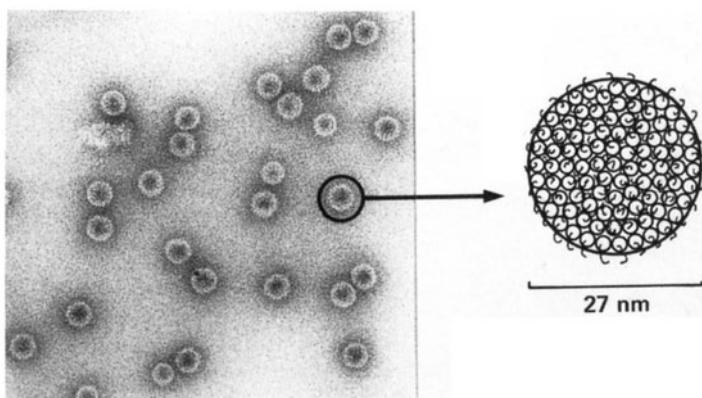
**Fig. 3.15.** Electron micrograph and diagram representation of hepatitis B core/peptide fusion particles. (E.M. by courtesy of Dr. H. Sailbil, University of Oxford.)

systems such as *E. coli*, yeast, insect, and mammalian cells. The HBc antigen protein has a molecular weight of 21.4K and is composed of 186 amino acids. The spherical core particles are 27 nm in diameter and are composed of ~300 copies of the protein (47). It has been shown that chimeric core proteins in which the foreign sequences have been fused at the N or C termini, or inserted internally, retain the ability to assemble into core particles (Fig. 3.15) (23a,37,80). The high immunogenicity of the particles appears to be due to a combination of their polymeric structure and the presence of strong helper T cell epitopes (23,60).

Early attempts to produce HBc fusion particles, in which the N terminus of the protein was extended to include the FMDV VP1 142–160 sequence, by expression in *E. coli* failed. For reasons that remain abscure this product was highly toxic to bacteria. However, sufficient material for immunogenicity experiments was made in HeLa cells by using recombinant vaccinia virus as a transient expression system. The results showed that on a weight for weight basis these recombinant particles approach inactivated FMDV particles in their ability to induce virus-neutralizing antibodies (24).

The fusion of peptide epitope sequences to the N terminus of HBc protein resulted in the formation of particles that could elicit high levels of antibody to the foreign determinant; in fact the responses are approximately 10-fold greater than those obtained with an equivalent weight of peptide coupled to KLH carrier protein. However, the majority of the antibody response was directed against HBc-specific determinants (37). By a combination of predictions of the tertiary folding pattern of the core protein (4) and epitope mapping of anti-HBc antibodies (77) a region of the protein has been identified that appears to be an immunodominant feature, the el loop. Recently, hybrid constructs have been produced by
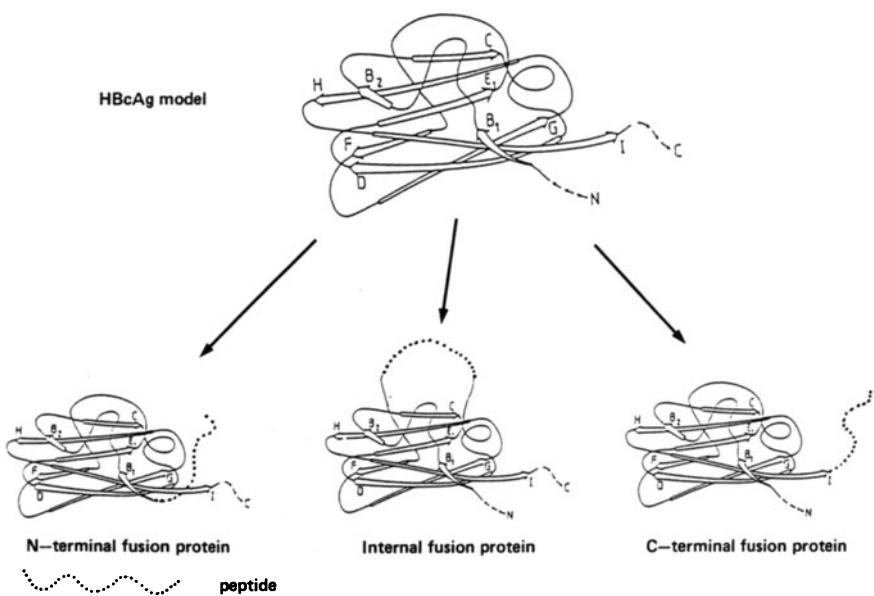
**Fig. 3.16.** Predicted folding of hepatitis B core protein showing fusion sites that have resulted in assembly competent chimeric particles (4).

inserting foreign peptide sequences within this loop (Fig. 3.16) and the resulting particles have proved to be approximately 10-fold more effective in inducing antipeptide responses than N terminal fusion constructs (15). Paradoxically these e1 loop insertion constructs bearing the FMDV VP1 142–160 sequence can be expressed to high levels in *E. coli*. The particles are highly effective in inducing FMDV neutralizing responses in guinea pigs and their immunogenicity in the target species, cattle and pigs, is currently under investigation.

## 12. Conclusions

Peptide vaccines against FMD have been found to be particularly successful experimentally, perhaps more so than in any other system described so far. Protection against virulent virus challenge can be routinely achieved in guinea pigs and has been demonstrated in the major target species, cattle and pigs. Clearly there are major problems to be overcome before this approach can become a practical reality, if ever. Examples of these are:

1. The question of MHC restriction and the development of constructs that will be broadly effective within and between species.

2. The significance and, if necessary, manipulation of the antibody isotype response.
3. The maximization of the affinity of peptide induced antibodies for virus, perhaps by manipulation of the peptide conformation.
4. The problem of selection of neutralization resistant variant viruses; is this more significant with peptide than whole virus vaccines and if so can it be overcome or forstalled by using, for example, novel cross-reactive peptides or mixtures.
5. Can the immunogenicity of chemically synthesized peptide vaccines be further improved; fusion protein constructs produced by bacterial fermentation techniques look promising but lack some of the theoretical advantages of a totally synthetic product.
6. Can formulations be developed that would provide longer lived immunity by incorporating a proportion of the immunogen within triggered release capsules.

The possibility of producing peptide vaccines has resulted from fundamental studies on the nature of FMDV and its antigenic structure. The demonstration of the feasibility of the approach has spawned further studies on the molecular structure of the virus and its relationship to antigenic character and the mechanisms of the host immune response. At the very least the quest for synthetic vaccines provides the rationale for exciting research.

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CHAPTER 4

# Vaccination against Animal Retroviruses

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## 1. Introduction

Retroviruses were among the earliest known viruses, discovered by
Ellerman and Bang in 1908 and by P. Rous in 1910 as filterable agents
causing leukemias or sarcomas in chickens, respectively. For many years,
however, they had a small following in the scientific community, due in
part to the lack of reliable cell culture systems and appropriate bio-
chemical techniques to characterize these transmissible agents. These
attitudes began to shift by the late 1960s with the discoveries of viruses,
later proved to be retroviruses, that cause a variety of naturally occurring
disorders in several animal species. They can be characterized as (a)
diseases with uncontrolled growth of cells of various types and origins, (b)
disease with the loss of certain cell types, and (c) disorders in which signs
and symptoms of inflammation and autoimmunity prevail. Some of these
viruses are capable of inducing several well-defined disease entities (see
BLV, FeLV, and FeLV) (3). In the last decade the pace of progress in
retrovirology has been further accelerated and amplified by the discovery
of new connections between retroviruses and human diseases: (a) dis-
covery of human retroviruses that cause adult T-cell leukemia/lymphoma
and acquired immunodeficiency syndrome (AIDS) and (b) identification
of human oncogenes related to retroviral transforming genes.

Vaccination against animal retroviruses will be viewed here essentially
from two potentially attractive perspectives: (a) it might be indicated as a
procedure to control important veterinary pathogens such as BLV, FeLV,
and EIAV and (b) an efficient vaccine tested in animal models like SIV
will play a central role in AIDS research in the coming years.

## 2. Genome Organization and Biological Cycle of Retroviruses

Despite the variety of their interactions with the host, all retroviruses
are quite similar in virion structure, genome organization, and mode of
replication (28). Retrovirus virions consist of a lipid-containing envelope

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and are 80–130 nm in diameter. Their surface is decorated by a single protein structure, probably a trimer of two protein subunits, products of the *env* gene. The internal nucleocapsid, or core, is an ill-defined, roughly spherical to conical structure made up of the three or four products of the *gag* gene. Also included in the core are several proteins that have important catalytic roles during replication. These include a protease and two products of the *pol* gene: the reverse transcriptase converting the genetic information from single-stranded RNA to double-stranded DNA, and integrase, necessary for covalently joining virus to cell DNA to form the provirus. The genome consists of two (usually identical) molecules of single-stranded RNA, ranging from about 7 to 10 kb in length, modified in ways reminiscent of cell mRNAs, including capping at the 5′ end and polyadenylation at the 3′ end. The order of the genes encoding structural proteins is invariably *gag–pol–env*. These replicative genes are flanked at both ends by regulatory sequences. During reverse transcription these sequences are in a way resulting in the presence of identical units, called long terminal repeats (LTR) at both ends of the integrated viral genome. A number of other genes involved in regulation of virus expression are present in some virus groups. These viruses code for proteins that act on the LTR and stimulate transcription. These regulators are called trans-acting transcriptional activators (TAT).

   Retroviruses have an unusual cycle of replication. Within the cell, the viral genome RNA, released from the envelope, serves as template for the synthesis of viral double-stranded DNA. This copy moves to the nucleus and becomes *integrated* in the cellular DNA as *provirus*. It is then used for transcription, generating RNA copies using host-cell systems as RNA polymerase, some of which are the full length genomes of progeny virions and others are processed to mRNAs and proteins sometimes aided by the presence of specific viral gene regulatory products. Virion assembly proceeds by encapsidation of the genome, association of the nucleocapsids with the cell membrane, and release of the virion by budding and probably also by membrane fusion in cell–cell interactions.

## 3. Classification of Retroviridae

Retroviruses have been initially divided into three subfamilies, based primarily on pathogenicity rather than on genome relationships. Viruses are further described according to the following: (a) virion structure (types A to D and others according to their morphology and budding characteristics; (b) utilization of particular cell receptors with respect to their host range (xenotropic, ecotropic, amphotropic); (c) life-style, whether endogenous (i.e., passed from parent to offspring as a provirus integrated into the germline) or exogenous; (d) presence or absence of an

**Table 4.1.** Retrovirus subfamilies, hosts, and diseases.[a]

| Subfamily | Host | Disease |
|---|---|---|
| Oncoviruses | Man, animals, birds, reptiles | Cancers, neurological diseases |
| Lentiviruses | | |
| Visna-maedi virus (VISNA) | Sheep, goat | Pneumonia, neurological diseases |
| Progressive pneumonia virus (PPV) | Sheep, goat | Pneumonia |
| Caprine arthritis encephalitis virus (CAEV) | Goat, sheep | Arthritis, pneumonia, neurological disease |
| Zwoegerziekte | Sheep | Pneumonia, neurological diseases |
| Equine infectious anemia virus (EIAV) | Horse | Fever, anemia |
| Human immunodeficiency virus (HIV) | Man | Immune deficiency, neurological diseases, Kaposi sarcoma, lymphoma |
| Simian immunodeficiency virus (SIV) | Non human primates (except macaque, the Asian old world primates) | No symptoms or immunodeficiency (varies with the species) |
| Feline immunodeficiency virus (FIV) | Cat | Immunodeficiency |
| Bovine immunodeficiency virus (BIV) | Cow | ? |
| Spumaviruses | Man, animals | ? |

[a] For more details, see (28).

oncogene (v-*onc*, a gene of cellular origin c-*onc* acquired by events of recombination); and (e) other pathogenic properties such as the absence of replication of some defective virus in the absence of simultaneous infection with a closely related, replication-competent virus. Based on nucleotide sequence relationship, structure and biological differences, the family Retroviridae has been divided into three subfamilies and groups containing some well-known strains (28,188,190,194) (Table 4.1).

## A. Oncoviruses

Viruses originally isolated as tumor-inducing agents, as well as related viruses, are traditionally placed into the subfamily Oncovirinae. This family includes five groups that are not closely related to one another. The avian leukosis-sarcoma virus (ALSV) group includes both exogenous and closely related endogenous viruses of birds. Viruses of this group have C-type virions and genomes that encode only virion structural genes (*gag, pol, env*), although many isolates of exogenous viruses are further modified by the presence of oncogenes, such as *src* in Rous sarcoma virus

(RSV) and *erb*-A and *erb*-B in avian erythroblastosis virus (AEV). The B-type virus group includes as infectious agents only the mouse mammary tumor virus (MMTV), isolated as both endogenous and exogenous but vertically transmitted viruses.

The mammalian C-type virus group includes a large number of endogenous and exogenous viruses and are represented by isolates from many groups of mammals, including rodents, carnivores, and primates, as well as some exogenous viruses of birds [i.e., the reticuloendotheliosis virus (REV)]. Feline leukemia viruses (FeLV) are arbitrarily classified into subgroups A through C, with endogenous viruses belonging to subgroup C. FeLV is an exogenous oncovirus that causes T-lymphoma, sarcoma, immunodeficiency and many other diseases.

The D-type virus group includes exogenous isolates from primates, such as the Mason–Pfizer virus, isolated from a mammary carcinoma of a rhesus monkey but of uncertain pathogenicity. Isolates include a virus associated with simian acquired immunodeficiency syndrome (SAIDS) in some captive monkey colonies. No oncogene-containing isolates have been described.

The HTLV-BLV group includes exogenous viruses associated with B-cell lymphoma in cattle and with T-cell lymphoma, as well as with some neurological diseases in humans and some wasting syndrome in rabbits. No endogenous relatives of oncogene-containing viruses of this group are known. In addition to genes encoding virion proteins, these viruses encode at least two nonvirion proteins important for gene expression.

## B. Lentiviruses

This subfamily includes exogenous viruses responsible for a variety of neurological and immunological diseases. HIV in humans is indirectly implicated in Kaposi sarcoma. The prototype members of this family were the "slow" viruses visna, equine infectious anemia (EIAV), and caprine arthritis-encephalitis (CAEV). HIV and the related simian (SIV), feline (FIV), and bovine (BIV) immunodeficiency viruses also belong to this group. Genomes of these viruses are characterized by a complex combination of genes in addition to *gag*, *pol*, and *env* (6).

## C. Spumaviruses

The spumaviruses or foamy viruses are the least well-characterized of the retroviruses. They have been isolated as agents that cause vacuolation ("foaming") of cells in culture from a number of mammalian species, including monkeys, cattle, cats, and humans. Persistent infection with these viruses is not associated with any known disease.

## 4. Animal Retroviruses as Naturally Occurring Viruses and as Model Systems

The hallmark of all of the three subfamilies of retroviruses is the establishment of permanent infection as integrated provirus in their hosts, which then become healthy or asymptomatic carriers for a prolonged period of time during which they are infectious for others. Within the oncovirus subfamily there are two distinct groups of leukemia viruses: those of chickens, mice, and cats and those of man and cattle. In the first group, viremia is clearly established and some animals are known to recover from infection and are subsequently immune to challenge. By contrast, people infected with HTLV or cattle infected with BLV do not appear to recover spontaneously. In the group of lentiviruses recovery is also never observed and viral persistence is the rule. Latency and variability have been revealed as the two mechanisms to escape the immune response of infected hosts and to establish persistent infection. Latency corresponds to a restricted synthesis of antigenic viral proteins; the reservoirs of viral genetic material invisible to the immune system in cells such as macrophages have been described as a "Trojan horse strategy." Variability, a direct consequence of the high number of mutations that occur during the viral replication cycle, allows the sequential appearance of antigenic variants that escape preestablished neutralizing or cytotoxic immune response. This strategy has been shown essentially for lentiviruses (VISNA, EAIV, HIV, etc.) but for HIV and VISNA, it has been shown also that the initial virus subtype or clone persists throughout the duration of the infection.

Since there is no evidence that the immune response to natural infection by these viruses is able to eliminate the infection, doubts have been expressed about the possibility that vaccines may induce protection. However, it still remains to be seen whether the generation of a strong immune response by vaccination will prevent infection following challenge by BLV, HTLV-1, or lentiviruses.

We shall discuss here retroviruses from each group described above:

1. BLV will be described in details as a animal model where latency, no viremia, and no recovery exist
2. FeLV will be cited as an animal model where viremia is prevalent and where recovery and successful vaccination have been achieved
3. SIV will be cited as an animal model of lentiviruses. EIAV will be briefly alluded to as it represents an illustrative case of generation of new variants concomitant to waves of fever. The new virus variant is not neutralized by the preexisting immune reaction.

The success of vaccination depends probably on a better knowledge of the pathogenesis induced by these viruses and of vaccination strategies used.

# 5. Oncoviruses

## A. Bovine Leukemia Virus (BLV)

Bovine leukemia (lymphoma, lymphosarcoma) is a contagious disease induced by bovine leukemia virus (BLV), a retrovirus exogenous to the bovine species. It is a chronic disease, evolving over extended periods (1–8 years). BLV is now recognized as the etiologic agent of enzootic bovine leukosis (EBL) (21–24).

### Pathogenesis

Bovine leukemia virus particles were first observed by Miller et al. in 1969 in short-term cultures of peripheral blood lymphocytes of BLV (+) animals in persistent lymphocytosis (PL). Numerous attempts to observe the virus in body fluids of PL animals or in tumors supposed to be of the enzootic bovine leukosis type failed until the discovery that viremia can only be monitored in the first 10–12 days postinfection preceding the appearance and permanency of antivirus neutralizing antibodies. The permanency of anti-BLV antibody proves the existence of a permanent antigenic stimulation, mediated via viral proteins and particles produced by lymphocytes of the B cell lineage and perhaps other cell types. If the long terms of BLV infection are considered, cattle seem to fall into three groups. The first, and largest of these groups, consists of those animals that develop a persistent infection and immune response but remain normal in all other respects. The second group, representing perhaps 30 to 35% of all BLV-infected cattle, develops a persistent lymphocytosis. The lymphocytosis is due to an expansion of the B-lymphocyte population. Some of these B cells carry the BLV information but others do not and it has been suggested that they are a population that is expanded because it is responding to the infection. A third, and much smaller group, consists of those animals that develop lymphosarcoma or tumors.

Numerous experimental data have established major features of the BLV pathogenesis:

1. BLV is the etiological agent of bovine and ovine leukemia. All sheep infected by BLV die in the tumor phase of the disease (unless they disappeared too early for unrelated causes). Control animals, housed together with the uninfected ones showed neither sign of infection nor onset of tumors.
2. Infection, once established, lasts for life. Take of infection is linked to the infectious dose. Moreover, the smaller the infectious dose, the longer the latency before onset of the neoplastic phase (98,99). It appears thus that the future of the animal is sealed at infection.
3. As in the case of other retroviruses, the surface glycoprotein (gp51 for BLV) serves as the cell attachment protein and the hydrophobic N-

terminus of the transmembrane glycoprotein gp30 is responsible for membrane fusion. Recent results from our lab suggest also that both BLV glycoproteins, gp51 and gp30, play a crucial role in cell fusion and that the replacement of the BLV fusion peptide by its SIV counterpart does not modify the fusion capacity of BLV gp30.

4. The nature of the host-cell receptor for BLV is not currently known, although it appears to be distinct from CD4 surface glycoprotein for HIV. In cow and sheep, the target cell of the virus express the pan-T CD5 marker after short term culture of infected lymphocytes. Persistent lymphocytosis represents a spectacular enlargment of a compartment containing BLV-infected CD5$^+$ B cells (23,24,151).

5. It appears that transformation by BLV does not occur by transduction of viral oncogenes or by viral insertion and cis-activation of cellular proto-oncogenes. Rather, the proviral structure includes genes *tax* and *rex* necessary for tumor induction, most probably via interaction of their protein products with the products of normal genes. Rare circumstances lead the target cell (a pre-B-lymphocyte) to a disruption point of normal hematopoeisis as for non-viral human leukemias and lymphomas (169) and to a point of no return on the way to full transformation.

6. Tumors are monoclonal, referring to the site(s) of BLV integration and, in a given animal, derive from a single transformed cell. Abnormalities in the structure of chromosomes and in their number (aneuploidy) are frequently observed in BLV-induced bovine leukemia and in cultured tumor cells from cow and sheep.

7. Only a few cells in the tumors express BLV antigens. Some sheep tumor cells grown in culture have silent BLV proviruses and some tumors have a truncated provirus unable to code for viral proteins, indications that no BLV information is mandatory to maintain the transformed state (184).

8. BLV when injected into rabbits induces profound perturbations of the white cell compartment (neutropenia, lymphopenia) together with diarrhea, wasting syndrome followed by death while the antibody titer dropped to zero (22,151).

**Transmission of BLV**

BLV infection has a worldwide distribution. In temperate climates the virus spreads mainly via iatrogenic transfer of infected lymphocytes. In warm climates and in areas heavily populated by hematophageous insects, there are indications of insect-borne propagation of the virus. Transmission of BLV infection has been the subject of many field observations and experimental trials. Cases of natural infection are documented in cattle, sheep, capybara, and water buffalo. The infection can be experimentally transmitted to goats, pigs, rabbits, rhesus monkeys,

chimpanzees, and buffaloes (22,97,151). It has been amply documented that horizontal transmission is the rule, including the transplacental route, which amounts to 15% of infections in the offsprings of BLV-positive dams and that infected cells are the best potential vehicles of infectious BLV particles. Consequently, the concentration of BLV-infected cells in the transmitted fluid (blood in most cases) is expected to play a major role in the success or failure of BLV transmission. As few as 926 lymphocytes from a highly infectious animal have been shown to transmit infection to serologically negative sheep (98).

## Protection against BLV Infection

Importance of gp51 in Immune Response

All the data available demonstrate the importance of the external envelope glycoprotein gp51 (51,000 Da) in the immunological response of naturally or experimentally infected animals:

1. BLV envelope glycoprotein gp51 (51,000 Da) is the first viral antigen reacted against by the newly infected host. Antibodies are detectable 2 to 5 weeks after experimental infection. Antibodies against the gp51 glycoprotein are generally present in higher titers and appear earlier than antibodies raised against the major internal protein p24 (24,000 Da) (10,96,97). This indicates that viral proteins especially gp51 are potent antigens and that the presence of anti-BLV antibodies is indeed a faithful marker of the presence of the virus, throughout the life of infected animals.
2. Anti-gp51 antibodies produced after natural or experimental BLV infections of cattle and sheep display several antiviral activities: they neutralize virus infectivity (pseudotypes) and syncytia inducing activity, and exhibit a strong cytolytic effect on BLV-producing cells in the presence of rabbit complement.
3. Sheep or calves immunized passively with various doses of immuno-globulins obtained from infected sheep serum (82) or colostrum (96) or from infected cow colostrum (86) successfully resist an infectious challenge provided they had sufficiently high anti-gp51 antibody titres.

In order to characterize regions of gp51 which could potentially act as efficient BLV subunit vaccine or as an efficient probe for diagnosis, approaches based on the use of monoclonal antibodies and synthetic peptides have been used to precisely identify crucial epitopes involved in the biological activities of BLV gp51. Molecular dissection of gp51 with monoclonal antibodies has allowed the definition of 8 distinct antigenic sites and 2 overlapping sites: A, B, C, D, E, F, G, H and B' and D'. Sites F, G, H were shown to be involved in the biological activities of the virus, namely infectivity and syncytia neutralization tests; monoclonal anti-bodies against site G displayed complement cytotoxic activity for BLV-

infected cells (15,16). Only MAbs to F, G, H were competed for by sera of BLV-infected cattle or recently infected sheep in competition assays for gp51 binding (17). These three epitopes are localized on the NH2-half of the polypeptide backbone; they have a three-dimensional structure and depend upon accurate and important glycosylation of the polypeptide backbone (18,87). In fact, the gp51 antigen contains only 268 aa polypeptide backbone (30,572 Da) (159); the yeast-expressed gp51 is partially glycosylated into heterodisperse protein molecules and is poorly recognized by sera from BLV-infected cows (87).

On the other hand, ELISA tests involving BLV virions disrupted with nonionic detergent or purified gp51 as antigens in a sandwich assay, where the same MAb was chosen as binding MAb and as conjugate, suggest the existence of oligomeric ENV complexes for the native structure where F, G, H were maximally reactive (152). The antigenicity of such oligomers may thus be relevant to the design of diagnostic procedures and subunit BLV ENV vaccines. A synthetic peptide approach indicated also that only the protein segment extending between aminoacids 39 and 157 carried epitopes involved in virus infectivity (149). In other experiments involving heterologous expression of *env* gene fragments in *Escherichia coli* and *Saccharomyces cerevisiae*, it appeared that the fragment extending from aa 56 to aa 105 was clearly recognized in ELISA or in WB by bovine antibodies from cattle naturally infected with BLV.

The question of BLV variability among field isolates is also relevant to the understanding of BLV evolution and spread, and is of paramount importance for the design of an efficient diagnostic procedure or an efficient broadly protective vaccine. ELISA assays allowed the selection of BLV gp51 variants carrying $F^-$, $G^-$, or $H^-$ mutations. Until now no variant lacking F, G, and H could be identified, suggesting that this situation is most probably incompatible with the possibility for the virus to achieve infection. It appeared at the molecular level that sequence variations among widely different isolates were limited and confined to point mutations sometimes leading to only 12 amino acid substitutions (30,100,148). Considering the various facts reported above, it follows that procedures that make use of native, biologically important determinants of gp51 constitute a prerequisite for the design of an efficient BLV eradication program, including diagnosis of the disease, and/or vaccination campaigns.

## Diagnosis of Enzootic Bovine Leukosis

EBL should be distinguished from sporadic bovine leukosis (SBL), a disease that occurs mainly in young animals. In SBL, lesions have the same appearance as in EBL, but no virus could be retained as etiologic agent. The diagnosis of EBL may thus be confirmed by a specific laboratory test for BLV. Absence of viremia and importance of the gp51 antigen (see above) prompted the investigators to develop an early

detection of BLV infection based on serological screening for anti-gp51 antibodies. Agar-gel immunodiffusion test (AGID) has been widely used to detect infected animals and eradicate the disease in field conditions. For routine purposes enzyme-linked immunosorbent assay (ELISA) involving one or two monoclonal antibodies appears for routine purposes as the most practical, sensitive, and specific system presently available for large scale serological detection of BLV infection, where samples with low amounts of antibodies (individual milk) or pooled per herd (bulk milk, pooled sera) are used (147,150).

Recently, monoclonal anti-p24 antibodies were produced by several groups. By using the p24 internal antigen, we have developed ELISA tests (competition and indirect) that enable us to distinguish between infection (having antibodies to the p24 antigen) and immunization with a vaccine consisting of the envelope gp51 glycoprotein. The polymerase chain reaction (PCR) is regarded as a useful and rapid method for the detection of BLV and provides an alternative to conventional serological techniques. Preliminary results indicated that the PCR is a useful tool for the direct detection of BLV in eradication programs and in challenge experiments after vaccination.

## Prophylactic Approach

The existing ways to confront BLV infection are as follows:

1. Diagnosis followed by direct culling of infected animals and strict surveillance of the herd including successive serological tests. This has been used in Europe. It is an efficient but costly solution.
2. Diagnosis followed by segregation of the infected animals from uninfected ones within a herd and subsequently culling the infected cows. This method could be used for selected herds but is unpractical nationwide.
3. Zona pellucida-intact bovine embryos can be transferred from bovine leukemia virus-infected donors, including those bred by BLV-infected bulls, without risk of transmitting BLV, provided they are properly washed prior to transfer. As a result, elimination of BLV infection is thus easily feasible even from herds with very high genetic value where culling of infected recipients would represent an important financial loss.
4. Vaccination would be the method of choice from a cost and practical point of view, once a safe and reliable vaccine exists.

## Vaccination against BLV Infection

A reliable protective BLV vaccine would have to be noninfectious, nononcogenic, and prevent persistent infection and should induce an antibody response that could be distinguished from the response induced by BLV infected animals. Since antibodies to BLV gp51 are neutralizing

*in vitro* to both virus infectivity and release and include subclasses with cytolytic activities, a vaccine resulting in the production of antibodies to gp51 should be protective to virus infection and spread.

*Past.* The first attempts to establish immune protection to BLV infection was carried out by Miller and Van der Maaten in 1978 (108). That study was very limited but repeated and expanded in 1983 (109,111). They used whole virus as immunogen prepared from cell culture supernatant of the persistently infected fetal lamb kidney cell line (FLK). Either acetylethylenimine or binary ethylenimine were used for inactivation. Selection of these chemicals was based on research with foot-and-mouth disease virus, which showed that viral nucleic acid was affected, whereas antigenicity of proteins was not. An aluminium hydroxyde gel was used as adjuvant. The problem with oil adjuvants as Freund's adjuvant is that the intense tissue reaction may cause a problem in animals that are intended for slaughter. Each dose of vaccine contained 0.3 to 0.4 mg of viral glycoprotein. Virus challenge was accomplished by subcutaneous inoculation of infected lymphocytes. The number of lymphocytes used for challenge has varied from 2500 to 4,000,000.

Three different systems were used for evaluating the results of vaccine challenge: syncytium induction assay (SIA) in cat cells, glycoprotein antigen production in lymphocyte culture, as detected by radioimmunoassay (RIA), and bioassay in sheep, the most sensitive indicator of BLV infection. The trial in 1978 was limited to four animals but three of them appeared to be protected by the vaccine. The second study was less successful. One reason may have been that they used a 100-fold higher dose of infected lymphocytes for the challenge. Another factor was that the virus inactivation treatment apparently was inadequate because one of four vaccinated cattle that were not challenged became infected. Even though it was difficult to interpret in these conditions the protective effect of this vaccine it seems that immunization altered the course of BLV infection in many animals. When they compared SIA results for the year following challenge, it appeared that even though vaccinated animals became infected the number of infected lymphocytes in their blood was reduced.

In another experiment conducted in Romania by Pätrascu et al. (136), better results were achieved using inactivated whole virus with an oil adjuvant which may have improved the immune response; they showed complete protection in 18 of 20 animals after challenge. However, the challenge was given intramuscularly and might not have been as effective as in the subcutaneous inoculation procedure. The carbohydrate component of gp51 is necessary for the antigen to be recognized by antibodies of infected animals (87,146). Using just the protein as a vaccine cattle might be protected without causing them to seroconvert. This serological reaction is a disadvantage in terms of the potential usefulness of such a

vaccine because vaccinated cattle could not be easily differentiated from infected cattle. However, as tested by Miller et al. in 1984 antigen treated with glycosidases did not prevent infection (110).

In 1984, Onuma et al. described the vaccination of sheep with several different BLV preparations (132). Gp51 and p24 antigens purified from the virus, glutaraldehyde-fixed fetal lamb kidney (FLK) cells chronically infected with BLV and sheep fibroblasts transformed with BLV (SF-28 cells) were used to inoculate 12 sheeps. Each sheep was given three injections, the first in Freund's complete adjuvant. For the challenge 3 weeks after last inoculation, they used 10,000 infected lymphocytes, given subcutaneously. The sheep vaccinated with p24 antigen or with SF28 cells did not make antibody to glycoprotein antigen, and were not protected against the challenge. In contrast, sheep vaccinated with glycoprotein or with FLK cells made antibodies to glycoprotein and seemed to be protected. It should be noted that syncytium assay was used to check for BLV infection and a more sensitive evaluation, such as bioassay, might have shown that some of the sheep were not protected.

Two other vaccination trials were performed in Russia by Parfanovich and her colleagues (135) and in Japan by Kono et al. (82). The Russian workers purified virus on sucrose gradients and then tested several methylated amino acids as inactivating agents. Aluminium hydroxyde was used as an adjuvant and two injections were given. The challenge inoculum was either cell-free BLV or infected lymphocytes but in the results it is not clear which was used in various experiments. The authors concluded that vaccinated cattle were completely protected against challenge, but this probably could be considered questionable because of the method used to test vaccinees for infection. Blood lymphocytes were cultured with phytohemagglutinin and then examined by electron microscopy for virus-like particles, and most people would agree that such an assay is relatively insensitive. The Russian group also did some vaccine trials in sheep but they are difficult to interpret because the controls shown are from experiments done previously in other laboratories. The Japanese group purified BLV from FLK supernatant by ammonium sulfate precipitation and further treatment with 0.1% Triton X-100. Eight sheep were injected 3 times at 2 weeks intervals with an immunogen–Freund's complete adjuvant mixture. Two weeks after the last injection, sheep were challenged with BLV-infected sheep lymphocytes and remained uninfected. All sheep were challenged again 45 weeks after the first immunization when their gp51 antibody titers had significantly dropped and all animals were infected.

These preliminary experiments clearly suggest that induction of neutralizing antibody response is a faithful parameter for short-term protection to BLV infection, provided sheep have high enough anti-gp51 titers. They also seem to indicate that protection does not last for more than a few months.

Some experiments have also been performed in which attempts were made to use a nonviral immunogen to protect animals. Theilen et al. used as antigen a lymphoblastoid cell line BL-3 derived from a case of calf lymphosarcoma (non-BLV infected). Two or three injections of these cells, mixed with aluminium hydroxyde were given to young cattle and the treated animals were challenged with infected lymphocytes. Results of the first two experiments (180,184) showed that 7 out of 9 vaccinees were protected against BLV challenge although only 7 of 9 nonvaccinated controls became infected after challenge. Moreover, in a third experiment cited by Miller (111), a control group was added that received only aluminium hydroxyde: the adjuvant was just as protective alone for 3 of 12 animals. A different lymphoid cell line BL-20, also from a calf lymphosarcoma, was used by Roberts et al. (164) to immunize sheep. Although a slight protective effect was observed in the period shortly after vaccination, a subsequent study in cattle showed no protection (165). Cellular extracts or plasma membranes obtained from lymph nodes of BLV-infected cattle, or BL-3 cells were used more recently by Ristau et al. to immunize sheep and calves. No BLV infection was induced in sheep using bovine BLV-infected lymphocytes as an inoculum, 11 and 18 months after vaccination. BLV infection was achieved, however, in four vaccinated sheep by injection of BLV-infected sheep lymphocytes, 18 months following vaccination (161). The same protocol using tumor cell extracts or BL3 cells, when applied to calves did not lead to protection against a challenge of BLV-infected bovine lymphocytes (162,163).

*Present.* Modern adjuvants exhibit reduced side-effects and significantly enhance immunogenicity of the antigen. Another advance is a better knowledge of the immunogenicity of an antigen: complex or multimers are more immunogenic than soluble antigens. Such criteria could be fulfilled by incorporation of gp51 into the immunostimulating complexes (ISCOMS) which contain as unique adjuvant, Quil A, a glycoside extracted from the bark of the South American tree *Quilaja saponaria Molina* (116).

The use of ISCOM technology for presentation of gp51 has proven to induce considerably higher response in mice than purified gp51 (107). But further work to study the immunoprotection of BLV-ISCOMs in sheep was unsuccessful, when a very high dose of infected lymphocytes was used for the challenge (unpublished results). In this first experiment, purified whole BLV was used for the preparation of ISCOMs. The virus was solubilized by nonionic detergents *n*-octylglucoside or Tween-20, which did not denature the neutralizing epitopes F, G, and H, while the detergents Triton X-100 and MEGA-10 made the neutralizing epitopes unreactive to the corresponding monoclonal antibody. The presence of all viral proteins in the BLV-ISCOMs made difficult the distinction between vaccinated and infected animals. An other major problem of the BLV-

ISCOMs is that as found with other retroviruses (FeLV, HIV), the BLV outer glycoprotein is poorly recovered during the purification of the virus. In the search for alternative methods for the purification of BLV and recovery of gp51, three procedures were applied to purify gp51 as a monomeric antigen.

First, Buck et al. (19) used polyethylene glycol (PEG) precipitation to concentrate the virus from the culture medium: PEG precipitation is considered as a mild method for concentration of proteins and organelles, their biological activities usually being retained. The precipitation phenomenon occurring with PEG partly obeys the principles involved in two-phase extractions with aqueous systems; this procedure has been applied recently to concentrate and purify outer envelope proteins BLV gp51 (62) and FeLV gp70 (61). Second, lectin chromatography can be utilized for the purification of viral envelope glycoprotein: lectin from *Lens culinaris* and concanavalin A have been used for the purification of gp51 from purified BLV preparations or from cell culture supernatant, respectively (132,146). Lectins can also be used as probes to reveal sugar structures present in viral glycopeptides, as for the external glycoprotein gp120 of HIV when expressed in different cell lines (60). Third, immunoaffinity chromatography is a key step for the purification of viral envelope protein when monoclonal antibodies are available such as for BLV gp51.

In the case of ISCOMs, hydrophobic interactions hold together the viral protein and the iscom-matrix. The purified gp51 lacking an accessible hydrophobic region, it now seems possible to overcome the problem by forcing gp51 to expose hydrophobic areas hidden in the molecule by low pH treatment, as worked out with bovine serum albumin (115). The purified gp51-ISCOM when inoculated into four calves induced high antibody response (M. Merza, unpublished results). It remains to be seen whether its immunogenicity does protect animals against challenge infection.

An other advance in the recent years has come from the use of recombinant DNA technology to express viral proteins in large quantities in prokaryotic or eukaryotic cells such as yeast, insect, and mammalian cells or to develop synthetic vaccines. Expression of the core antigen of hepatitis B virus (HBC Ag) in *E. coli* leads to the highly efficient synthesis of capsids that are morphologically and immunologically indistinguishable from viral core particles. The usefulness of recombinant HBcAg as a carrier for foreign oligopeptide sequences has been suggested and special vectors have been constructed to express and expose foreign oligopeptides, such as aminoacids 56–103 of gp51 (13). Yet no information is available concerning the immunogenicity of this construct in rabbit, sheep, or cow.

Recombinant vaccinia virus appears particularly efficient at producing the gp51 antigen in a native configuration, with accurate addition of the

saccharide moiety and without further extraction, purification, and denaturation of oligomeric structures. Several recombinant vaccinia viruses containing in part (gp51 alone) or in full (gp51 and gp30) the *env* gene of BLV have been constructed and used in rabbits to study their immunogenicity or in sheep to confer protection against experimental BLV challenge. No transmission to contact control animals and no side effects were observed suggesting that live modified vaccinia viruses can be used as expression vehicles for BLV antigens to confer protection against the disease. Considering the worldwide distribution of BLV and the variability of BLV envelope, three isolates have been used for the constructions: Australian provirus isolated from a sheep tumor (84), Belgian provirus from bovine tumor T15-2 (153), and Japanese provirus from bovine tumor BLV-1 (127). The BLV *env* gene is cloned generally into the hemagglutinin HA locus of vaccinia virus, except for the Australian isolate where the thymidine kinase (TK) locus was used.

Several facts contribute to the efficacy of the recombinants used:

1. No significant neutralizing antibodies have been observed in rabbit or in sheep after inoculation of recombinant vaccinia virus containing the genetic information for gp51 alone (84,153). The ability to raise high titer neutralizing antibody was shown to be linked to the presence of the native configuration of epitopes F, G, and H. These determinants were thus in their native configuration only when presented on the external gp51 coexpressed with the transmembrane gp30.
2. The recombinants that used stronger promoters induce higher antibody titers than did weak promoters as the modified or unmodified 7.5-kD promoter (84,127,128). In fact good expression of BLV glycoproteins was obtained by the relatively strong promoters H6 (early/late promoter) (153), ATI (cowpox virus A-type inclusion promoter) (129), and PFE/L (fowlpox virus early/late promoter) (84). Higher expression of BLV glycoproteins was driven by an even stronger triple promoter element consisting of the vaccinia virus H6 promoter, the cowpox virus ATI promoter and the vaccinia virus HA promoter (153).
3. Until now, two vaccination trials have been performed in sheep. In the first experiment two different constructs harboring the BLV *env* gene (gp51 and gp30, noncleaved precursor pr 72) downstream the triple promoter have been used (153). Briefly, animals were injected with live recombinants intradermally and subcutaneously at 2 spots on both sides of the spinal cord and were immunized twice at 6-weeks intervals. Six weeks later, challenge was performed with high doses of bovine infected lymphocytes (1500 infectious doses). Animals that became infected after challenge showed high anti-p24 antibody titers, high neutralizing antibody titers in a pseudotype inhibition test, and no decrease of anti-gp51 antibody titers. Moreover, BLV could be

recovered from their lymphocytes after short-term culture. In contrast, absence of anti-p24 antibodies and decrease of anti-gp51 antibody titers reflected protection against BLV infection. Virus could not be recovered after short-term culture of peripheral white cells. In the second experiment sheep were vaccinated with recombinants harboring the BLV *env* gene (gp51 and gp30) downstream of the ATI promoter; recombinant-infected cells inactivated with 0.4% $\beta$-propiolactone were used as boosters three times at 10, 14, and 17 weeks after recombinants inoculation. At 20 weeks postinoculation, all sheep were challenged with high doses of bovine-infected lymphocytes (129). Although vaccination did not completely prevent infection itself, it significantly suppressed the growth of BLV in peripheral blood lymphocytes infected after challenge as assessed by syncytia assay.

In conclusion, high neutralizing titers seemed to parallel protection of animals against a BLV infection. As suggested earlier when the recombinant was able to activate helper T cells without inducing detectable anti-gp51 antibodies (127), it could be that the neutralizing titer is not the crucial parameter but reflects an overall efficacy of the protective response. The cell-mediated immune responses were assumed to play a major role in the protective immunity when vaccination induced protective immunity that could suppress the growth of BLV in carrier animals without correlation with neutralizing antibody titers.

*Future.* Studies on the "processing" of antigen led to the demonstration of the role of major histocompatibility antigens (MHC) in presentation of antigenic fragments or peptides to the T cell. Recent studies exploited a successful vaccination protocol with RLV (Rauscher leukemia virus complex) in inbred mice to analyze the T-cell response capable of protecting more than 90% of mice against a lethal challenge with live RLV. In this system, cellular immunity alone is sufficient for protection and requires both $CD4^+$ and $CD8^+$ immune T cells, as Th (helper), Tc (cytotoxic), and Td (delayed-type hypersensitivy) (65).

Retroviruses of murine, avian, feline, bovine, and human are immunosuppressive in their hosts. It has been shown that the transmembrane proteins of retroviruses all have regions of homology spanning up to 26 amino acids residues. Synthetic peptides encompassing a portion of this region of homology suppress the proliferation of lymphocytes *in vitro* (105). On the other hand the discovery of molecular mimicry of a cellular growth factor (erythropoietin) by a viral glycoprotein (gp55 of the spleen focus-forming virus SFFV) (91) is a provocative stimulus to search structures that could perturb or suppress the host immune responses to vaccines.

In order to develop an ideal vaccine against BLV infection, molecular dissection of BLV proteins are in progress to identify T cell epitopes that associate with MHC antigens to induce T cell proliferation. Hydrophobic

cluster analysis is a very efficient method to analyze and compare protein sequence (88) and identify undesirable structures and sequences that could alter the efficacy of a subunit vaccine. The ideal vaccine against BLV infection should (a) generate large numbers of memory T- and B-lymphocytes to overcome variability of BLV isolates; (b) be capable of being processed to induce T cell responses to a sufficient number of T cell epitopes to overcome genetic variability between hosts and also to overcome *in vivo* selection of virus mutants that are resistant to recognition by cytotoxic T cells, as described recently for the lymphocytic choriomeningitis virus, a negative strand RNA virus (143); (c) result in persistence of antigen so that B memory cells are continuously recruited to produce circulating neutralizing antibody; and (d) neutralize free virus, prevent cell fusion and syncytia formation, and eliminate cells that are already infected.

## B.  Feline Leukemia Virus (FeLV)

Feline leukemia viruses (FeLV) first discovered in 1964 by Jarrett and co-workers are naturally occurring, contagiously transmitted typical-type C retroviruses of domestic cats. Study of FeLV provided the first evidence that horizontally transmitted retroviruses were the predominant cause of leukemia in animals in natural conditions. Naturally occurring FeLV-associated fibrosarcomas and lymphomas have also been a source for the identification of viral oncogenes. Furthermore, study of FeLV provided early evidence that immunosuppressive diseases appeared to be caused by retrovirus infections and provided an important paradigm in seeking a retroviral etiology for AIDS in humans. Finally FeLV is the first retrovirus for which a vaccine was developed and is now in use (83,95,117,123,126).

**Pathogenesis**

After FeLV has entered the oral or nasal cavity, the virus first replicates in lymphoïd tissue. From there it spreads in a cell-associated state to the bone marrow, where neutrophils and thrombocytes are preferential target cells. When bone marrow cells become infected, viremia develops and the virus spreads readily to the spleen, lymph nodes, intestine, urinary bladder, and salivary glands, where it is shed in high amounts. FeLVs are endemic in free-roaming, urban domestic cats, and it has been estimated that up to 50% become infected at some point in their life time. Up to 30% of these cats become persistently viremic (progressors), particularly those which are repeatedly exposed or exposed at an early age, and remain generally virus neutralizing-antibody (VNA) negative. Once established, this progressor (persistent viremic) state rarely is reversed and mortality is nearly 100% within 3 years after diagnosis. Up to 80% of these cats succumb to degenerative diseases such as aplastic anemia or

immunodeficiency. However, a substantial minority develop leukemia or lymphoma, myeloproliferative disease, fibrosarcoma, or degenerative neurological syndromes. The majority of adult cats exposed to FeLV experience regressive infection, curtail virus replication, and develop serum antibody to the virus. Some of this latter population harbor latent FeLV infection, which persists for months to years in bone marrow cells. Latent FeLV infections may on occasion be reactivated, transmitted congenitally, or be involved in the genesis of "virus-negative" leukemia or aplastic anemia.

FeLV isolates have to date been assigned to three subgroups A, B, C according to their host range *in vitro* or the seroreactivity of their external glycoprotein gp70. FeLV-A is the subgroup most frequently isolated, whereas FeLV-B occurs only in association with FeLV-A. FeLV-C is rarely found and then either together with FeLV-A, or with FeLV-A and FeLV-B. After experimental infection both FeLV-A and FeLV-B induce tumors, but FeLV-C seems to be associated with aplastic anaemia. FeLV-A isolates are generally minimally pathogenic, FeLV-B isolates are strongly age restricted and are inconsistent in their pathogenicities, and FeLV-C isolates are also strongly age restricted but consistently induce fatal aplastic anemia.

Molecular analyses of FeLV genomes, their gene products, and the genetic mechanisms underlying the development of FeLV-associated diseases have been conducted in several laboratories, utilizing both naturally occurring and experimentally induced infections. Some insight has been provided into the divergent mechanisms of disease induction, yet the proposed mechanisms remain incompletely understood. They include molecular and biological events such as oncogene transduction to form the replication defective feline sarcoma virus (FrSV), provirus integration near an oncogene as c-*myc* and recombinant viruses derived from FeLV and c-*myc*, recombination of FeLV-A with endogene FeLV *env* sequences to obtain FeLV-B and C subtypes, genetic events within viral genome such as punctual mutations and insertion or deletion mutations, presence of specific variants, termed FeLV-FAIDS shown to induce feline AIDS (FAIDS) in 100% of SPF cats (158), and immunosuppressive effects associated with different events such as lysis of FeLV-infected neutrophils, FeLV-soluble circulating immune complexes, and soluble circulating antigens such as p15E reported to contain an immunosuppressive peptide (see BLV).

**Transmission of FeLV**

FeLVs are transmitted from carrier cats in the saliva, in milk, or across the placenta. Cats that are exposed to the virus by contact either become persistently infected or recover and are immune. Recovery is strongly associated with the presence of VNA in the serum, which are directed

toward antigenic determinants on the surface glycoprotein gp70. Passive transfer of VNA, either naturally in the colostrum to the kittens of recovered mothers, or by passive administration of antibodies, confers resistance to challenge with high doses of FeLV. Hence there is good reason to believe that cats can be made immune if they can be induced to produce VNA. This is not to say that other immune mechanisms are not involved in recovery from infection; indeed it is likely that cytotoxic T cells are responsible for the elimination of FeLV-infected cells from transiently infected cats. Nevertheless, there is no doubt that antibodies are sufficient for protection against experimental FeLV challenge (71).

**Vaccination against FeLV Infection**

Over the past 15 years there have been numerous reports dealing with FeLV vaccination (63,71,90,95,137,187). These include the use of crude vaccines made up of soluble products of virus-infected cells, whole live or dead FeLV-infected tumor cells, inactivated whole FeLV, and low-dose live-virus and viral envelope subunit. Some of these vaccines have protected cats against infection with virulent virus, while others have not.

The first successful vaccine available in practice was initiated in 1980 by Olsen and his group and is a "subunit" vaccine based on cell culture techniques involving the FL-74 cell line, which produces the FeLV-ABC. The commercially produced vaccine "Leukocell" is a filtrate of FL-74 cells cultured in serum-free media that was then chemically inactivated and combined with "adjuvant material" instead of Freund's complete adjuvant. This vaccine was shown to protect a proportion, but not 100%, of vaccinated cats against viremia and tumor development under experimental and field conditions (58,103,138). Similar results were obtained by Jarrett (71) where kittens were vaccinated with two doses of para-formaldehyde-inactivated FeLV vaccine: in four separate experiments about 80% of the vaccinated kittens were protected. An other commercially available vaccine is based on a nonglycosylated protein including the 45K protein core of external viral glycoprotein gp70 and the first 34 amino acids from the transmembrane protein p15E expressed in *E. coli*. The vaccine consists of the purified protein adsorbed on to aluminum hydroxyde and used in conjunction with a novel saponin adjuvant purified from the tree *Quillaja saponaria Molina*. Doubts have been expressed about the immunogenicity of vaccines based on nonglycosylated forms of proteins that are normally glycosylated. However, cats immunized with this formulation developed a strong humoral immune response, including VNA and feline oncornavirus-associated cell membrane antigen (FOCMA) antibodies and were protected from viral infection (101).

Recently attempts have been made to develop subunit vaccines that induce VNA in all cats to ensure complete protection against FeLV

challenge. Candidate vaccine based on isolated FeLV-gp70 has not proven successful. It induced antibodies detectable by ELISA but not virus-neutralizing antibodies in immunized kittens, which were more apt to become persistently viremic following virulent FeLV challenge exposure than nonvaccinates (139). Synthetic peptides have been used recently as candidate antigenic sites for incorporation into a synthetic vaccine: among this potential antigenic sites, two domains of gp70 and two domains of p15E were identified to induce infection-enhancing anti-bodies (124). An other candidate vaccine has not proved successful: recombinant live-vaccinia virus encoding gp70 was not immunogenic in cats (52). Other workers sought to develop live-virus vectors derived from the feline-specific virus, feline herpesvirus 1 (FHV) and expressing the ENV and GAG proteins of FeLV (29).

The most promising of new approaches has been the use of ISCOMs. For FeLV the gp70 is extracted from purified virus with a suitable deter-gent and is incorporated into ISCOMs. Cats inoculated with FeLV-ISCOMs are resistant to challenge with virulent virus (133); the ISCOM preparation elicits virus-neutralizing antibodies in the majority of the vaccinated cats and a clear booster response in seropositive animals under field conditions (134,187). All these data thus suggest that the ISCOM could be of great value in the construction of safe and effective nucleic acid-free FeLV vaccine and justify efforts toward the development of a highly performant method for the recovery of FeLV envelope protein from cell culture (61).

Finally the generation of an antiidiotype vaccine is another approach to developing a FeLV vaccine. Until now, preliminary experiments have been performed in mice and rabbits and look very promising (187). In the future it may be possible to exploit the use of antiidiotype such as the use of synthetic peptides to prevent certain undesirable autoimmune reaction or to turn off unwanted effects of vaccines due to immunosuppressive structures and induction of infection-enhancing antibodies.

## C. Avian Retroviruses

As seen above (Section 3A), this group includes both exogenous and closely related endogenous viruses of birds. These viruses induce in chickens and other birds a large variety of tumors including sarcoma, lymphoma, lymphoid leukosis osteopetrosis, and reticuloendotheliosis (28,32,43). For these viruses the ultimate neoplastic transforming event is very rare. Although neutralizing antibody can clear virus from the chicken it is not known whether humoral antibody can prevent the development of tumors once transformation is initiated. Also little is known about the effect of cellular immunity on the development of tumors. Recently work with avian retroviruses illustrates the status of germ line engineering to introduce useful genes for "genetic vaccination."

Salter and co-workers (167) established a line of chickens producing an envelope protein from a leukemia-inducing retrovirus vector. These chickens became resistant to infection with the leukemia-inducing virus. In an other experiment, Bosselman and co-workers (14) infected chicken embryos directly with replication-defective vector derived from reticuloendotheliosis virus (REV).

## D. Conclusion

Increasing knowledge should clarify the immunological requirements for a vaccine to give long-lasting immunity against animal oncoviruses. These include (a) the contribution of different types of immune responses to the prevention and control of the retroviral infection, (b) the replication cycle regulation, (c) the genetic variability of retroviruses, and (d) the cell fusion mechanisms. Prevention and control may include neutralizing antienvelope antibodies, antienvelope antibodies plus complement, antibody-dependent cellular cytotoxicity, natural killer cells, and cytotoxic T-lymphocytes (CTL). Until now the major protective antigens of oncoviruses are considered to be their glycosylated envelope proteins.

Several experiments suggest that the development of latent reservoirs could be blocked by an immune response directed against structural internal proteins or viral products responsible for latency or expressed at the surface of latently infected cells. These last products could be encoded by negative or differential regulatory genes such as *nef* or *rev* for lentiviruses (6) and such as *tat* or *rex* for retrovirus such as BLV. In fact it has been mentioned recently that structurally related HTLV-1 *gag* and *px* gene products are recognized by CTL in a rat model. Preliminary experiments also suggested that immunization of rats with *gag* or *rex* recombinants vaccinia resulted in the generation of CTL capable of lysing syngenic HTLV-1 infected cells (178). Humans were also able to develop CTL against retroviral regulatory protein: circulating CTL specific for HTLV-1 *px* products have been identified in patients with HTLV-1-associated neurological disease (70). Some peptides derived from these structural regulatory proteins could thus act as efficient target epitopes for CTL.

# 6. Lentiviruses

## A. Introduction: Vaccination and Its Problem

In his recent review "Vaccination against SIV infection and disease," Murray Gardner (49) states "clearly, the mechanisms and qualities of protective immunity that vaccines must induce lasting protection against these lentivirus infections of animal and human remain to be deter-

mined." Getting to assumptions about the immune parameters required, the author continues: "Therefore, to prevent SIV or HIV infection, the vaccine reduction of immunological memory and associated T- and B-cell priming will be critical not only for producing neutralizing antibody but also through the activation of CMI (Cell Mediated Immunity) directed at the infected cells." We fully share these views and want to add, besides induction of strong cellular and humoral immunity, a third major component, namely protection of the immune system of the host from immunosuppression. Down-regulation of the immune response is one of the obvious consequences of HIV/SIV/FIV infection. It is already evident before the decrease in T4 cells (T-helper cell repletion) and is an early element in immunopathology. This observation corroborates the finding by Luria et al. (94) that the HIV-1 NEF protein interferes with a signal emanating from the T-cell receptor complex that induces IL-2 gene expression. Preliminary observations by Zagury's group in symptomatic HIV human patients indicate that induction of an immune response against $\alpha$-interferon (IFN-$\alpha$), a well recognized cytostatic agent, might be a way to alleviate immunosuppression. As a consequence of the treatment, delayed type hypersensitivity (DTH) and T4 cell count increase significantly (D. Zagury, VIth Int. Conf. on AIDS, Firenze).

Vaccination and immunotherapy against AIDS are complicated issues. AIDS researchers could solve the problems, provided (a) the capacity of immune response remains intact and (b) the immune response has been educated to recognize critical and rather well-conserved epitopes. We believe that an attitude of cautious optimism must prevail, even if the virus can hide and mutate at high rate. The ability to dissect the biology and the complex molecular regulation of the virus (hence its vulnerability) and present knowledge of the regulatory mechanisms of immune reaction (hence the possibility to maintain if fully operational) are reasons for optimism. The reader is referred for further reading to excellent reviews and overviews published by experts in the human model and various systems (9,11,12,35,48,49,53,57,81,85,137,170,172).

1. Lentiviruses establish infection in immunoprivileged sites like the brain, compartments of the bone marrow, and the epididymis.
2. Virtually all type C and type D retroviruses as well as lentiviruses contain an immunosuppressive 17-mer peptide in their transmembrane envelope protein. That sequence is evolutionary conserved and its action (inhibition of mitogen and antigen simulation) is interspecies specific. There is suggestive evidence that a high titer of binding antibodies to this peptide sequence correlates with positive prognosis (80).
3. Gp120 envelope protein of HIV-I inhibits lytic activity of NK cells from normal donors.

4. HIV-I-infected humans do not develop complement-activating cytotoxic antibodies, in contrast to both HIV-infected healthy chimpanzees (122) and to the situation encountered in animals infected by potentially oncogenic retroviruses, such as BLV (145).

5. As for other lentiviruses, the theme of restricted virus gene expression is the dominant motif of HIV/SIV/FIV infections. The virus can remain latent in lymphocytes (66,144) and macrophages. In the latter cells, virus particles can be formed and accumulate in cytoplasmic vesicles (44). They are released by bursts, a mechanism that probably allows the infected producing cell to largely avoid to be the victim of immune surveillance.

6. Vaccination of rhesus monkeys with detergent-inactivated whole virus or formalin-inactivated whole virus or subunit preparations induced infection-enhancing antibodies that disappeared or decreased after challenge in protected animals. They increased in unprotected animals (113).

7. HIV, SIV, and retroviruses in general exhibit a rate of mutation between $10^{-3}$ and $10^{-4}$. This rate corresponds to the error rate per site of HIV-reverse transcriptase and is comparable to the mutation rate observed in the replication of single-stranded RNA viruses (foot-and-mouth disease virus, Rous sarcoma virus, influenza virus NS gene) (179). As suspected by Nowak (125), "human immune response against HIV favors variation in some parts of the viral genome. If this is true then there is an optimal mutation rate which maximizes the probality of producing new resistant mutants due to errors in viral replication. This optimal mutation rate is in good agreement with the measured replication accuracy of the HIV-I reverse transcriptase." The rates of variation and thus the speed of evolution cited hereabove could be compared to the error rate of $10^{-9}$–$10^{-10}$ encountered in DNA polymerization, and thus in the progeny of human T lymphotropic viruses (HTLV-I), simian T lymphotropic viruses (STLV-I), or bovine leukemia virus (BLV), retroviruses that essentially propagate as proviruses and not as free particles. It follows that retroviruses replicating as a virus are difficult to counteract by vaccination or by the design of drugs due to the appearance of escape or resistant mutants.

## B. Simian Immunodeficiency Viruses (SIVs)

Simian immunodeficiency viruses have been identified and isolated from several species of nonhuman primates (38). These viruses naturally occur as nonpathogenic lentiviruses, among African old world primates, mainly African green monkeys, *Cercopithecus aethiops* and sooty mangabey monkeys, *Cercocebus atys*. Although Asian old world primates are not naturally infected with SIVs, they are clearly susceptible to infection with

SIV and to SIV induced AIDS-like disease. Indeed the SIVmac isolates were recovered from captive macaques with immunodeficiency and clinical signs typical of the AIDS related complex. Experimental infection of naive macaques with SIV isolates induces an immunodeficiency syndrome remarkably similar to AIDS in humans and very often leads to death in a period of several months. SIVs and HIVs do not share only pathogenic similarities in macaques and humans. They also have common genetic, immunogenic, and biological properties. SIV infection of macaques is consequently of main importance for AIDS research as a model for pathogenesis, treatment, and prevention of HIV-induced AIDS disease in humans.

In this chapter we shall focus on the characterization of the SIV groups in relation with their human counterparts HIV-1 and HIV-2. Recent advances in vaccination trials in the SIV macaque model will be discussed and analysed at the light of results obtained from other relevant animal models. Several excellent reviews devoted to these areas have been published earlier (9,12,35,38,49,53).

## SIVs in Different Monkey Species

### SIV in Green Monkeys

In the wild, the main identified reservoir of SIV is the green monkey (*Cercopithecus aethiops*). These old world primates are scattered mainly in four regions of Africa (Kenya, Sub-Sahara, Ethiopia, and South Africa). In their natural habitat, green monkeys are highly infected with SIV since 20–50% of them have antibodies to SIV (74,130). However, green monkeys either naturally or experimentally infected do not develop any disease. SIV from African green monkeys (SIVagm) have been isolated in culture (34,55,72,83,130) and molecular clones have been obtained [SIVagmTYO-1 (45), SIVagm3 (4), SIVagm155-4 (72)] and sequenced (45,72). One of these clones (SIVagm3) is biologically active *in vitro* and *in vivo* (4) in homologous and heterologous (pig-tailed monkeys) species. Clone SIVagmTYO-1 was considered as the representative of a SIVagm group distinct from other SIVs and HIVs since SIVagmTYO-1 DNA hybridized with SIVagm isolates of Kenyan and Ethiopian origins but not with the other SIVs and HIVs in stringent conditions (45,130). However, restriction endonuclease mapping, cross-hybridization, and limited nucleotide sequence data have revealed a high degree of genetic heterogeneity among SIVagm isolates exceeding divergence between individual isolates of other SIVs or HIVs (4,73,92).

### SIV in Sooty Mangabey Monkeys

Among African old world primates, sooty mangabeys (*Cercocebus atys*) were also shown to be infected by SIV (SIVsmm) that could be isolated in culture (46,93,118).

   The extent of infection of sooty mangabeys in their native habitat (Central and Western Africa) is unknown, but in captivity they are extensively infected. The SIVsmm virus, like SIVagm, does not cause clinical immunodeficiency or disease following natural infection of the host from which it was isolated. However, infection of rhesus macaques (*Macaca mulata*) led to induction of AIDS-like disease and isolation of SIVsmm Delta B670 (118). This isolate was used, as whole inactivated virus, for the successful vaccination of macaques against both homologous and heterologous SIV challenge. Infection of a pig-tailed macaque (*Macaca nemestrina*) with another SIVsmm isolate [SIVsmm 9 (46)] led to acute disease and death at 14 months postinfection (47). This SIVsmm isolate passed in macaque (PBj14) determined an acutely fatal disease in pig-tailed or rhesus macaques or seronegative mangabeys characterized by development of bloody mucoid diarrhea within several days of inoculation and peripheral T-cell depletion. However, healthy SIVsmm-infected mangabeys survived a lethal challenge with the PBj14 isolate (47). This picture holds for biological clones and even for the molecular clone SIVsmm-PBj14 obtained by PCR amplification of cellular DNA from macaque PBMC infected 7 days earlier with a biologically cloned virus SIVsmm-PBj-bc13 (39). The complete sequence of SIVsmm clones are now available (39,64). Analysis of sequence similarities shows that SIVsmm forms with SIVmac and HIV-2 a subgroup of closely related viruses (64).

## SIV in Mandrills

A third species of African old world primates, mandrills (*Papio Sphinx*) from western equatorial Africa, were also shown to be infected with SIV. Healthy wild caught mandrills from Gabon were indeed shown to be infected with a SIV isolate (SIVmnd) that was cloned (182) and sequenced (183). Genomic comparisons show that SIVmnd is part of a distinct subgroup of SIVs.

## SIV in Macaque Monkeys

SIV viruses were first isolated from captive rhesus monkeys (*Macaca mulata*) (33,118). SIVs were also isolated from other macaques: cynomolgus (*Macaca fascicularis*) (77), pig-tailed (*Macaca nemestrina*) (6,7), and stump-tailed (*Macaca arctoides*) (20). Contrary to African old world primates, macaques, the Asian old world primates, are clearly susceptible to SIV-induced AIDS-like disease. Indeed, the SIVmac isolates were recovered from animal with immunodeficiency and clinical signs typical of the AIDS-related complex (opportunistic infections, diarrhea, wasting, and lymphoid depletion) and experimental infection of naive macaques with SIVmac isolates induces an immunodeficiency syndrome very often leading to death in a period of several months (5,7,89). In addition, SIV

infection of macaques was also associated with lymphomas and lympho-proliferative diseases (6,27,33). However, macaque do not seem to be naturally infected with SIV and infection in captivity is a rare event. Some of these SIVmac have been cloned (26,77) and sequenced (26) and tested for *in vivo* infectivity (121). One of these infectious molecular clone, SIVmac239, induces in rhesus monkeys a disease remarkably similar to AIDS in humans (78). Comparison of nucleotide sequences shows that SIVmac are members of a subgroup of closely related lenti-viruses including SIVsmm and HIV-2 (more than 84% amino acid identify in the *pol*-encoded proteins between each member of the sub-group) and clearly distinguishable from the 3 other subgroups, SIVagm, SIVmnd, and HIV-1 (55–60% amino acid identify in the *pol* encoded proteins between prototypes of each subgroup).

## SIV in Chimpanzees

A lentivirus, SIVcpz, has been isolated from a wild chimpanzee (*Pan troglodytes*) in Gabon (33). The genetic organization of SIVcpz is similar to that of HIV-1 as judged by the nucleotide sequence of a molecular clone. Contrary to the other SIVs and HIV-2 but similarly to HIV-1, SIVcpz has a *vpu* gene.

## Similarities between SIVs and HIVs

SIVs share common features with other lentiviruses and particularly with human immunodeficiency viruses type 1 and 2. These common features include morphogenic, genomic, biologic, antigenic, and pathogenic properties (190).

## Morphogenesis

SIVs bud at the cell membrane as crescent and particles with a diameter of 100–120 nm are released from the cells. Mature particles consist of a condensed core that appears tubular-prismatic or centrosymmetric on electron micrographs depending on the plane of section, and an envelope with prominent knobs [SIVagm (130), SIVmnd (182), SIVmac (6,33,118), SIvsmm (118)].

## Genomic Structure

Beside the standard retroviral *gag*, *pol*, and *env* genes, the genome of SIVs includes additional open reading frames, a common feature of lentiviruses. Among these additional genes, the *tat* gene involved in transactivation of virus replication and the *rev* gene that selectively enhances the expression of *gag* and *env* are generally maintained in SIVs (3,171,185). Another regulatory gene present in all simian and human immunodeficiency virus genomes, the *nef* gene, was reported to be required for full pathogenesis, when a SIVmac strain was used to infect

rhesus macaques (79). The *vpx* gene is only found in SIVs (all SIVs except SIVmnd) and HIV-2 genomes and the *vpu* gene is only found in HIV-1 and SIVcpz genomes.

Antigenic variation during persistent infection is a common property of immunodeficiency viruses. Infection of rhesus monkeys with a molecularly cloned SIVmac strain led to evaluation of the genetic changes in the *env* gene that are fixed during *in vivo* divergence. Alignment of the HIV-1 and SIVs envelope amino acid sequences shows that among the 5 hypervariable regions of the HIV-1 envelope glycoprotein, the V3 region, corresponding to the HIV-1 principal neutralization determinant, is strikingly poorly variable in SIVmac (20).

## Biologic Properties

The human CD4 glycoprotein and its closely related simian analogs are the cellular receptors for human and simian immunodeficiency viruses (83,106,168). SIVs show a tropism for T4 lymphocytes and macrophages and macrophages are the main sites for replication of SIV *in vivo* (160). SIVs induce severe cytopathic effect in CD4 positive cells in culture (33,130,182) and giant cells in lymph nodes, lung, and other organs (5,160).

## Antigenic Characteristics

SIVs viral proteins show size similarity and cross-reactivity with sera from SIV-infected monkeys and patients with AIDS: *gag*-related proteins of SIVs and HIVs possess marked cross-reactivity but their envelope glycoproteins show only weak common antigenicity (74,75,130,182).

## Pathogenesis

When infecting susceptible hosts (*Macaca mulata*, *fascicularis*, or *nemestrina*) SIVmac and SIVsmm can induce an AIDS-like disease with opportunistic infections, diarrhea, immunological abnormalities, including decrease in CD4 cell number, and, in 50% of macaques dying from SIV infection, encephalitis. All these characteristics are commonly observed in human AIDS. Specificities of simian AIDS are association with the development of lymphoma and formation of syncytia (giant cell disease) in lymph nodes, lung, spleen, and other organs. Human and simian immunodeficiency viruses also show the common feature of viral persistence in individuals developing even strong humoral and cellular immune responses toward the virus.

## SIVs Infection of Nonhuman Primates as a Model for Vaccination against Human AIDS

Successful vaccination of macaques against challenge infection with SIV has been first obtained using whole inactivated virus (25,37,119). Vaccine

preparations were made of SIVmac251 (25,37) or SIVsmmdeltaB670 (119) inactivated with triton (37), formalin (119) or $\beta$-propiolactone (25) and injected by intravenous or intramuscular routes with muramyl dipeptide adjuvant.

These first studies stressed the importance of the challenge doses: protection was obtained only with 10–200 animal infectious doses given im or iv; higher doses led to breakdown of protection (37). Vaccine formulations using inactivated SIVmac infected cells and "Quil-A" adjuvant also led to protection against challenge with low doses of the homologous SIV (173). Absence of protection was observed with high dose challenge ($10^2$–$10^3$ id) given either intravenously or by exposition via the genital mucosa after vaccination protocols using psoralen-UV-light-inactivated whole SIV with threonyl muramyl dipeptide (175). Animals vaccinated and showing protection after challenged with SIVdeltaB670 (119) or SIvmac251 (25) were subsequently submitted to a second challenge with the heterologous SIV isolates (SIVmac251 and SIVdeltaB670 differ by about 10% in outer envelope amino acid sequence). Cross-protection was observed (50), a result that was confirmed after immunization with SIVmac 251 and direct challenge with SIVdeltaB870 (31). In one study, infection of monkeys with an attenuated molecular clone SIVmacIA11 (102) failed to protect against infection with pathogenic SIV virus but prevented severe, early disease and prolonged the lives of the immunized monkeys. In most reported vaccination trials that failed to protect against infection, viral replication and disease symptoms were delayed (37,119). However, in infection by the genital mucosa route (175) the vaccinated animal died significantly sooner than infected controls.

In all studies showing protection, challenge was given at the height of the antibody response, 2 to 4 weeks after the final boost. The duration of the protection has still to be estimated and possibly improved. Other aspects to be analyzed are (a) protection against challenge with more divergent SIV strains, given via one of the main natural routes of infection, i.e., the vaginal or rectal mucosa; (b) protection from cell-associated virus; (c) determination of the viral proteins that are crucial to protection; (d) dissection of viral antigen in T and B cell epitopes and careful identification of protection structures (constant structures required for target cell recognition, virus-cell fusion, an obvious candidate); and (e) in depth analysis of the progressive reduction of the immune response and attempts to prevent the fatal outcome. A first step to these goals has been done by comparing the efficacy of a glycoprotein-enriched and a glycoprotein-depleted subunit vaccine prepared by fractionation of gradient-purified SIVdeltaB670 by lentil lectin affinity chromatography (120) and by using recombinant-made SIVmne glycoprotein (67). Scores of protected animals are better with gp-enriched (2 animals protected out of 4) than with gp-depleted (0/4) preparations. This result stresses the possible

need of envelope glycoprotein for induction of protective immune responses. Of more than passing interest is the observation that rhesus macaques that show T4 cell proliferation after vaccination with a tween-ether disrupted whole virus preparation do resist the challenge with the same virus strain. On the contrary, lack of T4 cell response is predictive of infection upon challenge (G. Hunsmann, private communication). A summary of published vaccination trials is given in Tables 4.2 and 4.3.

## C.  Human Immunodeficiency Virus (HIV) in Chimpanzees

The dramatic outcome of HIV-I infection in man and the continuous expansion of the AIDS epidemic (estimated number of HIV-infected individuals in the year 2000 being around 50 millions) lead investigators to search for an animal model that could represent a valid replica of the human situation. Inoculation of HIV-I resulted in infection only in the chimpanzee; many other species were not susceptible (2,41). However, among the 120 animals, HIV-I carriers worldwide, only two have shown transient lymphadenopathy. None has shown early persistent signs of immunodeficiency, within the first 7 years of infection. This situation strongly suggests that the chimpanzee, even if exquisitely sensitive to HIV infection, is probably resistant to disease. The data of Gibbs et al. (51) even indicate that two chimpanzees, infected in 1983, "eliminated" the virus in 1987, at about the time when they were vaccinated with a core-enriched HIV-I vaccine and then challenged with an HIV-I inoculum whose infectivity has been verified. These two animals remained virus-negative by peripheral blood mononuclear cell cocultivation for more than 2 years of observation after challenge. Even if the role of the vaccine preparation administered is difficult to assess, it looks like the chimpanzee might very well be able to eliminate the resident HIV-I from its sanctuaries (41).

It therefore appears that the interplay between the chimpanzee and HIV-I significantly differs from the human situation. Besides economical, ecological, and ethical reasons, scientific arguments militate against the use of chimpanzees as the best model for vaccine research even if the virus involved is HIV-I itself. Such a conclusion does not mean that data obtained in this system are not transposable to man. They should be transposed with caution and with emphasis on understanding the reasons of the differential behavior of man and chimpanzee in their interaction with the virus.

Protection against challenge with a limited dose of HIV-I has been reported by Berman et al. (8) and Girard et al. (54). It should be mentioned that (a) the challenge dose is limited, (b) challenge is performed at short time (a few weeks) after the last boost, and (c) challenge at later times were not tried so far. Both groups of authors emphasize the apparent necessity for high neutralizing antibody titers as a requisite for

**Table 4.2.** Vaccine studies in the macaque: homologous challenge following active immunization (intravenous route, 1–4 weeks after final boost).[a]

| Vaccine type | Agent | Adjuvant | Schedule (weeks) | Challenge[b] | Protection? | Reference |
|---|---|---|---|---|---|---|
| **Killed infected cell** | | | | | | |
| Glutaraldehyde | SIVmac | Quil A | 0,4,8,36 | 10 | 4/4 | Lancet 336:1538 (1990) |
| Glutaraldehyde | SIVmac | Quil A | 0,4,8,16 | 10[l] | 4/4 | Lancet 336:1538 (1990) |
| **Attenuated virus** | | | | | | |
| 1A11 genomic clone | SIVmac | (None) | 0,30 | 200[c] | 0/3[d] | J. Virol 64:3694 (1990) |
| **Inactivated virus** | | | | | | |
| Formalin | SIVmac | tMDP | 0,3,6, | ±10[6] | 0/4[d] | PNAS 86:6353 (1989) |
| Formalin | SIVsm | tMDP±alum | 0,4,10,72 | 10 | 8/9 | Science 246:1293 (1990) |
| Psoralen-UV | SIVmac | tMDP | 0,12,20,34 | 200 | 0/4[d] | J. Virol. 64:2290 (190) |
| β-Propiolactone | SIVmac | None | 0,4,12,17,38 | 200 | 1/3 | AIDS Res. Hum. Retr. 6:1239 (1990) |
| β-Propiolactone | SIVmac | Incomplete Freund | 0,4,12,17,38 | 200 | 1/2 | AIDS Res. Hum. Retr. 6:1239 (1990) |
| β-Propiolactone | SIVmac | tMDP | 0,4,12,17,38 | 200 | 3/3 | AIDS Res. Hum. Retr. 6:1239 (1990) |
| Triton X-100 | SIVmac | tMDP | 0,3,6,23,25 | 1000 | 1/2[d] | PNAS 86:6353 (1989) |
| Triton X-100 | SIVmac | tMDP | 0,3,6,42,45,47 | 200 | 1/4 | PNAS 86:6353 (1989) |
| Triton X-100 | HIV-2 | Incomplete Freund | 0,5,8,11,105 | 100 | 2/2 | AIDS Res. Hum. Retr. 7:271 (1991) |
| MEGA-10 | HIV-2 | ISCOM | 0,5,52,75 | 100 | 0/2 | AIDS Res. Hum. Retr. 7:271 (1991) |
| **Subunit proteins** | | | | | | |
| env-enriched (viral) | SIVsm | tMDP | 0,5,58 | 10 | 2/4 | AIDS 5:655 (1991) |
| env-enriched (viral) | SIVsm | tMDP | 0,5,57 | 10 | 0/4 | AIDS 5:655 (1991) |
| **Epitope approach** | | | | | | |
| β-gal fusion protein | SIVmne | Complete Freund | 0,3,5,55 | 100 | 3/3[e] | PNAS in press |

[a] Reproduced with the kind permission of A. Schultz, Vaccine Research and Development Branch, Division of AIDS, NIAID, NIH, Bethesda, Maryland, and J. Warren, The EMMES Corporation, Potomac, Maryland.
[b] Approximate 50% animal infectious dose ($ID_{50}$); different stocks make strict comparisons unreliable.
[c] Challenged at week 43.
[d] Infected animals nonetheless showed prolonged survival.
[e] Blood/node failed to transmit to naive animals.

**Table 4.3.** Vaccine studies in the macaque.[a]

| Vaccine type | Agent | Adjuvant | Schedule (weeks) | Challenge[b] | Protection? | Reference |
|---|---|---|---|---|---|---|
| Heterologous challenge following active immunization (intravenous route, 2–4 weeks after final boost) | | | | | | |
| Attenuated virus | | | | | | |
| Transient infection | HIV-2 | (None) | 0, challenge at week 24 | 10 SIVsm | 0/3[c] | AIDS 4:783 (1990) |
| Inactivated virus | | | | | | |
| β-Propiolactone | SIVmac | None | 0,4,12,17,38,[40],[d] 62 | 10 SIVsm | 3/3 | AIDS Res. Hum. Retr. 6:1239 (1990) |
| Homologous challenge following active immunization (mucosal challenge route) | | | | | | |
| Inactivated virus | | | | | | |
| Psoralen-UV | SIVmac | tMDP | 0,12,20,34 | 2 urethral ID$_{50}$ | 0/2 | J. Virol 64:2290 (1990) |
| Psoralen-UV | SIVmac | tMDP | 0,12,20,34 | 2 vaginal ID$_{50}$ | 0/2 | J. Virol 64:2290 (1990) |

[a] Reproduced with the kind permission of A. Schultz, Vaccine Research and Development Branch, Division of AIDS, NIAID, NIH, Bethesda, Maryland, and J. Warren, The EMMES Corporation, Potomac, Maryland.
[b] Approximate 50% animal infectious dose (ID$_{50}$); different stocks make strict comparisons unreliable.
[c] Infected animals nonetheless showed prolonged survival.
[d] Timing of successful protection against intravenous homologous virus challenge.

**Table 4.4.** Vaccine studies in the chimpanzee: active immunization only.[a]

| Vaccine type | Agent | Adjuvant | Schedule (weeks) | Challenge | Protection? | Reference |
|---|---|---|---|---|---|---|
| **Live recombinant virus** | | | | | | |
| Vaccinia-env | LAV/IIIB | (None) | 0,8 | $3.16 \times 10^5$ TCID$_{50}$ | 0/2 | Nature 328:721 (1987) |
| Vaccinia-env | LAV/IIIB | (None) | 0,8 | $3.0 \times 10^5$ TCID$_{50}$ | 0/4 | "AIDS Vaccine Research and Clinical Trials", Chapter 9 (Putney, Bolognesi eds) (1990) (M. Dekker, NY) |
| Vaccinia-env | LAV/IIIB | (None) | 0,8 | 100 TCID$_{50}$ | 0/2 | "AIDS Vaccine research and Clinical Trials", Chapter 9 (Putney, Bolognesi eds) (1990) (M. Dekker, NY) |
| **Inactivated virus** | | | | | | |
| β-Propiolactone,[60] Co[b] | HZ321 | Incomplete Freund | 0,36,45 | Week 62, 40 TCID$_{50}$ | 0/1 | PNAS 88:3348 (1991) |
| **Subunit proteins** | | | | | | |
| rgp 120 (CHO cells) | LAV/IIIB | Alum | 0,4,10,14,28 | Week 32, 100 TCID$_{50}$ | 0/2 | PNAS 85:5200 (1988) |
| gp 120 (HIV virus) | LAV/IIIB | Alum | 0,4,8,20,35 | Week 37, 400 TCID$_{50}$ | 0/1 | J. Virol 63:5046 (1989) |
| gp 120 (HIV virus) | LAV/IIIB | Alum | 0,4,8,20,35 | Week 37, 40 TCID$_{50}$ | 0/1 | J. Virol 63:5046 (1989) |
| rgp 160 (CHO cell) | LAV/IIIB | Alum | 0,4,32 | Week 35, 40 TCID$_{50}$ | 0/2 | Nature 345:622 (1990) |
| rgp 120 (CHO cells) | LAV/IIIB | Alum | 0,4,32 | Week 35, 40 TCID$_{50}$ | 2/2 | Nature 345:622 (1990) |
| Pr 55$^{gag}$ (yeast) | LAV/IIIB | Alum | 0,4,24 | Week 28, 80 TCID$_{50}$ | 0/1[c] | ARHR 6:1247 (1990) |

| Combination | | | | | | |
|---|---|---|---|---|---|---|
| BPL, formalinized HIV | LAV/IIIB | tMDP | 0,4,8,29,91 | | | |
| rgp 160 (CHO cell) | LAV/IIIB | (None) | 33,41,48,52,54 | | | |
| V3-KLH[d] | LAV/IIIB | (None) | 72,75,93 | Week 98, 100 TCID$_{50}$ | 1/1[c] | PNAS 88:542 (1991) |
| Vaccinia-env | LAV/IIIB | (None) | 0,8,22 | | | |
| Vaccinia-env | LAV/IIIB | (None) | (?) | | | |
| rgp 160 + p18$^{gag}$ + p27$^{nef}$ + p23$^{vif}$ | LAV/IIIB | tMDP | 48,54,58,81,86,88,114,124 | | | |
| V3-KLH[b] | LAV/IIIB | (None) | 105,108,126 | Week 131, 100 TCID$_{50}$ | 0/1[c,e] | PNAS 88:542 (1991) |
| rgp 160 + p18$^{gag}$ | LAV/IIIB (Mixture) | tMDP | 0,6,10,21,33,38,66,76 79,83,87,102 | | | |
| V3 peptides | | tMDP | | Week 106, 100 TCID$_{50}$ | 1/1 | PNAS 88:542 (1991) |

[a] Reproduced with the kind permission of A. Schultz, Vaccine Research and Development Branch, Division of AIDS, NIAID, NIH, Bethesda, Maryland, and J. Warren, The EMMES Corporation, Potomac, Maryland.
[b] Vaccine product was virtually free of envelope proteins.
[c] No control chimpanzee was challenged at the same time.
[d] Keyhole limpet hemocyanin.
[e] Animal was apparently protected and virus-free for 8 months.

protection against homologous challenge with cell-free virus. They, however, suspect, as did Gibbs et al. (51), that CMI plays a major role in preventing or eliminating virus infection.

One of the experimental animals vaccinated and challenged by Girard et al. (54) appeared to be protected for 7 months but actually was infected from time of challenge, despite repeatedly negative tests for virus isolation and detection by PCR. As stated by the authors, this observation is worrisome and underscores the fact that HIV can be sequestered such that it defies detection by both virologic and serologic criteria.

A summary of published vaccination trials is given in Table 4.4.

## D. Feline Immunodeficiency Virus (FIV)

Feline immunodeficiency virus (formerly feline T-lymphotropic lentivirus or FTLV) was first isolated from a group of cats in Petaluma, California in 1986. The virus is a typical lentivirus in gross and structural morphology. It replicates preferentially but not exclusively in feline T-lymphoblastoid cells, where it causes a characteristic cytopathic effect (95,140) leading to immunologic abnormalities (1). It has a prevalence of 1.2% in low-risk animals and 14% in high-risk groups (191).

The disease states seen in FIV-infected cats include (a) generalized lymphadenopathy, fever, malaise, and leukopenia usually associated with the initial stage of infection, (b) a period of relative normalcy that is not clinically evident but may be detected on routine screening, including antigen and/or antibodies detection, and (c) the terminal or AIDS-like stage of illness. This stage is usually characterized by a number of chronic infections of a secondary or opportunistic nature and/or neurologic disease.

There is no statistical linkage between FIV and feline leukemia virus (FeLV) infections in nature. The FeLV infection rate in FIV-infected animals is the same as it is for non-FIV-infected cats. However, FIV-infected cats with preexistent FeLV infections developed severe immunodepression, anorexia, fever, diarrhea, dehydration, weight loss, and leukopenia 4 to 6 weeks of the onset of signs, whereas cats infected only with FIV developed much milder self-limiting gross and hematologic abnormalities (95). A preexistent FeLV infection enhances thus the expression and spread of FIV in the body, increases the severity of both the resulting transient primary and chronic secondary stages of FIV infection and may be an important aspect in selecting appropriate vaccine strategies which might be used against FIV (141).

Infectious molecular clones of FIV are available. They show 55 to 65% sequence identity in the 5' 1.5-kb fragment of the *pol* gene with *pol* genes of SIV, HIV-1, HIV-2, EIAV, visna virus, and CAEV. There is also serological cross reactivity of rabbit antibodies to CAEV and visna virus with FIV core protein p28 (131,176).

From the experience acquired with FeLV, Dr. O. Jarrett recently proposed to use FIV-ISCOM to test the efficacy of vaccination against FIV (71). In addition to its veterinary importance FIV may become as SIV a useful model to study AIDS in man. FIV infection has several characteristics in common with HIV infection and cats are, in contrast to primates, easily available.

## E. Equine Infectious Anemia Virus (EIAV)

Equine infectious anemia virus causes disease in horses and related species. The clinical entity was described as early as 1843 and shown to be transmissible by Carre and Vallee in 1904 (104). It is characterized by symptoms of fever, glomerulonephritis, and anemia. The agent is a typical lentivirus. Proviruses are cloned and sequenced and show major similarities with immunodeficiency viruses of man and primates (192).

Equine infectious anemia (EIA) is a recrudescent disease where a chronic phase with episodes (bouts) of fever is followed by a phase of inapparent infection with no symptoms. In experimental infection, the chronic phase consists of one to four waves of fever, corresponding each to appearance of a virus variant, invading the whole individual (viremia).

Most infected horses bring virus replication and disease under control through a strong cellular and humoral immune response (142). Neutralizing antibody titers broaden with time and eventually reach a plateau. They appear after the wave of viremia, which is not concomitant with the episode of fever, an observation suggesting that their role may be secondary to non-specific immune response (NK cells) and cell mediated immunity (114). Persistence of antibody titers requires persistent stimulation by the virus, which again points to virus persistence and spread in the face of a significant host immune response. The basis for persistence is unknown but most probably involves restriction of viral gene expression, which enables virus-infected cells to evade elimination by the immune system. The stage of inapparent infection can last for years and represents a state of immunological control since virus replication cannot be readily detected unless the immunological status of the host has been significantly altered (heavy stress, immune suppression). Horses with combined immunodeficiency lack functional T and B lymphocytes and cannot manifest antigen specific immune responses and, thus, terminate viremia.

It follows that EIA is a somehow simplified model for AIDS. Virus-induced immunosuppression is mild if existent, allowing a clear picture of host–virus interaction to emerge. Virus persistence in monocytes/macrophages and generation of mutants at a rate seen among lentiviruses induce a rapid and efficient reaction from both arms of the immune system. A state of equilibrium is rapidly reached in which the host controls but cannot eradicate the virus. Only events of immunosuppres-

sion (mimicked experimentally by cyclophosphamide or dexomethasone treatment) will break the fragile equilibrium and allow eruption of uncontrolled viremia, anemia, liver and kidney inflammation, and death of the infected host.

Protection against EIA has been a matter of concern since many years. The advent of techniques of genetic engineering with possible identification of mutations and T and B cell epitopes (and promiscuous epitopes) together with imaginative approaches in the areas of adjuvant research and modes of antigen presentation have brought new impulse in the field of vaccination against EIAV. We summarize here the preliminary data reported by Rushlow et al. (166). The protocol used allowed protection against infection by a virus strain homologous to the vaccination one, but could not induce protection against infection by an heterologous virus. In most cases, protection against disease was achieved (Table 4.5).

Obviously, as in other lentivirus systems, a thorough and careful investigation of constant and crucial epitopes must be carried out. Modern vaccinology is progressing and will derive very significant information from the EIAV system.

## F.  AIDS Virus and Host: What Is the Future?

The virus replicates at the site of entry, for example, the rectogenital mucosa, and subsequently spreads via the bloodstream from where it colonizes the individual. The infected host mounts a rapid and strong immune response with both nonspecific components, such as phagocytic cells and specific cellular and humoral elements. These defense mechanisms are effective against free virus and virus-expressing cells but are apparently unable to eradicate the infectious agent, hidden in a latent stage in macrophages. This Trojan horse mechanism of spread is remarkably illustrated in the EIAV system where waves of fever correspond to appearance of a new virus variant, non neutralized by the preexisting immune reaction.

Virus persists in many organs, including the central nervous system and causes local inflammatory reactions directed at infected cells. In the EIAV system, the virus hides in macrophages and kills cells of the erythroid lineage. The immune system remains largely intact and a large proportion of infected horses will cope with the disease provided the immune system is not altered and remains able to control all generated virus variants. In the HIV:SIV systems, the virus invades cells mostly if not solely via the CD4 surface marker. Virus infection and expression lead to cell killing and the progressive paralysis of the immune system with destruction of the $CD4^+$ T cell compartment. At the same time as the immune system collapses, new virus variants appear and spread. The inevitable consequence is an abundant virus proliferation and spread on the ruins of the immune system.

**Table 4.5.** Vaccine studies in the pony.[a,b]

| Vaccine type | Agent | Adjuvant | Schedule | Challenge | Protection |
|---|---|---|---|---|---|
| 1 dose = 1 mg whole killed formalin | EIAV prototype Wyoming strain cell-adapted | MDP | 0,2,4,5 to 9 doses | iv $10^6$ TCID 3 weeks post last boost homologous | 12/13 |
| | | | | iv $10^6$ animal infectious doses heterologous | 0/13 against infection |
| | | | | | 11/13 against disease |
| 1 dose = 1 mg whole killed formalin | EIAV prototype Wyoming cell-adapted | MDP | 0,2,6 | iv $10^6$ TCID 5 weeks post last boost homologous | 3/3 against infection |
| | | | | iv $10^{3.5}$ horse infectious doses heterologous | 0/3 against infection |
| | | | | | 3/3 against disease |
| 1 dose = 200 μg subunit enriched in *env* glycoprotein | EIAV prototype Wyoming cell-adapted | MDP | 0,2,6 | iv $10^6$ TCID 5 weeks post last boost homologous | 4/4 against infection |
| | | | | iv $10^{3.5}$ horse infectious doses heterologous | 0/4 against infection |
| | | | | | 3/4 against disease |

[a]The table is derived from data presented by Rushlow et al. (166).
[b]MDP, muramyl dipeptide; iv, intravenous; TCID, tissue culture infectious dose.

Virus-producing cells export the TAT protein, a powerful transactivator, and, at the same time, a growth factor for the spindle cells that will make up the Kaposi sarcoma (42). The dramatic incidence of lymphomas in the surviving AIDS patient perhaps proceeds from the same type of mechanism. AIDS-associated Kaposi sarcomas or B cell lymphomas are virus-induced cancers in which no trace of the etiologic agent can be found.

This rapid survey of the host–virus interplay in AIDS has definite implications and predictions for the design of a vaccine whether as a preventive treatment or as a therapeutic intervention:

1. The vaccine preparation should include envelope and internal proteins and be able to strongly stimulate CMI. T cell proliferation in the presence of the vaccine preparation is a good prognostic factor for protection (G. Hunsmann, personal communication).

2. Preparation of antigens should allow presentation of conserved epitopes in order to teach the immune system to strongly react against structures shared by all virus variants (40). The CD4 reactive region (loop between cysteine 418 and 445) of envelope gp120, the carboxyl end of gp120 and the fusogenic segment of gp41, the carboxyl end of gp41 (presence of lytic peptides), the integrase, might fulfill this requirement). Well conserved regions of poor immunogenicity should be associated with peptide sequences recognized as T-cell epitopes by the host to protect (177).

3. In therapeutic interventions, the virus load will be reduced by any available means and care taken to alleviate immunosuppression and restore the immune system with, at the same time, therapeutic vaccination with conserved epitopes.

4. The exact contribution of the two compartments of the immune sytem, cellular and humoral, in the successful reaction of the host against the invading retrovirus is not settled at present. Arguments for a major role of CMI can be found, in the work of Hom et al. (65) involving Rauscher leukemia virus. The authors clearly show that both $CD4^+$ and $CD8^+$ cells contribute to the host's successful defence. The case of SIVsm infected mangabeys that resist infection with the highly pathogenic isolate PBj14 in the absence of neutralizing antibodies calls strongly upon a decisive contribution of cell-mediated immunity. Analogous emphasis on the CMI compartment derives from the data of Hunsmann and colleagues (personal communication), where T cell proliferation in the presence of the vaccine preparation predicts resistance to challenge.

The data reported by Gibbs et al. (51) extend and confirm the major role played by the cell compartment: HIV-I-infected chimpanzees cleared their infection and remained virus negative by peripheral blood mononuclear cell cocultivation for more than 2 years of observation after

challenge. Such a "cure" occurred in the absence of any detectable level of neutralizing antibody in one animal and a low level in the second. Also, Miller et al. (112) have shown that monkeys that develop a major histocompatibility complex-restricted cytotoxic T-cell response to simian immunodeficiency virus *gag* encoded proteins appear to produce less virus and live longer than animals that do not.

In turn, the capacity of antivirus antibody, with high neutralizing titer, has been demonstrated to prevent infection by the homologous virus in cynomolgous monkeys (155–157). Successful passive immunization had been reported previously to protect against murine, feline and bovine oncoviruses (36,69,82,96). Recently, Kataoka et al. (76) have shown that human antibodies to human T-lymphotropic virus type I (HTLV-I) prevented virus infection in rabbits. Previously, passive immunization against HIV-I infection in chimpanzee had failed (154). Tentative explanations for success or failure probably reside in neutralizing antibody titers, the use of cell-free virus inoculum, and homology between the antibody-inducing and the challenging virus.

The present notions of homologous and heterologous challenge are vague and await extensive sequencing of SIV genomes and dissection of SIV proteins into T and B cell epitopes. Sequence comparisons (point mutations, appearance or elimination of glycosylation sites in SIV ENV proteins, for example, may eliminate the neutralizing capacity of a serum) and cross-reactivity studies among induced T cells and antibodies will tell to what extent challenge virus is homologous to the vaccinating one. From the paucity of presently available observations, it is evident that both arms of the immune response may be important. One or the other is considered as more important according to the conditions of the experiments, the immune parameters measured, and the taste of the investigator. For example, in the chimpanzee system, Gibbs et al. (51) emphasize the role of the cell compartment in the apparent "cure" from viral infection manifested by 2 animals, while the reported data of Berman et al. (8) and Girard et al. (54) suggest that neutralizing antibodies are effective in preventing experimental infection.

Indeed, it should be recalled that the humoral response depends upon T-helper cells and that a peptide determinant serving as a T-helper cell epitope enhances the immune response of an unrelated peptide region and even overcomes its poor immunogenicity (177).

5.  The fight against lentiviruses sometimes takes advantage of the highly sophisticated biology of the viruses involved. As a result of evolution, HIV-I is bound to enter the cell via a sequence of molecular steps, the first one being through high affinity interaction with CD4. Linking the HIV binding domains of CD4 to an IgG Fc region resulted in an immunoadhesin (CD4-IgG) with affinity for gp120 and long half-life in the blood. Pretreatment of a chimpanzee with CD4-IgG prevented

infection with cell-free HIV-I. It is foreseen and predicted that the treatment might find a remarkable application to prevent infection at birth of babies delivered by infected mothers (186).

6. Another attempt to take advantage of the sophistication of AIDS viruses is the so-called intracellular vaccination. Elements peculiar to AIDS viruses such as the regulatory proteins NEF (56), TAT, REV, VPX, VPU, and target elements of these proteins might provide remarkable keys to disrupt the virus life cycle. For example, over-expression of tar sequences (the RNA region, that is recognized by the TAT protein) through stable transfection of a well-tailored plasmid might behave as an efficient trap for TAT molecules (a TAT trap) and a powerful silencer of virus expression (174). Introduction of antisense expression vectors in the stem cells and their progeny proceeds from the same mode of thinking. If indeed, the NEF protein is necessary to maintain high virus load and development of AIDS (79), *nef* antisense expression should be a first choice candidate especially if one considers the role of NEF in the paralysis of the T-helper cell function (94). If by all possible means, eradication of virus fails, it remains plausible that strong reduction of virus expression and thus maintenance of the integrity of the immune system will provide the infected person with a normal life span? It may well be that a combination of immune therapy, drug therapy, and perhaps intracellular vaccination will have to be applied in order to dominate the highly sophisticated machinery of AIDS viruses.

The ideas outlined above are for a large part theoretical views. A number of research groups in the world are developing the necessary technology to identify and grow bone marrow stem cells. Together with the impressive progress made in human gene therapy, the knowledge acquired in HIV-I molecular biology allows the authors to think that science will overcome and that cells of the hematologic lineage, made resistant to HIV-I, might represent the ultimate tool to eradicate HIV from the infected host. Even though much remains to be done, continuous progress of science is paving the road toward decisive success in the battle against AIDS and full harvest of knowledge in the biology of man.

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CHAPTER 5

# Vaccines against Rabies Virus

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## 1. Introduction

Rabies is one of the oldest recognized diseases. It most probably originated
on the African continent and diffusion in North Africa was followed
by the spread throughout Europe and Asia. The disease subsequently
appeared on the North American continent possibly by transmission
through circumpolar animals such as foxes and wolves, though the
importation of rabid dogs from Europe is also probable. Today rabies is a
disease of major importance, present on all five continents (59,111).

   There are two main epidemiological features of rabies: urban rabies
where stray dogs are responsible for the maintenance of the disease and
its transmission to man, and wildlife rabies in which rabies is maintained
by wildlife vectors. Here the term vector means the animal species most
susceptible to rabies in a region at a given time, solely responsible for
maintaining the infection. All other species are victims, even if they are
able to transmit rabies. Consequently, their destruction or immunization
has no effect on the disease cycle. Whereas rabies among domestic
animals can be controlled by appropriate prophylactic measures, it poses
a bigger problem in wildlife and, until 1960, the only available mean was
considered to be the reduction of vector populations. For instance, in
Europe, the main vector of the current epidemic of sylvatic rabies is the
red fox (85). Control measures by reducing fox population were only
temporarily effective and did not stop the spread of the disease. For this
reason, other methods such as oral immunization of foxes against rabies
needed to be assessed.

   The main development last few years in antirabies vaccines was mainly
in the field of improvement of wildlife vectors vaccination. Wildlife
vaccination poses several specific problems. Large populations of free
ranging wild animals cannot be vaccinated by parenteral route and the
oral route is the only appropriate one for such purpose (17). We must
therefore use live viruses either attenuated rabies virus or recombinant
viruses bearing gene coding for potent rabies virus immunogens.

This chapter will describe the new advances in conventional vaccines both for human and veterinary uses and focus on recent developments in rabies virus expression by biotechnological means and in wildlife (vector) vaccination.

## 2. Historical Background

The infectious nature of rabies was demonstrated only at the beginning of the nineteenth century by Zinke (1804) who used the saliva of a rabid dog to transmit the disease. Further studies by Galtier (1879) showed that he could protect sheep against rabies by previous intravenous injection of infected material (53) and were followed by the discoveries of Pasteur. Pasteur was able to demonstrate that saliva is not the only source of virus and that the nervous tissue of rabid animals can be used to transmit the disease (78). This was followed by the demonstration that the virulence of the virus can be modified by successive passages in monkeys (79). The attenuated virus thus obtained was capable of protecting dogs against experimental inoculation with a fully virulent virus ("Street virus").

In 1885, Pasteur reported the results of the first postexposure vacci-nation ever performed in man; this first cure in man had been preceded by numerous and conclusive experiments in dogs (80). The first human patient, Joseph Meister, had been bitten by a rabid dog and vaccination was carried out on July 6, 1885. For this purpose Pasteur used repeated injections with a suspension of fragments of spinal cord derived from rabbits experimentally infected with a virus adapted by several passages in experimental animals (fixed virus). Following storage in dry air at room temperature, the virulence of these preparations was found to decrease rapidly and was normally lost after 15 days. Thirteen injections of preparations of increasing virulence were injected over a 10-day period, including 5 "totally virulent" cords. Roux, the initiator with Pasteur of the inactivation technique of rabies virus through aging of spinal cords, later developed a technique that used glycerol to stabilize the virulence of the virus (93), a procedure that was used until 1953 by the Institut Pasteur for the preparation of rabies vaccine for human use. As mentioned, the first human vaccination published by Louis Pasteur was a postexposure one. Postexposure treatments of humans now require several vaccine injections when badly exposed.

In veterinary medicine, preventive vaccination is the rule. Most of the current vaccines confer partial protection against some rabies-related virus infections. Only inactived vaccines have been licensed for humans. Since the end of previous century, antirabies vaccines have been improved. Some countries do still use vaccines produced in nervous tissues. Those vaccines are not safe because of their myelin content; foreign myelin may be responsible for hypersensitivity reactions of the recipient leading to

paralysis (108). Since rabies virus can be grown in cell culture, most of the vaccines now used for humans are derived from cell culture and perfectly safe. They can be used both for preventive vaccination or for postexposure treatment. Attenuated virus vaccines have been widely used previously for immunization of domestic animals; all of them are still pathogenic for some animal species and cases of vaccine-induced rabies were often reported (81). Humans exposed to attenuated vaccine strain for veterinary use are treated in the same way as after wild virus exposure (56) since, with the exception of HEP Flury strain (63), we have no informations concerning the degree of attenuation for man.

The emergence of new potent inactivated vaccines for veterinary use has led to the cession of attenuated vaccines.


## 3. Rabies and Rabies-Related Viruses

Rhabdoviridae are enveloped RNA viruses characterized by their shape (Greek-rod "Rhabdos"), and by the presence of helical nucleoapsids that are infectious and enclosed in a lipid envelope bearing surface projections (132). The genome is a single molecule of negative sense single-stranded RNA that is noninfectious and is transcribed into 5 mRNAs each of which codes for a single protein. The gene order in the genus *Vesiculovirus* is 3'-N-NS-M-G-L-5', representing, respectively, the nucleocapsid protein N, the nonstructural protein NS, the matrix protein M, the envelope glycoprotein G, and a large protein L.

About 80 members of the family infect vertebrates, mostly mammals, but also fishes and reptiles, and several of the viruses that infect vertebrates also infect invertebrates. Others in the family infect plant and/or vertebrates. The family contains 2 genera: the genus *Vesiculovirus*, derived from the name of the virus causing vesicular stomatitis (VSV), a disease of domestic animals observed in the Americas, and the genus *Lyssavirus*, named after rabies (Greek "Lyssa"-madness). The Indiana serotype of vesicular stomatitis virus is the type species of the vesiculoviruses, whereas rabies virus is the type species of the lyssaviruses. In addition, a number of other viruses of vertebrates, invertebrates, and plants are placed within the family but have not been placed in a genus. Certain rhabdoviruses have been isolated only from blood-suckling arthropods, but some of these are believed to infect vertebrates on the basis of serological survey.

Among the Rhabdoviridae, lyssaviruses are of special interest. Rabies has been known for centuries as a disease of humans and of animals and for many years rabies virus was thought to be unique. It is now clear that antigenic and pathogenic variations exist within rabies virus strains, and the existence of several distinct rabies-related viruses is now recognized.

The genome of rabies virus that has been completely sequenced contains 11,935 nucleotides (117). The presence of a pseudogene between the G and L cistrons implies that rabies virus is evolutionarily intermediate in the Rhabdoviridae family (19); apart from that, the gene order is similar to that of the vesiculoviruses. The helical core of ribonucleoprotein (RNP) contains RNA complexed with about 1800 molecules of nucleoprotein N, 30–60 molecules of transcriptase L, and 950 molecules of phosphoprotein NS. The nucleocapsid structure is surrounded with an envelope of about 1500 molecules of membrane protein M, through which project the surface spikes of the only transmembrane protein, the glycosylated G protein, present at 1800 molecules. Purified N protein elicits group-reactive antibodies detectable by complement fixation, immunofluorescence, and immunoprecipitation that react with rabies and the rabies-related viruses.

Nevertheless, the immune response against N protein plays a minor protective role. Purified G protein elicits neutralizing antibodies. Glycoprotein can, alone, elicit a protective immunity as it has been shown by using a vaccinia recombinant virus containing the rabies glycoprotein gene (58,129). The antigenic determinants of glycoprotein vary as shown with monoclonal antibodies (60).

Pathogenicity of rabies virus is partially related to the G glycoprotein since the introduction of a mutation at arginine 333 of this protein (except lysine) by selection of mutants resisting neutralization by appropriate monoclonal antibodies renders the virus avirulent for mice and other species (67,118). The sequence of amino acids of the glycoprotein deduced from the nucleotides sequence contains residues and shows analogies with sequences of curaromimetic neurotoxins extracted from snake venom (68).

The 5 rabies-related viruses already described are all African viruses. Lagos bat virus isolated from fruit bats (*Eidolon helvum*). Kotonkan and Mokola viruses were isolated first from *Culicoides*, and then from a child and from shrews (*Crocidura species*). Obodhiang virus was isolated from mosquitoes, whereas Duvenhage virus was isolated from a man who died after having been bitten by a cat. The new virus isolated from European insectivorous bats although closely related, differs somewhat antigenically from Duvenhage virus (60).

These viruses can be distinguished from rabies virus using both conventional serums or monoclonal antibodies. The complete nucleotides sequence of Mokola virus, the most divergent from rabies virus, has been obtained (18,19). The sequence homology between its glycoprotein and the rabies (Lyssavirus type 1) one is only 58%, whereas it is more than 94% between the closely related type 1 rabies viruses.

Homology is not randomly distributed since there are as many as 24 consecutive identical amino acids within the transmembrane domain of the protein, particularly between residues 77 and 157. It is interesting to

note that the arginine in position equivalent to 333 of rabies virus glyco-protein is absent in Mokola.

Although the dog is the principal source of infection for humans where urban rabies exists and on a worldwide basis, the reservoir of rabies is often to be found in wildlife. For instance, in western Europe, the red fox (*Vulpes vulpes*) is the main vector, whereas it is the striped skunk (*Mephitis mephitis*), the red fox, or the raccoon (*Procyon lotor*) in the United States; the situation is more complicated in Africa. Vampire bats (such as *Desmodus rotundus*) are responsible for cattle rabies as well as human cases in Latin America. Rabies is usually acquired through the bite of an infected animal, but aerosol transmission has also been described. Viral entry may be via acetylcholine receptors or via rhabdovirus common receptor-like. Multiplication takes first place locally at motor end-plates and later in myocytes, subsequently spreading to the central nervous system (CNS) via axons of peripheral nerves (30). The incubation period can range from a few days to several years. The disease pattern may be of a classical "furious rabies" or "dumb" (paralytic) rabies.

# 4. Protection to Rabies Virus Infection

## A. Genetic Control of Resistance to Rabies

The first demonstrations that different strains of mice varied in their susceptibility to rabies virus infection were reported independently in 1940 by Johnson and Leach and Habel (70). Those findings were confirmed later on. This resistance is a dominant trait, controlled by 1 or 2 genes and not linked to the H-2 locus. The mechanisms involved in the genetic variability observed are not yet elucidated. Several factors could play a role, including differences between the multiplication rates of defective interfering particles (126). The amount of infectious virus needed to reproduce the disease experimentally differs markedly according to the virus strain and the species (12,14).

## B. Immune Response during Rabies-Virus Infection

Natural rabies infection is characterized by an often long incubation period, the absence of serological evidence of infection before disease onset, and the usually fatal issue of the disease when declared. Nevertheless recovery from CNS infection can occur (ableit rarely) in both humans and animals. Therefore informations on the mechanisms involved in protective immunity have mainly been gained through vaccination studies.

Rhabdovirus do readily induce interferon production in the host and are fairly susceptible to interferon action. Interferon production could

be involved in the protection conferred by the curative vaccination procedure discovered by Louis Pasteur. A paradoxical phenomenon may be observed when using high doses of infectious virus for challenge infection. Animals infected with high doses resist to challenge whereas those infected with lower doses succumb. This paradoxical phenomenon may be linked to a better interferon response in the first case.

Vaccination during the incubation period, if not repeated, leads to the so-called "early death phenomenon," which is to say the reduction of the incubation period as compared to control infected animals. The rabies virus glycoprotein can alone produce this phenomenon since it can be reproduced by a recombinant vaccinia virus expressing the rabies virus glycoprotein gene (23). Protection against rabies can be achieved by passive transfer of specific antibodies.

Many questions remain to be answered concerning the role of the various immunological mechanisms involved in the protection against rabies virus infection. For instance, the importance of cell-mediated immunity in the protective mechanisms has not yet been fully evaluated. Even if there is an excellent correlation between antibody titers and protection, nevertheless, vaccinated animals devoid of detectable neutralizing antibodies may resist a challenge infection that kills the controls. Moreover, the protective mechanisms following postexposure treatment of humans with rabies vaccine is very likely to involve T cells (72). Among the many questions concerning the role of immune mechanisms in protection against rabies, perhaps the most important one from a practical point of view is whether it is necessary to consider the capacity of a vaccine to stimulate T cells in order to develop the next generation of veterinary and human rabies vaccines.

As already mentioned, rabies virus infection is characterized by a silent progression of rabies virus in the nervous system during the incubation period, without any detectable humoral immune response. Moreover rabies virus infection can lead to asymptomatic carriers of the infection that excrete the virus; it has been observed in dogs in Africa (3,46). Clinically normal animals may be infective for a few days before the onset of clinical disease.

## 5. Problems Associated with the Use of Conventional Antirabies Vaccines

Since the end of the previous century when rabies vaccines were prepared in the nervous system of rabbits, rabies vaccines have been significantly improved. However, some countries still use vaccines produced in the nervous tissue of animals. These vaccines are not safe because of their myelin content; foreign myelin may be responsible for the induction of hypersensitivity reactions of the recipient, leading to paralysis. Because

myelination is delayed in neonatal mice, the use of suckling mouse brain reduces this risk (51), but some reactions may still occur and thus such vaccines are best avoided. Since rabies virus can be grown in cell culture, most of the vaccines used today are derived from cell cultures and are perfectly safe. In humans, they can be used both for preventive vaccination or for postexposure treatment in conjunction with specific immuno-globulin therapy.

Attenuated virus vaccines have been widely used in the past for immunization of domestic animals. However, all of them still had some residual pathogenicity for some species, and cases of vaccine-induced rabies occasionally occurred, often in cats (9,38,43,123). The use of monoclonal antibodies has made it much easier to distinguish such cases from infection with wild-type virus. Humans exposed to attenuated vaccine strain for veterinary use are treated in the same way as after wild virus exposure. Safe and potent inactivated vaccines have been since developed for veterinary use, and these have now largely superseded attenuated vaccines.

Rabies virus strains have been adapted to cell cultures so that large amounts of virus can be produced without the hazards associated with vaccines prepared in nervous tissue, and adjuvants have increased the immunogenicity of such vaccines. In animals, only preventive vaccination is generally carried out. In some countries, postexposure vaccination of domestic animals may be allowed but only if the animal has previously been vaccinated.

## 6. Vaccines for Human Use

In 1881, Pasteur and Roux isolated a strain of rabies virus from the brain of a rabid cow and, after 90 successive inoculations into rabbits via the intracerebral route, noted that the incubation period has been reduced from 15 to 7 days and remained constant ("fixed virus"). They also found that when stored in a dry atmosphere at room temperature the spinal cord of rabbits inoculated with this fixed virus rapidly lost virulence. By injecting dogs subcutaneously with a suspension of infected spinal cords stored for various periods, and starting with the cord stored for the longest time, not only did the dog resist inoculation of virulent cord but they were also protected against inoculation with street virus. The basic principles of Pasteur have formed the basis of all subsequent vaccines.

The best characterized live rabies vaccine for human use is the FERMI vaccine (47). This was prepared from a suspension of sheep or goat brains inoculated with a fixed virus strain, to which was added a small quantity of phenol to inactivate the virus partially. Due to the persistence of virulent viral particles, such vaccines were liable to induce vaccinal rabies and their use today is forbidden. Semple in 1911 prepared a vaccine

similar to the FERMI one, except that rabbit brains were used and the virus was completely inactivated with phenol.

Until 1980, this type of vaccine represented 60% of all rabies vaccines used throughout the world. A variant of this vaccine was introduced with inactivation of the virus with $\beta$-propiolactone. To decrease the number of postvaccinal reactions attributable to myelin, vaccines have subsequently been prepared from the brain of newborn mice (51). To minimize the risks of vaccinal complications a number of vaccines have been prepared from virus grown in tissue culture in the complete absence of nervous tissue. For instance, vaccines have been prepared from virus multiplied in human diploid cells (124,125,127). At present, they represent the most elaborate form of human rabies vaccine and can be used both preventively and curatively. The fixed virus PM (Pitman Moore) strain is multiplied in a semicontinuous human fibroblast cell line (WI-38 or MRC-5) and inactivated with $\beta$-propiolactone. The activity of this vaccine is higher than then previous ones and the number of injections necessary for postexposure treatment has been reduced from 14–21 to only 5 or 6 (90). Inactivated vaccine produced in VERO cells has recently been licensed.

## 7. Vaccines for Veterinary Use

Although Pasteur demonstrated the possibility of vaccinating dogs between 1884 and 1885, it was only in the 1920s that domestic animal vaccination was developed and used in practice. The first vaccine widely used was the SEMPLE type (119). Later on, attenuated live virus and inactivated virus vaccine were developed. The adaptation of the fixed Flury strain to chicken embryos (61) led to the selection of two strains differing in the number of passages: the low egg passage (LEP; 50 passages) (62) and the high egg passage (HEP; 183 passages) (63). The HEP strain has lost its neurotropism, and the two strains differ from one another in their level of attenuation. The HEP strain can be used for the vaccination of dogs, cats, and cattle, but the less attenuated LEP strain is strictly reserved for use in dogs. Other strains, such as the ERA one have also been developed (1,2). Unfortunately, as already mentioned, cases of vaccine rabies have been reported after the use of such vaccines. Thus, live virus vaccines are now forbidden in many countries. Like vaccines for use in humans, vaccines for veterinary use have been prepared from sheep brain, from newborn mouse brain, from embryos or, as most frequently the case today, from virus grown in cell culture. The virus is commonly inactivated with $\beta$-propiolactone and associated with an adjuvant such as aluminium hydroxide (104). Inactivated vaccines produced in cell culture may be derived from already attenuated strains. Most of them confer a long term protection and have the great advantage of being multispecies vaccines.

# 8. Vaccines for Wildlife

## A. Conventionnally Attenuated Vaccines

A method of vaccinating wild animals against rabies was developed in the United States (7,33,130,131) and in Europe (15,71,97,120) and was used for the first time in the field in October 1978 in Switzerland (106,107). Since then, the method has been recognized as extremely effective in several countries (99). There has been much recent research on technical adjustments, particularly the type of vaccine used. For a long time, most researches concentrated on the parenteral or oral administration of vaccines already widely used in domestic animals, particularly strain SAD ("Streat Alabama Dufferin") of rabies virus (17).

There have been attempts to immunize foxes parenterally, after capture within their dens (82). Field trials were conducted in Switzerland in 1976 and subsequently in Germany with inactivated vaccines (105), but it proved impossible to immunize more than 40% of the population, and attempts were soon abandoned. Furthermore, the cost of this procedure was prohibitive. Research then focused on oral administration, the only procedure truely applicable in the field. Some inactivated vaccines have been tried (20), but in most cases, an attenuated strain of rabies virus was used. The strains most often used are standard SAD (10,11), strain SADB19 (98–100) a variant of SAD, the derived ERA (from the names Gaynor, Rokitniki, and Abelseth), Flury low egg passage (LEP), Flury high egg passage (HEP), or its variant strain "675" (42,50,57).

In early experiments on immunization, the vaccine strain was administered either directly into the mouth of the animal or after incorporation into a bait. Various baits have been tried, including young mice, chicken heads or, more frequently, baits manufactured from fats and proteins, moulded by heat. The final choice depends on compatibility between bait and vaccine, stability in the environment, ease of handling and storage, attractiveness to the target species, and, of course, cost. Vaccine may be incorporated in the bait directly or more frequently, within a plastic capsule containing 1–2 ml of fluid vaccine. In western Europe, chicken heads have been used extensively (100,107), as well as manufactured baits. Only in the USSR has the vaccine been injected directly into chicken heads (102). The results of trials in experimental stations have been evaluated by the titer of rabies antibodies after vaccination and/or challenge infection with virulent rabies virus (13). The general conclusions were that only strain SAD, the derived strains SADB19 (developed in Germany), and perhaps strain Vnukovo 32 (developed in eastern Europe) are capable of protecting 100% of foxes without prior concentration of cell culture fluid. The titer of these strains of virus may easily exceed $10^7$ $TCID_{50}$ per ml of harvested fluid, which is the dose needed to immunize a fox. In no case has an orally administered inactivated vaccine

elicited an immune response capable of protecting an animal (5,73). Hence there has been unanimous choice of strain SAD for the initial field trials.

The main shortcoming of strain SAD is that it can harm nontarget species that might eat the vaccine (particularly certain members of the families Muridae, Mustelidae, and Felidae), which has raised concern (31,32,120). However, it has proved impossible to passage this virus serially in such species, and it is not excreted by animals that develop vaccine rabies (106,121,122). This strain can be distinguished from wild strains by using monoclonal antibodies (128).

In the United States, initial experimental trials with the CVS and Flury LEP strains, given orally to foxes (*Vulpes fulva*), failed to give conclusive results (8). These strains were abandoned in favor of strains SAD and ERA, which gave good results under experimental conditions (11,65). Experiments on other vectors of rabies in North America with the standard attenuated strains of rabies virus have given relatively poor results (8). Certain strains were pathogenic for certain species, such as the striped skunk (*Mephitis mephitis*), or they were ineffective in certain target species, such as the raccoon (*Procyon lotor*) (94,96). The research effort then turned to alternative solutions, such as the use of a carrier virus. The lack of safety of strain SAD for certain nontarget species has resulted in research being undertaken, even after the initial field trials commenced, to find another vaccine that is effective as well as perfectly safe. Some of the research concentrated on obtaining new, safer, attenuated strains.

## B. New Attenuated Strains of Rabies Virus

As already shown, the main problem with previous strains to be solved was the improvement of safety. New strains which have been tested are GSC, CVS, and mutants derived from them (thermosensitive-ts, $AVO_1$, etc.), as well as the SAG mutant derived from SAD (Bern). The unmodified CVS strain was tried only once by Baer and co-workers (6), who administered the virus by stomach tube to five foxes, only one of which formed antibodies and became resistant to challenge infection. Since then, numerous apathogenic mutants have been obtained from this strain. The first mutant was a thermosensitive (ts) strain that was soon abandoned because it was not immunogenic at the concentrations employed (29).

Other mutants have since been obtained (34,35,115), in which a single nucleotide in the glycoprotein gene was substituted, resulting in the replacement of arginine at position 333 by a leucine, isoleucine, glutamine, glycine, or serine residue (36,41,101). All mutations based on arginine 333 substitution rendered the virus apathogenic for mice, foxes, dogs, and several species of wild rodents (66,88). Unfortunately, administration of

mutants such as $AVO_1$ has failed to protect sufficient numbers of animals. Consequently, their use could not be considered. Since then, similar mutants (SAG) have been obtained from the Bern SAD strain, and seem to give promising results (48,67).

## C. Recombinant Vaccines

The spike forming glycoprotein of rabies virus is essential in eliciting rabies neutralizing antibodies and in conferring protection to rabies. Although induction of rabies neutralizing antibodies is essential for protection, it appears that cellular immunity is also required and that correct presentation of the glycoprotein is necessary to induce specific cytotoxic T cells. The most successful approach in addressing this requirement has been the use of live virus vectors. Recent technical advances have permitted the development of vaccinia virus as a cloning and expression vector (86,87). Expression of exogenous protein-coding sequences in vaccinia virus involves essentially two steps. First, the exogenous coding sequence is aligned with a vaccinia promoter and inserted *in vitro* at a site within a (nonessential) segment of vaccinia DNA cloned into a suitable bacterial plasmid replicon. Second, the flanking vaccinia sequences permit homologous recombination *in vivo* between the plasmid and the viral genome. Double reciprocal recombination results in transfer of the DNA insert from the plasmid to the viral genome, wherein it is propagated and expressed.

The cDNA coding for the rabies virus glycoprotein (524 amino acids) has been inserted into the thymidine-kinase (TK) gene of vaccinia virus, generating a selectable $TK^-$ virus called VVTGgRAB (58,129).

Similar recombinants have been prepared (44) using both vaccinia virus or a raccoon poxvirus (45) for raccoon oral vaccination. More recently adenovirus recombinants have been constructed, but the VVTGgRAB recombinant is the only one to be tested in the field so far.

The efficacy and the safety of the VVTGgRAB have been tested in the main target species for Western Europe and North America: fox, raccoon, and striped skunk (16,39,94,95,116). The results of experimental efficacy for foxes can be summarized as follows. All but one out of 26 adult captive foxes inoculated by various routes developed high titers of rabies virus-neutralizing antibodies and resisted wild rabies virus challenge on day 28 after vaccination. The duration of immunity conferred by VVTGgRAB ($10^8$ PFU; oral route) reached a minimum of 18 months, which is to say more than needed, since most of the foxes in the wild are under 24 months of age. Foxes receiving less than the recommended dose showed a clear dose-dependent response. A second administration of VVTGgRAB induces an increase of rabies virus neutralizing antibodies titers (booster effect). When administered to fox

cubs by the oral route ($10^{7.2}$ TCID$_{50}$), it induces significant levels of rabies virus antibodies and protects 92% of them (11/12) against rabies, with an immunity duration exceeding 12 months (21).

The efficacy of VVTGgRAB ($10^8$ TCID$_{50}$) contained in a new machine made baiting system has recently been tested (25). Thirty days after baiting, seroconversion to rabies was observed in 15/18 of the foxes and seroconversion to vaccinia in 14/18. Sixteen of the 18 baited foxes resisted a wild rabies virus challenge 90 days after baiting. These results demonstrate that the baiting-sachet system used permits a good release of the virus suspension into the mouth.

The absence of pathogenicity of the VVTGgRAB for the fox was observed whatever the dose of inoculation ($10^2$ to $10^{10}$ TCID$_{50}$) or the route of administration (oral, intramuscular, intraduodenal, subcutaneous, intradermic, conjunctival, or intranasal). In order to test for horizontal transmission, unvaccinated control animals have been held in close contact with vaccinated ones. No transmission of immunizing amounts of VVTGgRAB occurred in adult or young foxes with the exception of one adult fox bitten by a freshly inoculated one.

Foxes vaccinated during the incubation period, early after challenge with a wild virus, die after a shorter period of incubation as compared to unvaccinated controls (23). On the other hand, animals vaccinated belatedly after challenge died later than the control ones. These results show that "early" and "late" death phenomenons occur as a consequence of interactions between oral vaccination with VVTGgRAB and rabies infection, but preclude the risk of the emergence of asymptomatic carriers of wild rabies virus after vaccination. Similar results have been obtained with the raccoon and the skunk. The safety of the VVTGgRAB for the pregnant-female raccoons and the absence of epigenetic transmission to kits have been evaluated as well as the absence of the recombinant in the cerebrospinal fluid (54). There were no evidence of active *in utero* or lactogenic transmission of the recombinant.

Bait uptake surveillance and tetracycline (biomarker) detection controls performed after vaccination campaigns have proven that mustelids, wild boars (*Sus scrofa*), and domestic (feral or not) carnivora may ingest the vaccine baits. Moreover, a significant proportion of the baits is partially eaten by micromammals.

It is important to verify the safety of VVTGgRAB for nontarget species (both domestic and wild). Several nontarget wild species have been chosen for testing in Europe, because of their opportunistic feeding behavior and their presence in the areas where the vaccine must be distributed (24). Safety of the vaccine has been tested in daubenton bat (*Myotis daubentoni*), wild boar (*Sus scrofa*), eurasian badger (*Meles meles*), wood mouse (*Apodemus sylvaticus*), yellow-necked mouse (*Apodemus flavicolis*), bank vole (*Clethrionomys glareolus*), common vole (*Microtus arvalis*), field vole (*Microtus agrestis*), water vole (*Arvicola*

*terrestris*), common buzzard (*Buteo buteo*), kestrel (*Falco tinnunculus*), carrion crow (*Corvus corone*), magpie (*Pica pica*), and jay (*Garrulus glandarius*). Clinical signs and/or pox lesions were never observed in the vaccinated animals during the observation period (28 days minimum after vaccination). Similar experiments have been carried out with wild species from North America (4) including meadow vole (*Microtus pennsylvanicus*), wood chuck (*Marmota monax*), grey squirrel (*Sciurus carolinensis*), ring-billed gul (*Larus delawarensis*), red-tailed hawk (*Buteo jamaicensis*), great horned owl (*Bubo virginianus*), and coyote (*Canis latrans*) with similar results.

Experiments were also designed to determine the multiplication site in foxes of the recombinant virus as compared with that of the parental strain of vaccinia virus (VV), by virus isolation, titration, and indirect immunofluorescence. The polymerase chain reaction (PCR) was also used to detect specific virus DNA in several fox organs (114). Using those various techniques, VVTGgRAB or VV were detected during the first 48 hrs following vaccination by the oral route, but only in the tonsils, buccal mucosa, and soft palate. Similar results have been obtained by others with raccoons using virus isolation (95). No virus could be detected in salivary glands; the risk of transmission through saliva from one animal to another can therefore be neglected. Furthermore, the fact that VVTGgRAB multiplies only in restricted sites minimizes further the potential risk of recombination with other wild orthopoxviruses. No difference was observed between the multiplication sites of either VVTGgRAB or VV, demonstrating that recombination did not modify the tissue tropism of the virus. Virus was never detected in the brain.

## 9. Vaccination Campaigns against Wildlife Rabies

Following selection, first in the laboratory and then in experimental stations, the least dangerous and most effective live virus vaccines were used for field trials in Europe. The unpatented standard SAD strain produced in Bern (Switzerland) has been used from 1978 until now throughout the infected areas of this country, and also in certain regions of Germany (before 1985), Italy (in 1986), and France (in 1987). Baits were distributed at a density of 15–20/km$^2$ (106) with progressive and very satisfactory efficacy. Strain SADB19 was first used in 1985 in Germany, where it was patented. Austria, Belgium, France, Italy, and Luxemburg began to use it in 1986 for a while (22,83). It is still presently the most extensively used strain of vaccine used in the field. The disappearance of fox rabies from treated areas shows that the results achieved are at least as good as those obtained in Switzerland. This system may also be suitable for controlling rabies among raccoon dogs (*Nyctereutes procyonoides*). Several thousands of doses of SAG vaccine were also used in the field in Switzerland and France.

Taking into account all the available experimental data concerning the safety of the VVTGgRAB for target and nontarget species and its efficacy in foxes, initial, limited field trials of fox vaccination were authorized first by the Belgian (84), then by the French Public Health authorities. In the Belgian trial, on October 17 and 18, 1987, a total of 250 vaccine baits (chicken heads) were manually delivered on a $6\,km^2$ area situated in the central part of a military zone.

The VVTGgRAB safety having been confirmed by this small trial, the Belgian authorities agreed for an enlarged open field trial. This latter has been conducted in a $435\,km^2$ area in the southern part of the country. This vaccination area was chosen because it has the lowest human population average density (42 inhabitants/$km^2$) of the country combined with a high rabies incidence in foxes. Each bait used contained a suspension of $10^8$ TCID$_{50}$ of VVTGgRAB (volume 2.2 ml) within a plastic sachet and 150 mg tetracycline as a long-term biomarker of bait uptake. Fifteen to 20 baits were used per square kilometer. After the vaccination campaign, 222 dead wild animals belonging to 19 species were collected in the vaccination area.

Tetracycline was detected in foxes (61%), stone martens (*Martes foina*), domestic or feral cats (*Felis catus*), wood mice (*Apodemus* sp.), wild boars, and carrion crows, showing that those species are strong competitors of the foxes for the bait uptake. Twelve months of monitoring failed to detect any ecological hazard or public health concern. The vaccine was very stable even following natural freezing and thawing cycles (26). Three fox vaccination campaigns using VVTGgRAB were thereafter carried out in Belgium in November 1989 and April and October 1990 on an area of $2200\,km^2$ with a mean baiting density of 15 baits/$km^2$ (76). Field controls of bait uptake performed after these release has shown that more than 90% of the baits are taken by animals after 30 days. Rabies incidence has severely decreased in the treated area. Because of its efficacy, safety, and heat stability VVTGgRAB seems to offer an excellent alternative to the attenuated strains of rabies virus currently used in the field. The first, limited trial of raccoon vaccination against rabies has just been carried out in the United States (49). First trials of dog vaccination against rabies (urban rabies) using oral vaccination with baits are also under progress. Use of VVTGgRAB could lead to eradication of rabies from large area (27).

## 10. Other Developments

One other route to produce vaccines involves the elaboration of antigenic material based upon nonreplicating viral vectors expressing G protein, purified G protein, subfragments of G, or antiidiotypic antibodies raised against G (28,59).

## A. Avipox Viruses Recombinants

The natural productive host range of avipox viruses is limited to avian species. Nonetheless, abortive infection can be initiated *in vitro* in cell lines derived from nonavian species. The viruses have no observable effect on the cells after the first nonproductive passage and they cannot be adapted to growth in nonavian cell lines. Using avipox viruses recombinants, foreign antigens can be authentically synthesized, processed, and presented on the infected cell surface without infectious progeny virus being produced. Fowlpox and canarypox rabies recombinant viruses have been developed in which the gene coding for the rabies virus glycoprotein is expressed under the control of vaccinia virus promoters. They express an authentic immunologically recognizable rabies glycoprotein on the surface membranes of avian and nonavian cells. When inoculated to six nonavian species (mice, rats, rabbits, cats, dogs, and cattle), the fowlpox recombiant induces rabies-specific virus-neutralizing antibody 2 weeks after inoculation. In cattle, when revaccinated 2 months after the first inoculation, the animals are responding with increased rabies specific antibody levels indicating a typical booster effect (109). The route of inoculation does not appear to be important as comparable levels of antibodies are produced via intradermal, intramuscular or subcutaneous routes. The dose is important as mice receiving less than $10^{4.7}$ $TCID_{50}$ developed only a poor immune response as compared with mice receiving larger doses. Since the virus is not replicating to amplify the original, a minimal dose may be necessary to induce a significant immune response. When the virus is inactivated no immune response to the rabies glycoprotein is produced indicating that the response is due to *de novo* expression of the rabies glycoprotein in inoculated animal, and not to rabies antigen adventitiously carried on or internally associated with the virus inoculum. Protective activity was demonstrated by challenging inoculated animal species (mice, cats, dogs) with highly virulent rabies virus. In mice, the protective activity was shown to be related to the dose of recombinant used for immunization. In cats and dogs, using a unique dose of $10^8$ $TCID_{50}$ recombinant, all nonvaccinated control animals died showing rabies symptoms, whereas vaccinated animals survived a challenge done 94 days post vaccination.

To evaluate other avipox viruses as vectors, a canarypox vector expressing the rabies glycoprotein was constructed. Similar to the fowlpox rabies recombinant, the canarypox recombinant expresses an authentic immunologically recognizable rabies glycoprotein on the surface membranes of inoculated avian and nonavian cells. When inoculated to nonavian species (mice, cats, dogs) the canarypox recombinant is inducing rabies specific virus neutralizing antibodies (110). In order to compare its protective activity with the protective activity of the fowlpox recombinant, the mice $PD_{50}$ was evaluated using standard procedures. The results

indicated the following: by a unique foot pad administration with a range of dilutions of the recombinants, the canarypox virus recombinant was significantly better in eliciting neutralizing antibodies and was about 100 times more effective in protection ($PD_{50}$ 4.18 versus 6.17) than the recombinant fowlpox virus. The protective efficacy is, in this case, not significantly different from that of the vaccinia rabies recombinant.

In cats and dogs, the protection dose was determined using a unique administration of variable doses of the canarypox recombinant by subcutaneous route. All nonvaccinated control animals died after challenge showing rabies symptoms, whereas a dose-related survival was observed in vaccinated animals challenged 26 days postvaccination. Full protection in both cats and dogs was seen with a vaccine dose as low as $10^5$ $TCID_{50}$, and the calculated $PD_{50}$ was, in this experiment, 3.30 $\log_{10}$ $TCID_{50}$ for cats and 4.19 $\log_{10}$ $TCID_{50}$ for dogs.

None of the recombinants, either fowlpox or canarypox, was shown to be protective through oral administration to laboratory or target animal species, thus prohibiting their use for wildlife immunization.

## B. Antiidiotypic Antibodies

Antiidiotypic antibodies should mimic the structure of the original antigen and themselves be able to induce an immune response to that antigen (40). Reagan et al. (92) have raised polyclonal antisera in rabbits against 5 mouse antirabies monoclonal antibodies to obtain antiidiotypic preparations. This preparation was tested in mice for the ability to induce rabies-neutralizing antibodies. Two of 5 preparations induced significant titers of neutralizing antibodies though insufficient to protect against lethal challenge infection. Preimmunization with one of such antiserum followed by boosting with a nonprotective dose of standard rabies vaccine, yielded animals resisting challenge with low doses of rabies virus (64). Therefore, the use of antiidiotypic vaccines in the control of rabies seems, at present, unlikely to be appropriate for complete protection under field exposure.

## C. Iscoms and Liposomes

Cox et al. (37) reported that reconstituted vesicles containing viral lipids and G protein were capable of conferring protection to rabies. Thibodeau et al. (112) reconstituted liposomes in the presence of rabies G. Microscopic analysis revealed "immunosome" structures in which G protein anchored on the membrane formed a fringe around the particle. Inoculation of mice elicited a higher antibody response than obtained with purified G alone (89,112). The immunological potency of such preparations was, however, somewhat less than inactivated rabies virus.

Morein et al. (74) reported the aggregation of rabies G with glycoside Quil-A to yield an "iscom" (55,75). The protective immunity conferred was comparable to that obtained with standard vaccine (77).

## D. Expression in Baculovirus and Other Expression Systems

The baculovirus system has been recognized as a very efficient and safe eukaryotic expression vector (69). Préhaud and co-workers (91) have expressed rabies G protein in baculovirus. The protein produced in large yields could induce protection against challenge infection showing that the G protein obtained was very similar to the normal G protein of rabies virus. Baculovirus expression could be a good tool for the production of inactivated subunit vaccines.

# 11. Conclusions

Even if rabies virus vaccines have a long history of development, new improvements are still in progress. Oral vaccination of rabies reservoir seems very promising both for wildlife and urban rabies. In this respect, genetically engineered vaccines such as the recombinant vaccinia-rabies vaccine could provide a tool for the control of wildlife rabies and thereafter introduce a completely new approach for the fight against human and animal rabies (27).

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CHAPTER 6

# Vaccines against Morbillivirus Infections

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## 1. Introduction

Members of the genus *Morbillivirus* classified within the family of Paramyxoviridae are rinderpest virus (RPV), peste des petits ruminants (PPRV), canine distemper virus (CDV), and measles virus (MV) (16). Recently, other morbilliviruses have been isolated from aquatic mammals, like seals (phocid distemper virus, PDV) and dolphins (14,38). All viruses are highly contagious for their natural hosts and may cause serious, acute diseases that may result in high mortality rates. At present vaccination programs with live attenuated vaccines have proven to be the most effective approach to prevent RPV infection in cattle or domestic buffalo populations, PPRV infection in goats and sheep, CDV infection in dogs, and MV infection in humans. The use of live vaccines still has several disadvantages: they are less effective in the presence of maternal antibodies or intercurrent virus infections, may cause severe side-effects in immunocompromized hosts, and need a cold-chain during transport and storage. The use of live attenuated vaccines for use in wild species should not be advocated, as calamities in different wild species with live CDV vaccines have been reported (29). The introduction of Tween-ether- or formaldehyde-inactivated morbillivirus vaccines has been accompanied by serious problems. Animals or humans were shown to be insufficiently protected and in some cases illness following exposure to live virus was more severe than that in nonvaccinated individuals. As a result of new developments in biology and technology, also in the field of morbillivirus vaccines, the development is rapidly changing from the use of conventional methods to novel approaches for vaccine strategies.

In this chapter we describe the properties of conventionally produced morbillivirus vaccines and possibilities for novel generations of live recombinant morbillivirus vaccines and subunit vaccines.

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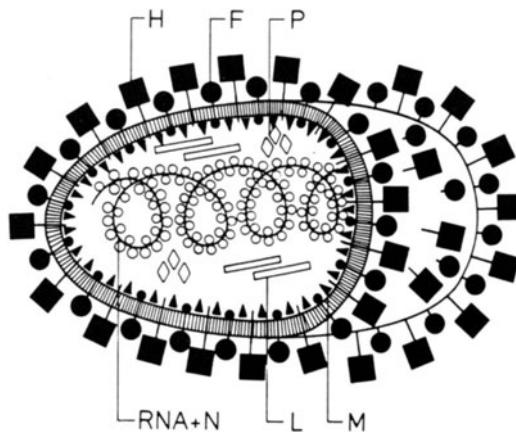
**Fig. 6.1.** Schematic representation of the structure of measles virion.

# 2. General Properties of Morbilliviruses

## A. Structural and biochemical properties

Morbilliviruses have a single stranded RNA molecule as the genome, which is associated with the nucleoprotein N, the major viral protein both in the virion and in the infected cell (Fig. 6.1). The phosphoprotein (P) and the large protein (L) are both minor structural proteins. A second major structural protein is the matrix protein (M). The M protein is either associated with the nucleocapsid or with the viral envelope, which carries surface projections and surrounds the nucleocapsid. The surface projections are formed by two other major structural proteins; the hemagglutinin (H) and the fusion protein (F), which are both glycosylated. The H protein is involved in the adsorption of the virus to the host cell receptor and only in the case of MV in hemagglutination activity. The F protein in its biologically active form consisting of two covalently coupled subunits $F_1$ and $F_2$, is responsible for virus–cell fusion, the formation of syncytia by infected cells, and only in the case of measles virus of hemolysing activity of the virus (44).

The nucleotide sequences of most of the structural proteins of MV, CDV, and RPV have been resolved now (13). The predicted amino acid sequences of the corresponding proteins show relatively high homology between the different morbilliviruses. The H proteins of the virulent RPV Kabete strain and of the Edmonston B strain of MV have 58.9% amino acid homology, whereas both these H proteins show an amino acid homology of 36% with the CDV H protein (9,58). The F proteins of RPV and MV are even identical for 81.3% and over 50% of the amino acid substitutions in the F protein is conservative (21). A comparison of the F

proteins of RPV and CDV shows 68.2% amino acid homology. Although MV is the official type species of the genus *Morbillivirus*, RPV has to be considered as the archvirus of the genus, as can be based on comparison of nucleotide and amino acid sequences and studies in which large panels of monoclonal antibodies are used (35,56).

## B. Biological Properties

The natural transmission of morbillivirus infections occurs by the airborne route (46,47,59). Infection initially takes place in the lymphoid cells of the upper respiratory tract, which subsequently causes a cell-associated viremia. The virus is then transported to all lymphatic tissues by infected B lymphocytes, T lymphocytes, and monocytes, by which the respective target organs become infected.

Viremia can result in marked leucopenia, that may be responsible for a reduced level of cell-mediated immunity during infection. Tissue damage caused by virus replication may lead to severe complications in the respiratory and/or gastrointestinal tract. How the disease develops after infection strongly depends on the virulence of the virus strain involved, the immune status of the animal, and the nature of secondary infections.

Available data about the present knowledge of susceptible domestic and wildlife animal species are summarized in Table 6.1. Natural infections by RPV occur only in even-toed ungulates belonging to the order Artiodactylia (46). Domestic animals, cattle, and buffaloes are most commonly affected. At present only goats and sheep from India are regularly infected with RPV. Asiatic domestic pigs have shown to be more susceptible than European pigs. Especially in regions where large domestic ruminants are kept, RPV infections in wildlife species have resulted in enormous losses. If RPV infections in domestic animals can be controlled by proper vaccination campaigns, it may be expected that rinderpest can be eradicated, since RPV infections in wildlife animals will most probably decrease simultaneously too (46).

Natural infections by PPRV occur mainly in goats and less often in sheep. Cattle and pigs can be infected but do not develop severe clinical symptoms. Species of wild ruminants and wild pigs may have RPV-specific antibodies in their sera, which may have been induced by PPRV. Eradication of PPRV can also be expected to be feasible, if suitable vaccination campaigns could be available in developing countries, where PPRV infection occurs predominantly (47).

The domestic dog is the first known natural host for CDV infections. Most terrestrial carnivores are susceptible to CDV infection. All animals in the Canidae, Mustelidae, and Procyanidae families can be infected with CDV (3). It has been suggested that an outbreak of distemper in Lake Baikal seals was caused by a virus closely related, if not identical to CDV (19,39,57). Cats can be infected but do not develop disease (1).

**Table 6.1.** Species susceptible to different morbilliviruses.[a]

| | Natural infection | | | Experimental infection | |
| | Domestic species | Wild species | | Domestic species | Wild species |
| --- | --- | --- | --- | --- | --- |
| RPV | Cattle Buffalo Pig Goat/Sheep | Buffalo Eland Giraffe Kudu Warthog Wildebeest (Africa) | Banteng Blackbuck Gaur Nilgai Sambhar (Asia) | Cattle Rabbit | |
| PPRV | Goat Sheep | | | Cattle Domestic pig | Deer |
| CDV | Dog | All animals of Canidae (Fox) Mustelidae (Ferret) Procyonidae (Raccoon) | | Dog Mouse Hamster Ferret Pig Cat | |
| PDV | | Seal | | Dog | |
| DMV | | Dolphin | | | |
| PMV | | Porpoise | | | |
| MV | Human Primates | | | Macaque Marmoset Mouse Hamster Rat | |

[a] Data from references 1, 3, 4, 17, 38, 42, 46, 47, and 54.

Since many wildlife species are susceptible to CDV and shed virus during acute infection, worldwide eradication of CDV seems to be virtually impossible.

Different seal species have shown to be a natural host for PDV, and dogs can be infected experimentally (40,57). Whether other domestic or wildlife species are susceptible to PDV infections is yet unknown. Recently morbillivirus infections in porpoises, and different dolphin species have also been documented (14,49,54). These new morbillivirus isolates [dolphin morbillivirus (DMV) and porpoise morbillivirus (PMV)] are serologically different from PDV and CDV (41). Eradication of PDV, DMV, and PMV infections from wildlife animals is unlikely to be feasible for obvious reasons.

Natural MV infections have been described only for humans. Monkeys contract the disease only after capture by humans (28). Since humans are the only natural reservoir for MV, eradication of MV should not be considered impossible.

## C. Antigenic Properties

Only one serotype has been identified of each of the members of the morbilliviruses, although antigenic differences between several isolates of each morbillivirus, that vary in their pathogenicities and/or tissue tropisms, have been observed.

Both the H and the F proteins are the major immunogens for the induction of biologically active antibodies, which may play an important role in prevention of or recovery from infection (32). Virus-neutralizing (VN) antibodies can be induced by both the glycoproteins. The H protein induces hemagglutination inhibiting (HI) antibodies and the F protein in its native form may also induce antibodies, which block the membrane fusion properties of the protein. These types of antibodies can be detected by fusion inhibition (FI) tests or in the case of MV by hemolysis inhibition (HLI) tests. HLI antibodies can further be divided into non-HI antibodies and HI antibodies, which inhibited hemolysis indirectly by blocking the attachment of MV to the erythrocytes. It has been shown that after regular MV infection non-HI HLI antibodies are induced, whereas after vaccination with inactivated MV preparations these non-HI HLI antibodies are not induced. It was suggested by Norrby et al. (34) that non-HI HLI antibodies play an important role in protection against MV infection.

As could be expected from their amino acid homologies, a close antigenic relationship between morbilliviruses has been demonstrated by cross-neutralization and cross-protection studies (2,22). Cross-reactive non-HI HLI antibodies can also be detected by the MV-HLI test in sera of animals, which have been infected with CDV, RPV, or PPRV. Similar to MV, natural infection or vaccination of dogs with live attenuated CDV vaccine resulted in the induction of biologically active antibodies directed against the F protein as was determined in the MV-HLI test, whereas vaccination with formaldehyde-, Tween-ether-, or heat-inactivated CDV vaccine failed to do so (4).

The establishment of large panels of monoclonal antibodies directed against RPV, CDV, PDV, and MV was helpful in demonstrating not only antigenic similarities, but also antigenic differences between the corresponding viral proteins of morbilliviruses. Cross-immunoprecipitation, cross-immunofluorescence and ELISA studies with monoclonal antibodies have shown a high degree of homology between B cell epitopes on the F and N proteins and to a lower degree of homology between the M and P proteins of the morbilliviruses. Pronounced antigenic differences between the H proteins of RPV, CDV, PDV, and MV have been observed (37,56).

Both the high degree of homology between amino acid sequences of the F proteins and the observed homology between B cell epitopes on the

F proteins suggests that the F protein is a major cross-protective protein in heterotypic vaccination among the morbilliviruses (4,11).

## 3. Natural Immunity to Morbillivirus Infections

For all morbilliviruses the induction of lifelong immunity to infection has been observed. As is the case for MV infection in humans, it has been shown for RPV and CDV infections in their respective hosts that VN antibodies directed against these viruses appear between 7 and 20 days after infection. The presence of VN antibodies usually persists for many years and in some cases even for life. For CDV infection in dogs it has been shown that recovery correlates with the early development of vigorous humoral and cell-mediated immune responses (1).

The appearance of cell-mediated immune responses upon RPV infection has only been studied limitedly. These studies concerned infection experiments in rabbits with lapinized strains of RPV (59). Whether cell-mediated immune responses in cattle play a major role in the recovery from RPV infection is unknown but likely. Cell-mediated immune responses as measured in CDV-specific CTL assays appear between 10 to 14 days after infection of dogs (5). In contrast to the humoral immune response, this cell-mediated immune response disappears in time. Initially, it has been shown rather difficult to determine MV-specific cell-mediated immune responses for MV several years after infection occurred. Recently, we have shown that in most individuals with a natural history of measles 20 years after infection MV-specific cell-mediated immune responses can be measured (52). MV-specific $CD4^+$ MHC class II- and $CD8^+$ MHC class I-restricted T cells, which both possess cytotoxic T cell activity, can be detected in individuals after infection (23,26,51,52,53). At least 1 year after recovery, $CD8^+$ cytotoxic T cells are abundantly present, whereas many years after infection $CD4^+$ T cells seem to be the major population of MV-specific T cells, although $CD8^+$ CTL could also still be demonstrated (51,53).

Although immune responses to some of the morbillivirus infections have been analyzed in more detail, still relatively little is known about the individual role of the humoral and cell-mediated immune mechanisms involved in the protection and recovery from infection. Consequently, it is also not fully understood what type of immunity has to be induced to protect against infection. Morbillivirus infections can be prevented effectively by high levels of circulating VN antibodies present at the time of infection: cattle can be protected against RPV infection by passively administered RPV-specific antibodies (46). Dogs and humans can similarly be protected by hyperimmune CDV sera and MV-specific $\gamma$-globulin, respectively. If VN antibody titers are too low to protect against infection, these antibodies may still play a beneficial role in prevention of

disease symptoms and final recovery from disease. Only in the case of RPV it has been reported that RPV antibodies can prevent disease when administered after infection, provided that the treatment is started before the erosive-mucosa phase of the disease (46). Cross-protection has been documented between RPV, PPRV, CDV, PDV, and/or MV after vaccination. However, passive transfer of RPV-specific antibodies to dogs does not protect dogs against CDV infection (46). This suggests that virus-specific cell-mediated immune responses may play an essential role in protection and recovery from infection.

## 4. Immunity Induced by Morbillivirus Vaccines

Since all known morbilliviruses are highly contagious and cause acute disease often with high mortality and morbidity rates in their respective nonimmune hosts, almost immediately after the discovery of the infectious agents of the disease, serious attempts have been made to develop effective vaccines that may control disease. In case of RPV infection in domestic cattle, several outbreaks, already reported in the eighteenth and nineteenth century, have caused enormous losses of animals all over the world (46). For economic reasons it has been very important to control the disease. Another reason for controlling morbillivirus infections is their risk for infections with high mortality in endangered wildlife species. The recent outbreak of PDV in harbor seals in the North and Baltic seas has caused a reduction of 70% of the population (38). Similar observations have been made earlier for CDV infections in raccoons or foxes (29).

For the development of safe and effective morbillivirus vaccines, it is important to define the minimal requirements for these vaccines. To control RPV and PPRV infections in domestic animals, the induction of protective herd immunity by vaccination has to be achieved. In case of CDV and MV infections each individual animal has to be protected against disease. For all morbillivirus vaccines, it is important that VN antibodies and most probably also biologically active HLI antibodies directed against the F protein are induced. These antibody titers should preferably be long-lasting at a protective level. If infection occurs, the presence of cell-mediated immune responses, mediated both by CD4$^+$ and CD8$^+$ T cells, may play an essential role in the outcome of infection and disease.

A phenomenon of morbilliviruses that may influence vaccine efficacy is the presence of high levels of maternal antibodies at the moment of vaccination. Young individuals with maternal antibodies are protected against infection. After several weeks these protective antibodies will disappear and the animals will become susceptible to infection. Preferably, a vaccine should induce protective immunity in the presence of

maternal antibodies. In general the occurrence of side effects shortly or long after vaccination should be avoided. In case of measles, it has been shown that in some of the individuals vaccinated with whole inactivated virus preparations, clinical symptoms after infection were more severe than in individuals who had not been vaccinated (18).

The first vaccines developed against CDV and RPV were based on whole inactivated organ materials. After virus isolation in tissue culture and improvement of the tissue culture systems, the first generation of attenuated vaccines has been developed and directly thereafter chemically or heat-inactivated whole virus preparations have been tested and used as vaccines. Recent developments in biology and technology have created possibilities for the formulation of new generations of vaccines.

## A. Live Attenuated Vaccines

The first demonstration of the possibility to vaccinate against morbillivirus infections came from Koch, who showed in 1899 that cattle can success-fully be immunized against rinderpest by administering bile from infected animals. RPV has been adapted to replication in goats and rabbits and has been attenuated by serial passage in these animals. These attenuated strains originally obtained from infected animal tissues are still used for the RPV vaccine that is most widely used (46). Rinderpest was also adapted to growth in the chorioallantoic membrane of embryonated chicken eggs. Vaccines based on these viruses are still widely used in Africa. The introduction of cell culture techniques in vaccine develop-ment has resulted in new generations of attenuated vaccines. The Kabete strain of RPV has been adapted to bovine kidney cell cultures and after 70 passages this strain only replicates in the lymphoid organs of vaccinated animals without being excreted by these animals. This strain is safe for cattle, goats, and sheep (43). No attenuated PPRV strain is commercially available yet for vaccination purposes. Therefore, sheep and goats have been effectively protected against PPRV infection with cell culture-adapted RPV vaccines (47).

Attenuated CDV vaccines have been developed in a similar way. The first attenuated vaccine was produced by serial passage of CDV in ferrets. This vaccine was not sufficiently attenuated for use in dogs. The adaptation of CDV to replication in eggs and later in cells of avian origin resulted in effective vaccines, which are safe for dogs and also proved to be safe for gray foxes (1,3). The subsequent adaptation of CDV to canine cell culture resulted also in a very effective vaccine (45). However, this vaccine was more virulent for dogs and some wildlife species than the CDV vaccine of avian origin.

The first attenuated measles vaccine (Edmonston B strain) was prepared only a few years after MV had been isolated and adapted to replication in *in vitro* cell culture systems (24). Although the first attenuated vaccines

against measles were quite efficacious, recently developed attenuated vaccine strains were claimed to induce less side reactions at a similar efficacy rate.

A major advantage of using live attenuated morbillivirus vaccines is that the nature of the induced immunity closely resembles that of immunity observed after recovery from natural infection. However, adaptation of the virus may lead to a too high level of attenuation of the vaccine strain, which may both qualitatively and quantitatively result in a suboptimal immune response. This phenomenon has, for example, been documented for CDV vaccines (1). For attenuated RPV, CDV, and MV vaccines it has been shown that biologically active antibody responses directed against both the H and F proteins are induced. VN antibody titers and also non HI-HLI antibody titers persist for long periods after vaccination. Probably due to the parenteral route of vaccination which is generally used, only low levels of virus-specific secretory IgA antibodies are induced as was demonstrated, for example, for RPV and MV vaccines (39).

Whether all essential components of cell-mediated immune responses are induced after vaccination with live attenuated morbillivirus vaccines has not been documented in detail. Since these types of vaccines cause *de novo* synthesis of viral antigens in the cell, it may be expected that apart from virus-specific MHC class II-restricted $CD4^+$ T cells, virus-specific MHC class I-restricted $CD8^+$ cytotoxic T cells are also induced.

Live attenuated CDV, RPV, and MV vaccines have been shown to induce protective immunity, which persists for many years or even life-long. Whether this long-lasting immunity is based on humoral and/or cell-mediated mechanisms and to what extent memory cells establish either of these mechanisms, have not been studied.

Although at present most of the currently used attenuated morbillivirus vaccines are able to control the spread of the infection and disease in their natural hosts, there are still some major disadvantages associated with their use.

A major problem in vaccination programs is interference of maternal antibody with the replication of the vaccine strain in young vaccinees. Levels of maternal antibodies in a young animal are directly related to the levels of antibodies present in the mother and are transferred trans-placentally and/or via the colostrum. Especially in highly infected populations, there is a need for vaccination at the youngest age possible. Since in such populations usually high levels of maternal antibodies occur, the optimal moment of vaccination of young individuals may be hard to determine.

To solve this problem, heterotypic vaccination experiments with MV or RPV attenuated vaccines have been carried out in young dogs to protect them from CDV infection. Although CDV-specific maternal antibodies in the young dogs have been shown not to interfere with the replication of

MV or RPV, dogs can partially be protected against canine distemper, but not against CDV infection by these heterotypic live vaccination procedures (4,33).

Although on theoretical grounds, it may be speculated that the efficacy of these live vaccines may be influenced by intercurrent infections with other viruses, this has so far not been documented. Another disadvantage of using attenuated vaccines is their potential virulence for endangered wild species. CDV vaccines have caused serious problems in c.q. red pandas, black-footed ferrets, and gray foxes (29). Therefore, during the recent outbreak of PDV infection in harbor seals, attenuated CDV vaccines were not chosen for protection of captive animals (40).

Finally the need for maintaining a cold-chain during transport and storage may present a serious problem. Vaccine preparations might lose too much of their infectivity after several hours at room temperature. The molecular basis for the attentuation of the live vaccines obtained by serial passages in animals, eggs, or cells has not been elucidated sofar.


## B.  Live Recombinant Viruses

The application of molecular biological techniques in vaccine development has resulted in new possibilities to construct effective and safe vaccines. Genes encoding for viral proteins, involved in the induction of protective immunity against infection, can be cloned and inserted into the genome of a vector. Vaccinia virus has been shown to be an important candidate vector (27). Vaccinia virus has a relatively broad host-range and a large genome in which foreign genes can be inserted without disturbing the replication properties of the virus. Furthermore, vaccinia virus has a long history as a relatively safe and effective vaccine successfully used for the eradication of small pox. After adsorption and penetration of the recombinant virus into the host cell, the foreign genes are expressed.

In contrast to attenuated tissue culture morbillivirus vaccines, vaccinia virus has been proven to be a very stable vaccine during transport and storage, which does not require refrigeration. Therefore, the use of a morbillivirus recombinant vaccinia virus (rVV) in developing countries may be more successful than the live attenuated morbillivirus vaccines. Apart from these advantages for using vaccinia virus as a vector, the pathogenicity of the virus as such still may constitute a serious risk for humans either directly through vaccination or indirectly through contacts with vaccinated animals (6).

Both MV- and RPV-rVVs have been tested for their efficacy in the induction of protective immunity. To solve the problems associated with the production and distribution of the RPV tissue culture vaccines, RPV-rVV vaccines have been constructed and tested for their efficacy in animal challenge models. Both the H and the F protein of the highly

virulent Kabete strain of RPV have been cloned into the vaccinia virus genome (60). The two recombinant viruses, rRVH and rRVF, express the RPV-H protein or the RPV-F protein in infected cells, respectively. Biochemical and antigenic characterization of the two proteins *in vitro* show no differences between the equivalent proteins produced in RPV-infected cells. Immunization experiments in cows have shown that after one vaccination with either rRVH or rRVF, RPV-specific VN antibodies are induced. However, VN antibody titers induced by rRVH alone or rRVH and rRVF together are almost 10-fold higher than those induced by rRVF alone. Similar observations have been made with rVVs that express the F protein of the RPV Plowright vaccine strain or the H protein of the lapinized strain after vaccination of cows or rabbits (7,8,50). Whether RPV-specific cell-mediated immune responses were induced after vaccination was not investigated in any of these studies. Upon challenge with virulent RPV all animals vaccinated with the recombinant viruses rRVH and rRVF or with the Plowright tissue culture vaccine as a control were completely protected against disease, whereas all animals in the nonvaccinated control groups died from massive hemorrhagic diarrhea at day 6 after challenge (60).

Animals vaccinated with rRVF alone survived, but did show clinical symptoms of RPV infection upon challenge with either the lapinized RPV strain for rabbits or a virulent strain for cows.

The induction of high levels of VN antibodies seems to occur more efficiently with rRVH than with rRVF. It is known that high levels of VN antibodies are sufficient to prevent animals or humans from infection. However, it is not yet known how long H protein-specific VN antibody titers will persist after vaccination. Consequently it is unknown what will happen after infection when protective immunity mediated by VN antibodies has disappeared. Although rRVF induce low levels of VN antibodies, animals still become infected after challenge with virulent virus. However, none of the animals develops clinical signs of rinderpest.

It has been shown that MV recombinant vaccinia viruses, which express either the F or the H protein, induce both VN antibodies and in the case of MV-F vaccinia virus also HLI antibodies. Protection induced by rVV-MV-F against a lethal challenge with MV has been demonstrated in mice (15).

In conclusion both the H and the F proteins play an important role in the induction of immunity to prevent infection by inducing VN antibodies and perhaps also to recover after infection.

In general a combination of both the F and H proteins of morbilliviruses expressed by rVVs might be the most promising vaccine candidate. Before the introduction of such a candidate in vaccination campaigns, there are still several questions that have to be answered. Is it possible to vaccinate young cows in the presence of maternal antibodies with recombinant vaccinia viruses and how long will protective immunity persist after

vaccination? Also the discussion of whether vaccinia virus is acceptable for eradication programs in view of public health risks and risks for wild species has not yet been finished. Whether individuals with a history of vaccinia vaccination can be effectively revaccinated is another issue that needs further investigation.

To overcome the problems associated with the use of vaccinia virus as a live vector, other viral vectors such as non-replicating avipox viruses, for example fowl pox and canary pox viruses, or adenoviruses and herpes viruses and bacterial vectors such as BCG and *Salmonella* are currently being investigated for their potential as live recombinant vaccines.

## C. Inactivated Whole Virus Vaccines

For RPV, CDV, and MV early attempts have been made to develop inactivated vaccines. In 1926 Laidlaw and Dunkin prepared a CDV vaccine by treatment of CDV derived from spleens of infected dogs with formaldehyde (25). Similarly, tissues of RPV-infected animals were treated with formaldehyde or chloroform.

It was shown that these types of vaccines induced only partial immunity for a short period of time after several doses. Also, when tissue culture systems were introduced to produce large amounts of virus, inactivation of purified, whole virus preparations by heat, formaldehyde, or Tween-Ether treatment did not result in vaccine preparations that induced long-lasting protective immunity. Illness following exposure to live MV often proved to be more severe in individuals vaccinated with inactivated vaccines than in nonvaccinated individuals (18). Norrby et al. (34) suggested that the lack of protection against MV infection was due to the absence of a F protein-specific biologically active antibody response after immunization. For whole inactivated CDV preparations similar observations have been made: in contrast to live attenuated vaccines after immunization with inactivated CDV, no biologically active non-HI-HLI antibodies could be observed (4). Another phenomenon observed after immunization with inactivated whole morbillivirus vaccines was the rapid decline of antibody titers directly after vaccination. Whether virus-specific cell-mediated immune responses were induced after vaccination has not been documented. Based on experience in other systems that indicate that proteins exogenously administered to antigen-presenting cells do not induce $CD8^+$ T cells, it can be speculated that these types of vaccines also have failed to induce virus-specific $CD8^+$ T cells. In general inactivated virus preparations may be expected to induce only virus-specific $CD4^+$ T cells.

## D. Viral Subunits

Since whole inactivated virus preparations have not been proven to be successful as safe and effective morbillivirus vaccines, the understanding of the role of the individual proteins of the virus and the way in which

they induce protective immunity is of major importance. Immunization experiments in dogs with either immunoaffinity chromatography purified CDV-F or CDV-H protein have shown that the F protein administered in Freund's complete adjuvant confers protection against distemper after challenge with virulent CDV, although animals were not protected from infection (36). Dogs immunized with the purified H protein of CDV did show clinical symptoms, but survived a challenge with virulent CDV. It has been suggested that the form of antigen presentation of the F protein may be very important for the development of a completely protective subunit morbillivirus vaccine (10,11).

In general it is known, that purified monomers of proteins are less immunogenic, and in certain cases even immunosuppressive, than multi-meric forms of the same proteins (30). Also the choice of adjuvant systems strongly influences the immune response induced by purified protein preparations. Iscom, a novel antigenic presentation form for membrane proteins, has been shown to be very effective in inducing high levels of biologically active antibodies, cell-mediated immunity, and protection in several viral systems (20,31). Since purified glycoproteins of human immunodeficiency virus and influenza A incorporated into iscom have shown to induce virus-specific CD8$^+$ T cells, iscom may also be a promising antigenic presentation form for the development of inactivated morbillivirus vaccines (48).

We started to study the immunogenic properties of whole MV iscoms, which contain apart from the F protein minor amounts of the H protein. MV iscoms were shown to induce MV-specific VN, HI, and non-HI-HLI antibodies in rats, whereas the whole inactivated MV preparation did induce VN and HI antibodies but no non-HI-HLI antibodies (31). To study the role of the MV-F protein in the induction of protective immunity in more detail we and others have subsequently incorporated immuno-affinity chromatography purified F protein into iscoms (MV-F iscoms) (10,55). It has been shown that these MV-F iscoms induce high levels of HLI antibodies in monkeys, mice, and rabbits but no MV-VN or MV-HI antibodies. We know now that after two immunizations with MV-F iscoms MV-HLI antibody titers persist for more than 3 years after the last immunization. The induction of MV-specific T cells after immunization with both MV-F iscoms and MV iscoms has been demonstrated by the measurement of a MV-specific DTH response in mice. MV-specific murine T cell clones with a CD4$^+$ phenotype have also been isolated from MV-F iscom or MV iscom immunized mice. Both preparations did induce pro-tection against intracerebral challenge with a lethal dose of a rodent adapted strain of MV (Table 6.2). Recently, it has been shown that MV-specific human T cell clones with a CD8$^+$ phenotype were able to recognize MV-F iscom presented in context of MHC class I molecules (61).

Immunization experiments have also been carried out in dogs with CDV iscoms, which have been prepared quite similarly to MV iscoms

**Table 6.2.** Protection induced by morbillivirus iscom.[a]

| Immunogen | Species | Challenge virus | Infection route | Protection |
|---|---|---|---|---|
| MV iscom | Mouse | MV | Intracerebral | +; 100% survival of infected mice |
|  | Dog | CDV | Intranasal | ±; less severe clinical signs, PBMC infected |
| MV-F iscom | Mouse | MV | Intracerebral | ±; 80% survival of infected mice |
|  | Dog | CDV | Intranasal | ±; less severe clinical signs, PBMC infected |
| CDV iscom | Dog | CDV | Intranasal | +; no clincal signs, no virus isolated from PBMC |
|  | Seal | PDV | Oculonasal and peritoneal | +; no clincal signs, no virus isolated |

[a] Data from references 10, 11, 31, 55, and 57.

(11). It has been shown that dogs immunized with CDV iscoms developed biologically active CDV-VN and MV-HLI antibodies but, in contrast to nonimmunized dogs, did not develop viremia or clinical signs of infection upon intranasal challenge with the virulent Snyder Hill strain of CDV (Table 6.2). Immunization of dogs with MV iscoms or MV-F iscoms resulted only in partial protection against CDV infection, confirming that this cross-protection was at least partly caused by epitopes shared by the F proteins of CDV and MV.

During the recent outbreak of PDV infection in seals, preventive vaccination of particular seal populations has been carried out with CDV iscoms (56). The absence of large-scale production systems for PDV, the close antigenic relationship with CDV, and the danger of insufficient attenuation of live CDV vaccines have been considerations to use an inactivated CDV iscom preparation. All seals admitted to a seal sanctuary in The Netherlands, which were seronegative in a CDV-VN assay, were vaccinated with CDV iscoms. All vaccinated animals developed CDV-VN antibodies and seemed to be protected since the survival rate increased from less than 10% to more than 90% within 2 months, whereas it remained under 30% in a nonvaccinated control group.

A protection experiment with PDV challenge was carried out in a seronegative, isolated group of harbor seals (57). All animals vaccinated with CDV iscom or with a candidate inactivated whole CDV vaccine (Duphar BV, Weesp, The Netherlands) developed high CDV-VN antibody titers. Upon challenge all vaccinated animals were shown to be protected, whereas two control animals developed severe clinical signs and died at 16 and 18 days, respectively, after infection (Table 6.2).

To mimic the *in vivo* situation of the presence of maternal antibody, we passively transferred homologous MV-specific antibodies to mice or monkeys. MV iscoms have been shown to effectively induce both

humoral and cell-mediated immune responses in the presence of these antibodies (12).

## 5. Concluding Remarks

All members of the genus *Morbillivirus* are highly contagious for their natural hosts and may cause serious acute diseases. High mortality rates are usually observed when the virus is introduced in a nonimmune population. The introduction of live vaccines has proven to be a generally effective approach to prevent morbillivirus infections. In industrialized countries there are only minor problems associated with the use of live attentuated vaccines. In developing countries, however, these problems are more severe. Novel approaches for vaccine strategies are now under investigation to construct stable, safe, and efficacious vaccines. It has been shown that with rVV expressing the glycoproteins of RPV or MV, protective immunity can be induced. Whether the induced protective immunity is long-lasting and can also be induced in the presence of maternal antibody is not clear at present. The safety aspects associated with these types of vaccines for both humans and animals are still a major point of concern. Other viral vectors such as avipox viruses, adenoviruses or herpes viruses or even bacterial vectors, which can be used for expressing the morbillivirus glycoproteins, may overcome some of the problems associated with the use of rVVs.

In general, inactivated vaccines are safer and should be preferred over live vaccines. However, whole inactivated morbilliviruses have proven to be insufficiently effective and occasionally even harmful. Both the quality and the quantity of the induced immune responses were poor. Novel approaches for presenting viral proteins to the immune system have been developed during the last decade. Studies concerning the potential of the iscom matrix for the construction of morbillivirus subunit vaccines have indicated that nonlive vaccines based on this form of presentation should be considered promising. Both glycoproteins incorporated into iscoms are able to induce virus-specific biologically active antibodies and virus-specific $CD4^+$ and $CD8^+$ T cells, which persist for a long period. In the presence of passively transferred homologous antibodies, MV iscoms were able to induce both humoral and cell-mediated immune responses. Protection experiments in several systems have shown that morbillivirus iscoms should be considered as serious subunit vaccine candidates.

In conclusion, we expect that the novel developments in the field of morbillivirus vaccines may result in a new generation of these vaccines in the near future.

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CHAPTER 7

# Control of Viral Diseases of Sheep and Goats: Conventional and Novel Vaccines*

E. Paul J. Gibbs

## 1. Introduction

It is axiomatic that vaccination remains the most effective approach to minimizing the economic losses associated with disease and controlling the spread of pathogens. The world population of sheep is estimated to be 1176 million and that of goats 526 million (for comparison the cattle population is 1281 million) (11). One would think that these populations are sufficiently large to sustain major research and development programs on vaccination of sheep and goats against viral diseases, but surprisingly, the literature on the development of viral vaccines for the two species is relatively small. Why is it that development of viral vaccines for sheep and goats receives comparatively little attention?

There is no simple answer to this question; several factors contribute to this situation and partly reflect the disparity of technical and financial resources between the industrialized and developing countries.

1. In general, the husbandry of sheep and goats is still traditional in both the industrialized and developing countries. Compared with cattle, swine, and poultry, sheep and goats are rarely maintained in feedlots or housed for long periods at a time. Consequently, sheep and goats are less subject to the respiratory and enteric problems associated with the intensive management of cattle, swine, and poultry. Thus, there are fewer disease problems and, as a corollary, less demand for vaccines. The market is seen as a poor return on investment by vaccine companies.
2. The control, and in many cases eradication, of "epidemic diseases," such as foot-and-mouth disease (FMD), in industrialized nations has been immensely successful. For example, FMD vaccination has recently been discontinued in the European Community. The focus of these programs has usually been on cattle, but sheep and goats, which are

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*This review of the literature was completed in March 1991.

often susceptible to the same diseases although less severely affected, have been indirect beneficiaries without the specific need for specialized vaccines. If the need arises for additional vaccines for use in sheep and goats in the industrialized nations, most governments and vaccine companies believe that the existing technology for cattle vaccines can be rapidly adopted.

3. Commercial vaccine manufacturers in the 1980s have often gone through many mergers to become multinational. They have invested heavily in new technology for vaccine development, but mainly within the industrialized nations. With the possible exception of FMD vaccines for cattle, they see little financial incentive for developing viral vaccines for the third world, especially for the poorer countries where, ironically, the major virus diseases of sheep and goats still occur.

4. Independence, since the 1960s, for many of the developing nations of the world, particularly those in Africa, has often led to an impoverished national veterinary service. Epidemic diseases, several of which are important to the sheep and goat industries, are often poorly controlled, principally, because technical resources and foreign currency are not available either to develop or purchase vaccines. The problem has been exacerbated further by the political philosophy of many of the industrialized countries of the world in the 1980s to reduce government spending substantially; this has resulted in research institutions focusing on the major animal diseases of national importance. Involvement in international research centers has been similarly reduced impairing their effectiveness to address problems in the developing world.

The above analysis is simplistic; examples to the contrary can be cited where close collaboration exists between industrialized and developing countries on vaccine development. This is particularly true when a viral disease, occurring in a developing country, is considered to have the potential to spread to the industrialized nations and cause a major epidemic. Diseases transmitted by arthropods, especially those that also cause disease in humans, e.g., Rift Valley fever, feature prominently in such cooperative "strategic" research and development.

The following section on conventional and new approaches to vaccination against viral diseases of sheep and goats should be viewed within the context of the above remarks. General reviews of viral diseases of sheep and goat are available (21).

The review is structured by examining available and prospective vaccines within the various virus families. The order in which the families are discussed follows that used in *Veterinary Virology* (12). The nomenclature for the different types of vaccines discussed in this review is summarized in Table 7.1.

**Table 7.1.** Categories of viral vaccines in production and development.[a]

In the market place

1. *Homogenates of infected tissues or unmodified live*: a crude type of vaccine that represents a controlled infection with virulent virus. Often given to an atypical host to induce cross-immunity to a related pathogen (Jennerian vaccination) or at a time in the production cycle when the infection causes least concern, e.g., orf. It is questionable whether some of these products can truly be called vaccines. Their use is limited, largely historical and sometimes unregulated.

2. *Inactivated vaccines*: whole virus, now usually grown in cell culture or embryonated eggs, inactivated with one of several inactivants, and often administered with an adjuvant, e.g., foot-and-mouth disease vaccines.

3. *Attenuated or modified live vaccines*: historically, attenuation was empirical and produced through repeated passage of the virus through atypical animal hosts or cell cultures; knowledge of gene expression has revealed that many of these attenuated vaccines have specific gene deletions. For viral diseases caused by DNA viruses, a new generation of attenuated vaccines in which specific genes have been excised from the DNA is now available. Using recombinant DNA technology, marker genes have been introduced into some of these gene-deleted vaccines to differentiate them from naturally occurring virus strains, e.g., pseudorabies vaccines in swine.

Predominantly in development/field evaluation

4. *Vectored vaccines*: an attenuated virus or bacterium, the DNA of which has gene inserts coding for the antigenic proteins of unrelated pathogens. When the vaccine replicates/multiplies in the host, the gene products induce immunity to their respective pathogens.

5. *Subunit or biosynthetic vaccines*: based on specific viral proteins originally isolated through purification procedures of viruses usually grown in cell cultures; now more commonly produced through recombinant DNA technology using *E. coli* and other expression systems. The vaccine contains only antigenic components that elicit a protective response.

6. *Reassortant and chimeric vaccines*: these are hybrid "live" viruses in which the chimera or reassortant is nonpathogenic, but contains those genes of the pathogen that code for antigenic proteins.

7. *Antiidiotype antibody vaccines*: relies upon the principle of antiantibodies mimicking antigen. A monoclonal antibody specific for an antigen associated with the pathogen is injected into an animal to induce an antiantibody or antiidiotype; when the antiidiotype is then injected as a vaccine, it induces antibody that cross-reacts with the original viral antigen.

8. *Chemically synthesized vaccines*: peptides produced synthetically in the laboratory from amino acids. The selection of the appropriate peptide structure from within the antigenic proteins of the virus is based on sequencing data, X-ray crystallography, and recognition by the immune system of the host.

[a] Modified from ref. 13.

## 2.  Viral Diseases of Sheep and Goats

### A.  Diseases Caused by DNA Viruses

#### i.  Papovaviridae

Papilloma viruses have been associated with benign skin growths in sheep and goats (15) and with precancerous lesions of the ears and genitalia of sheep in countries, such as Australia, where they are exposed to high levels of ultraviolet light (41). The genotype of at least one of the viruses associated with cutaneous disease in Australia is similar to bovine papilloma virus type 2. No commercial vaccines are available.

#### ii.  Adenoviridae

Adenoviruses have been isolated from outbreaks of respiratory disease and gastroenteritis in sheep and goats, but they have also been isolated from clinically normal animals (4). The viruses are generally distinct from those affecting cattle, but infection with bovine serotype 2 has been recorded; there are at least 6 ovine serotypes and 2 caprine serotypes (4,16). On farms where thousands of lambs are housed, and not at pasture, it is difficult to prevent adenovirus infections. While most infections are subclinical, infection is considered to predispose animals to infection with *Pasteurella hemolytica*. Accordingly, inactivated vaccines have been developed. These are commercially available in Hungary where losses associated with pneumoenteritis are reported to be markedly reduced once regular vaccination has been established within a flock.

   Although ovine and caprine adenoviruses can theoretically be used as viral vectors for vaccination against respiratory and enteric infections (and undoubtedly are in development), to my knowledge, there is none that has reached the point of field trials.

#### iii.  Herpesviridae

With the exception of sheep and goats, there is at least one major disease, in each of the major domestic species, that is caused by a herpes virus. Herpes viruses have been isolated from sheep and goats [caprine herpes viruses (CAV) 1 and 2]. In sheep, the isolates (CAV1) have been obtained from cases of pulmonary adenomatosis. The first isolates were obtained before the retrovirus etiology of pulmonary adenomatosis was determined. While not the cause of pulmonary adenomatosis it has been suggested that the two viruses may act synergistically to produce clinical disease. In goats, herpes viruses (CAV2) have been isolated from kids with generalized infections, but also from older goats affected with a wide range of clinical disease including pneumonia, genital lesions, and skin disease. While serological surveys indicate that herpes infections of sheep

and goats are of worldwide distribution, the incidence of clinical disease is not sufficient to justify vaccine development. As with adenoviruses, the herpesviruses of sheep and goats may, at some future time, be used as vaccine vectors. For many years sheep have been thought to be the reservoir of the causal agent of the European form of malignant catarrhal fever (MCF) of cattle. Recently, a third member of the caprine herpes virus has been isolated from sheep and is believed to be the causal agent of MCF (31). MCF is an interesting disease, but there is little economic justification for vaccine development in industrialized countries.

### iv. Poxviridae

In contrast with the herpes viruses, the pox viruses that affect sheep and goats are of great economic importance. The capripoxviruses of sheep and goat pox can cause high mortality; the parapoxvirus of orf (also known as contagious pustular dermatitis, contagious ecthyma, and scabby mouth), while rarely causing death, can be debilitating to young animals and a cause of considerable economic loss. Conventional vaccines are available for their control and recombinant vaccines are in development.

### a. Capripoxviruses

*Sheep and Goat Pox.* Because some outbreaks occur in which disease is seen only in one species, the two diseases are sometimes considered to be caused by two separate viruses. While host-specific strains occur, vaccines produced using either virus provide cross-protection; indeed sheep and goat pox viruses can even be used to protect cattle from the related virus of lumpy skin disease. Analyses of the DNA genomes of representative capripoxviruses reveal that the African sheep and cattle isolates are more closely related to each other than sheep pox virus is to goat pox virus (19). For practical purposes (i.e., in this context, vaccine development) goat and sheep pox viruses can be considered to be caused by the same virus. Although once found widely in Europe, the virus has been progressively eradicated, such that it is now restricted to Africa, the Middle East, and the Indian subcontinent (8,38). All ages of sheep and goats can be affected, but disease is most severe in young animals. While pustules of the skin are the most obvious clinical lesion, the disease is commonly generalized and lesions may be present in the lungs and abdominal organs. High mortality is associated with the generalized disease.

　　Historically, many different types of "vaccine" have been used but have not been critically evaluated. Attenuated vaccines (20) and formalin-inactivated virus previously adsorbed to aluminum hydroxide gel (9) have been described. Since attenuated vaccines are available for controlling lumpyskin disease and sheep pox and they are widely used in endemic areas, research at the Pirbright Laboratories of the Institute of Animal

Health in the UK has also focused on the use of these viruses as vaccine vectors (6). Research is currently in progress on the feasibility of a capripox vectored vaccine for peste des petits ruminants (see below).

b.  Parapoxviruses

*Orf.* This virus can spread rapidly within susceptible populations of sheep and goats, especially in lambs and kids. Lesions are usually present around the mouth affecting the lips and gums. In milking animals lesions may occur on the teats. Orf can prevent young lambs and kids from suckling, leading, in some cases, to death. In common with the bovine parapoxviruses of pseudocowpox and bovine papular stomatitis, animals that have recovered from orf may become reinfected in subsequent years. To minimize the economic losses associated with orf affecting animals during lambing and kidding seasons, the entire flock/herd can be "vaccinated" with unmodified live virus several weeks before the season begins. In reality, this constitutes a controlled infection rather than vaccination. The "vaccine" is commercially available in some countries as a suspension of virus in glycerol and is applied to the axilla or inguinal skin. Recent work has shown that some strains of orf virus when grown in cell culture can be attenuated and successfully used to protect sheep (29).

   Current research on the genome of orf virus may produce both a gene deleted modified life vaccine and an orf virus vectored vaccine for use in sheep (23).

## B.  Diseases Caused by RNA Viruses

### i.  Picornaviridae

Foot-and-mouth disease (FMD) virus (genus: *Aphthovirus*) is the only picornavirus that is known to cause clinical disease in sheep and goats. In contrast with the disease in cattle, FMD in sheep and goats is usually mild and characterized by foot lesions accompanied by lameness. The disease is no longer present in most of the industrialized nations where, in general, vaccination has now been discontinued. In view of the high infectivity of FMD virus, however, the risk of this disease being reintroduced into the disease-free countries is a constant threat. The economic importance of this disease to animal agriculture maintains a worldwide focus. One reflection of this can be seen by reviewing the veterinary literature on vaccine research and development; it is dominated by papers on FMD. A review of the field has been published recently (3). Probably without exception, all vaccines in current use are inactivated products. In 1987, before cessation of vaccination in the European Community, the FMD vaccine market was estimated to be $180 million—equivalent to a quarter of the total world market for veterinary biologicals, and bigger than the market for vaccines against any single human disease. Not

surprisingly, research on FMD vaccines is at the "cutting edge" of molecular biology and there are several promising discoveries, but currently there are no gene-deleted modified live, recombinant vectored, or synthetic peptide vaccines reported to be in field trials. Largely because of the mild nature of clinical FMD in sheep and goats and the cost of biannual vaccination with the inactivated vaccines, there is little incentive to vaccinate them, except when ring vaccination is necessary to eradicate disease from cattle and pigs. Sheep and goats generally respond well to multivalent FMD inactivated vaccines developed for cattle (28); thus, in contrast with swine, it has not been necessary to develop different adjuvants.

## ii. Togaviridae

Of the many viruses in the togavirus family, only the virus causing border disease of sheep is relevant to this review (1). This disease, which was first recognized in the border area of Wales and England, is characterized by newborn lambs being born with excessive coat hairiness, congenital deformities, and neurological deficits. The alternative name "hairy shaker disease" is an apt description. Infection of the lamb occurs *in utero*; infection in the ewe is subclinical. The virus is closely related to that of bovine virus diarrhea (BVD) and is antigenically related to hog cholera virus. The three viruses share a common host spectrum and interspecies transmissions occur frequently (24). Although modified live and inactivated BVD vaccines are commonly used in cattle, the incidence of border disease is not sufficiently high to justify vaccination of sheep.

## iii. Flaviviridae

*Louping Ill.* In recent years louping ill has been recognized to have a wider distribution than simply Scotland; the disease—an acute meningoencephalitis with a name acquired from the Scottish vernacular "loup" meaning to leap—has now been diagnosed in several geographically separate countries such as Norway, Spain, Bulgaria and Turkey (30). The virus has a wide vertebrate host range, but principally affects sheep; it is transmitted between sheep by the hard tick, *Ixodes ricinus*. Louping ill virus was first isolated as early as 1931 and a vaccine consisting of formalinized infected sheep brain was soon developed for use in Scotland. In 1937, there was a disasterous outbreak of scrapie in vaccinated sheep the origin of which was traced to contaminated vaccine. When, in a later incident, 3 laboratory workers became seriously ill with louping-ill infections, production of this vaccine was terminated.

The current vaccine is a formalin-inactivated product developed by growing the virus in BHK cell culture, concentrating the yield by ultrafiltration and then suspending it in an oil adjuvant. Systematic use of this vaccine can eradicate the virus from endemic areas of tick activity.

*Wesselbron Disease.* Like all flaviviruses, Wesselbron virus is principally transmitted by an arthropod (*Aedes* spp.), but contact and aerosol transmission are reported. Although the virus infects a wide range of species, disease is almost exclusively seen in sheep. It is characterized by fever, abortion, neonatal death, and a subsequent low incidence of congenital abnormalities in lambs.

The virus infects livestock in many countries of sub-Saharan Africa. In the Republic of South Africa, an attenuated vaccine is prepared from growing the virus in Vero cells (2). This vaccine may itself cause abortion in pregnant sheep.

### iv. Paramyxoviridae

The paramyxovirus family has three genera, the paramyxoviruses, the morbilliviruses, and the pneumoviruses. In each genus, there are important pathogens that infect sheep and goats.

### a. Paramyxoviruses

*Parainfluenza 3 (PI3) Infections.* PI3 virus was first isolated in the 1950s in the United States from cattle showing clinical signs of shipping fever. Infections with this virus have since been shown to be ubiquitous in cattle and many other ruminant species. It is generally assumed that cross-infection occurs between sheep and cattle. As with cattle, PI3 virus is seldom the only pathogen associated with an outbreak of respiratory disease in sheep; experimental studies have demonstrated that the virus usually causes a subclinical infection or mild respiratory disease characterized by coughing, fever, and nasal discharge. When PI3 virus is isolated from outbreaks of severe respiratory disease, *Pasteurella hemolytica* is invariably involved (38). Inactivated and attenuated PI3 vaccines are widely available and extensively used in combination with other vaccines to protect cattle against shipping fever. Attenuated vaccines developed for use in cattle have been administered intranasally to reduce successfully the incidence of respiratory disease in sheep at lambing time (32).

### b. Morbilliviruses

*Rinderpest and Peste des Petits Ruminants.* There are several historical reports of outbreaks of rinderpest in sheep and goats in Africa, Asia, and Europe, but most recently they are reported only from India. The early vaccines for protecting cattle against rinderpest were developed by passage of the virus in goats, thus it has been suggested that some outbreaks were attributable to the early vaccine strains (35). In the early 1940s, a hitherto unreported disease, resembling rinderpest, was seen in sheep and goats in the Ivory Coast in West Africa and was subsequently considered to be a strain of rinderpest adapted to, and pathogenic for, sheep and goats, but avirulent for cattle (34). This disease, which was named peste des petits

ruminants (PPR), was later shown to be caused by a distinct virus (17). Specific molecular diagnostic techniques were then developed for PPR that, when applied, revealed that the virus had a much wider geographic distribution than previously suspected (36). The disease is now recognized throughout the Sahelian countries and in the Middle East. The recent confirmation of PPR in India might clarify whether the previous reports of rinderpest were more likely to have been PPR (37).

Although the tissue culture attenuated rinderpest vaccine developed by Plowright has been successfully used in the field to protect sheep and goats from clinical PPR (5), this vaccine neither prevents infection nor completely suppresses virus excretion (17). An attenuated vaccine for PPR by passage of the virus through Vero cells has been reported (10). Since sheep and goat pox occur in the countries where PPR is also a problem, a recombinant vaccine against PPR using a capripox vector is attracting attention (6). The success of the vaccinia recombinant, expressing the HA or F gene of rinderpest virus, in protecting cattle against rinderpest is added incentive to develop such a vaccine (44).

### c. Pneumoviruses

*Ovine and Caprine Respiratory Syncytial Virus (RSV) Infections.* In contrast with cattle, the role of RSV in causing respiratory disease in small ruminants is far from clear (43). At present, there is no convincing evidence that it causes clinical disease. Antibody to the virus is widely found in sheep and an isolate distinct from bovine and human isolates of RSV has been reported from a goat. Vaccination against RSV is widely practiced in the cattle industry; thus, if RSV infections of small ruminants emerge as a clinical problem, it is probably safe to conclude that an effective vaccine could be quickly developed.

### v. Bunyaviridae

There are several viruses within this family of arthropod-borne viruses that are of significant economic and public health significance. As with many arthropod-borne viruses, the range of species infected is wide; sheep and goats feature prominently in the epidemiology of several of the viruses. The Bunyaviridae family is large; it is divided into several genera of which 3 contain viruses of particular importance for the sheep and goat industries of the world.

### a. Phleboviruses

*Rift Valley Fever.* This disease was first described in the Rift Valley in 1931, but it is known to infect animals throughout sub-Saharan Africa (22). An extensive epidemic of RVF occurred in Egypt in 1977 and in West Africa in 1987. The concern is that in the future the virus may spread beyond its "ancestral home" in Africa to the Middle East and

other countries. Experimental studies have shown that many species of mosquitoes in both the old and new worlds are potential biological vectors of the virus. Since humans are also susceptible to RVF and develop a viremia, the possibility of this virus speading to livestock in countries outside Africa, through infected travelers, cannot be ignored.

The acute form of the disease in young sheep is characterized by fever, inappetance, vomiting, and hemorrhagic diarrhea with mortality in up to 30% of those affected. In young lambs, a peracute form of RVF may occur in which listlessness, inappetance, and weakness may be the only signs preceding death. The mortality may be 100% in lambs less than 7 days old. In adult sheep the infection may be subclinical, but if the infected animal is pregnant the virus can cross the placenta and induce abortion. The incidence of abortion can be as high as 90–100%. The disease is similar in goats. Cattle are also susceptible to infection. The virus replicates to high titer in the liver causing necrotic foci, but is present in most tissues. Slaughtering animals for food can generate aerosols of the virus which are infective for humans in the immediate vicinity. In humans, the disease is usually seen as a severe febrile illness characterized by myalgia, headache, and retroorbital pain. Recovery in a week is normal, but in a few individuals jaundice, hematemesis, melana, and skin hemorrhage and even meningoencephalitis can develop. Some patients with the hemorrhagic or encephalitic form of the disease die.

In view of (a) the epidemic potential of this virus to spread beyond Africa and to infect not only domestic ruminants, but also humans, and (b) the potential use of this virus for biological warfare, the development of vaccines has been a priority of several veterinary laboratories around the world and also the Department of Defense in the United States. In Africa, an attenuated live virus vaccine is in use (Smithburn strain) and is highly effective in providing protection. Although approximately 50% of animals develop viremia, arthropod transmission to susceptible animals from vaccinates has not been proven. Of much greater concern is the undesirable feature of this vaccine to induce abortion. To overcome the abortigenic feature of the Smithburn vaccine, formalin-inactivated vaccines are available; two doses of the vaccine are required to provide immunity and annual booster doses are recommended.

It now seems likely that the disadvantages of the existing vaccines will soon be overcome through research in the United States. A mutagen attenuated live virus vaccine has recently been shown to be immunogenic and nonabortigenic when inoculated into pregnant ewes; further, the vaccine does not induce a high titer viremia (25).

## b. Bunyaviruses

*Akabane Disease.* There are several viruses, related to Akabane virus, that have been associated with epidemics of abnormal deliveries of calves

and lambs in Japan, Australia, Israel, and Turkey (18). The disease is characterized by abortions, stillbirths, premature births, and congenital arthrogryposis-hydranencephaly. While the term "Akabane disease" is technically inaccurate, the simplicity of this term finds favor over "congenital arthrogryposis-hydranencephaly syndrome." The disease is seasonal and is related to prior infection of pregnant cattle or sheep. The infection is acquired by the bite of an infected mosquito or *Culicoides*. Infection of animals other than as a developing fetus produces a sub-clinical infection. Viruses capable of causing similar disease are Aino virus in Australia and Cache Valley in the United States (7). In Japan, a formalin-inactivated, aluminum phosphate gel-adsorbed vaccine is licensed for use; in Australia a $\beta$-propiolactone-inactivated vaccine has been developed for use in cattle. A modified live virus vaccine, that is effective and safe in cattle, has been developed in Japan, but this vaccine should not be used in sheep since it can cause intrauterine infection of the fetus. In general, epidemics of "Akabane disease" do not occur sufficiently frequently in most parts of the world to justify a regular vaccination program.

c. Nairovirus

*Nairobi Sheep Disease.* This disease, which despite its name can be seen in both sheep and goats, is characterized by a febrile response followed by profuse diarrhea. The diarrhea may contain unchanged blood and affected animals may die 2–5 days after onset of signs. Pregnant ewes may abort. Goats are usually less severely affected. The causal virus of Nairobi sheep disease is closely related to Ganjam virus in India and Dugbe virus in West Africa and, in common with these viruses, is transmitted by hard ticks. The disease has acquired its name because sheep moved from areas of Kenya, where *Rhipicephalus appendiculatus* is absent, to the areas around Nairobi, where the tick is present, commonly develop the disease. Attenuated live virus vaccines and an inactivated oil-adjuvanted vaccine have been used on an experimental basis (40).

**vi. Retroviridae**

Currently there are 3 retroviruses that cause slow infections in sheep and goats. All are of economic importance and of interest to comparative pathologists. These are Maedi-Visna virus (or ovine progressive pneumonia virus), caprine arthritis encephalitis virus, and jaagsiekte virus (or ovine pulmonary adenomatosis virus). Maedi-visna and caprine encephalitis viruses are both classified as lentiviruses, while the classification of jaagsiekte virus is currently undetermined. Since maedi-visna and caprine arthritis encephalitis virus are classified in the same subfamily as the human immunodeficiency virus, there is keen interest in the development of effective vaccines against them. None is currently forthcoming.

Excellent reviews on the individual viruses in this family are available (26,27,42).

### vii. Reoviridae

There are 3 genera within this family, the orthoreoviruses, the orbiviruses, and the rotaviruses. Both the orbiviruses and rotaviruses contain pathogens of importance to the sheep industry.

### a. Orbiviruses

*Bluetongue.* Bluetongue virus (BTV) is an arbovirus that infects ruminants in many countries of the tropics and subtropics, and some, such as the United States, in the temperate zones. Currently, there are 24 known serotypes of the virus. The disease is characterized by congestion of the buccal and nasal mucosa and the coronary band of the hooves, stiffness due to muscle degeneration, and edema of the head and neck. Congenital abnormalities may occur in the fetuses of animals infected during pregnancy. Probably all ruminant species are susceptible to infection with BTV, but clinical disease is usually more severe in sheep. Humans are not susceptible to infection. Extensive epidemics of BT have occurred in which thousands of sheep have died, thus the disease is of particular concern to countries in Western Europe and Australasia that have large sheep industries to protect. Once BTV has been present in an area for several years, complete eradication seems impossible. In areas such as South Africa, Israel, and California, this means living with the disease while attempting to minimize losses. Since, as mentioned above, cattle and goats are commonly infected with the virus but rarely develop clinical disease, the use of vaccine has been to protect the sheep industry. Vaccination using attenuated vaccines against BT are widely used in South Africa. Attenuated vaccines can cause abortion, be transmitted by insect vectors, and may, when used as multivalent vaccines, generate recombinants (14). Unfortunately, despite research for several years on the development of inactivated vaccines, none is commercially available. $\beta$-Propriolactone and binary ethylenimine have shown promise as effective inactivants, but vaccines prepared using these inactivants have not progressed to field trials. Studies are currently underway on the insertion of cDNA gene sequences of the genome segments coding for the antigenic proteins of the virus into vaccinia and baculovirus expression systems. The early results using the baculovirus system are extremely promising (33) and are reviewed elsewhere in this book.

### b. Rotavirus

*Rotavirus Infections of Sheep.* Rotavirus infections are a common cause of diarrhea in young animals of most species, but there is no convincing

**Table 7.2.** Available and prospective vaccines for protection of sheep and goats against viral diseases.

| Virus family | Diseases[a] | Available vaccines[b] | Prospective vaccines |
|---|---|---|---|
| **DNA viruses** | | | |
| Adenoviridae | Respiratory and intestinal infections | Inactivated vaccines available in Hungary (4) | Similar to the poxviruses (see below) adenoviruses and herpesviruses may be developed as vaccine vectors for a range of vaccines |
| Herpesviridae | Respiratory and generalized disease in young kids | None | Similar to the poxviruses (see below) adenoviruses and herpesviruses may be developed as vaccine vectors for a range of vaccines |
| *Poxviridae* | | | |
| Capripoxviruses | Sheep and goat pox (skin and generalized disease) | Attenuated and inactivated available in endemic areas (9, 20) | Capripoxviruses are being experimentally evaluated as vaccine vectors for regions where sheep and goat pox occurs |
| Parapoxviruses | Orf (skin disease) | Unmodified/attenuated administered to dams several weeks before parturition (29) | Orf virus has potential as a vaccine vector worldwide (23) |
| **RNA viruses** | | | |
| Picornaviridae | Foot-and-mouth disease | Inactivated vaccines available (3) | Research on gene-deleted modified live, recombinant vectored and synthetic peptide is in progress (3) |
| Togaviridae | Border diseases | Attenuated and inactivated bovine virus diarrhea vaccines provide cross protection, but are seldom used | Limited economic importance restricts specific vaccine development |
| Flaviviridae | Louping ill Wesselbron disease | Inactivated vaccine (30) Attenuated vaccine (2) | |
| Paramyxoviridae Paramyxoviruses | Parainfluenza 3 virus respiratory infection | Attenuated vaccines, developed for cattle, given intranasally protect sheep (32) | |

| | | | |
|---|---|---|---|
| Morbilliviruses | Peste des petits ruminants | Attenuated rinderpest vaccine cross protects (17); a specific attenuated vaccine also available although not available commercially | A capripox vectored vaccine is in development (6) |
| Pneumoviruses | Respiratory syncytial virus infection | Attenuated vaccines for cattle available, but need to use in sheep and goats questionable | |
| Bunyaviridae Phleboviruses | Rift Valley fever | Attenuated and inactivated vaccines in use in Africa; the attenuated vaccine induces abortion in sheep | Mutagen attenuated vaccine that is immunogenic and nonabortigenic is in clinical trials (25) |
| Bunyaviruses | Akabane disease | Attenuated and inactivated vaccines available in Japan and Australia | |
| Nairoviruses | Nairobi sheep disease | Attenuated and inactivated vaccines available | |
| Retroviridae | Maedi/Visna, Caprine arthritis encephalitis, Jaagsiekte | None available nor forthcoming | |
| Reoviridae Orbiviruses | Bluetongue | Attenuated vaccines available, but can cause abortion in sheep | Synthesized capsids using the baculovirus expression system are immunogenic and hold great promise as novel vaccines (33) |
| Rotaviruses | Rotaviral diarrhea in neonates | Attenuated vaccines can be given to dam to boost passive immunity in young | Reassortant viruses are in development |

[a] Only diseases of economic importance to the goat and sheep industries are listed.
[b] Numbers in parentheses are references.

evidence that, under field conditions, cross-species infection is important. Plasma antibody levels in young lambs appear to have little effect in preventing rotavirus enteritis. Protection from infection is provided by the presence of antibody to the virus in the colostrum or milk while in the lumen of the gut. The frequent occurrence of rotavirus infections in lambs at the time when the antibody levels in the milk are declining (3–7 days after parturition) provides the logic for vaccinating the ewe *before* lambing to increase and prolong the secretion of antibody in the milk. This passive protection has two disadvantages: lambs must continuously be fed from an immunized ewe and they must be exposed and, hopefully, subclinically infected in order to develop active immunity. Oral administration of attenuated vaccines to young lambs soon after birth has successfully reduced disease but is affected by rotavirus antibody in the colostrum. To overcome what appears to be an almost intractable problem, the concept of vaccinating the fetus late in gestation by inoculating vaccine into the amniotic fluid has been investigated. While feasible, it is not presently a practical proposition.

### viii. Other Virus Families

Although there are several other viruses that infect sheep and/or goats, for example astroviruses, none is of sufficient importance to warrant development of vaccines. Certainly there is sufficient justification to develop a vaccine for scrapie, if only to reduce the risk of the agent being transferred to other species and causing problems such as bovine spongiform encephalopathy. However, since characterization of the etiological agent of scrapie remains elusive, speculation on the development of possible vaccines is beyond the scope of this review.

## 3. Conclusions

The available and prospective vaccines against economically important viral diseases of sheep and goats are summarized in Table 7.2. The above review of the viruses infecting sheep and goats and the availability (or in many cases lack) of vaccines to protect them draws attention to the different needs of the farmers of the industrialized and developing countries of the world. The increasing recognition of the economic importance of small ruminants, both within the industrialized and developing nations, is promoting greater attention to improving their health and productivity. It is axiomatic that vaccines be an important component in any program directed to improving health and productivity.

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CHAPTER 8

# Conventional and Contemporary Bacterial Veterinary Vaccines

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## 1. Introduction

The aim of this chapter is to review some of the conventional and contemporary methods used to vaccinate production animals against bacterial diseases. We confine our discussion to those veterinary diseases where recombinant DNA technology is beginning to play a part in vaccine development. The general objective of vaccinology is to achieve increasingly efficacious vaccines. An emerging trend toward this target is to identify those antigens capable of eliciting protective immunity then to present them to the animal in the optimal fashion. This concept has led first to the purification and presentation of single or mixed antigens, then to the production of recombinant antigens. Here we review the progress in bacterial veterinary vaccine development, from the use of simple bacterin preparations through to live delivery of recombinant antigens.

## 2. Conventional Vaccines

For the purpose of this chapter conventional vaccines are defined as those that have not been made using recombinant DNA (rDNA) techniques. In the past, control of a number of bacterial veterinary diseases (Table 8.1) was attempted by vaccination using a variety of often fairly crude antigen preparations (Table 8.2). Analysis of protective immune responses elicited by these vaccines revealed individual bacterial components capable of stimulating protective immunity, and thus useful for the preparation of recombinant subunit vaccines (Table 8.3). This section will describe examples of how vaccination using conventional bacterin and live-cell preparations have assisted in developing improved vaccines for bacterial diseases affecting livestock, and in identifying the crucial protective components required for effective subunit vaccines. In Section 3, the improvement in efficacy and production of such vaccines by rDNA is discussed. In the last section, the prospects of using genetically

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**Table 8.1.** Bacterial veterinary diseases.

| Disease | Common name(s) | Disease characteristics | Bacterial pathogen |
|---|---|---|---|
| Ovine footrot | Footrot | Interdigital inflammation, horn/hoof separation, lameness | *Bacteroides (Dichelobacter) nodosus* |
| Infectious bovine keratoconjunctivitis (IBK) | Pinkeye | Reddened eyes, conjunctivitis | *Moraxella bovis* |
| Bovine mastitis | Mastitis | Infection of the mammary gland | *Staphylococcus aureus* |
| Bovine brucellosis | Contagious abortion | Abortion, epididymitis | *Brucella abortus* |
| Bovine salmonellosis | Enteritis, ill thrift | Diarrhea | *Salmonella typhimurium* |
| Porcine enterotoxinogenic colibacillosis (ETCB) | Scours | Diarrhea | Enterotoxinogenic *Escherichia coli* (ETEC) |
| Porcine atrophic rhinitis | Turbinate atrophy or twisted nose | Atrophy of turbinate bones | *Bordetella bronchiseptica, Pasteurella multocida* |
| Bovine pneumonic pasteurellosis | Shipping fever | Pnemonia | *Pasteurella haemolytica* |
| Ovine-caseous lymphadentitis (CLA) | Cheesy gland | Abcessation of superficial lymph nodes | *Corynebacterium pseudotuberculosis* |

**Table 8.2.** Some antigen preparations assessed for use in conventional veterinary vaccines.[a]

| Disease | Antigen preparation | | | | | |
|---|---|---|---|---|---|---|
| | Bacterin | Live/cells | Capsular | Outer-membrane protein | Pili | Toxin |
| Ovine footrot | + | − | − | + | + | + |
| IBK (pinkeye) | + | − | − | − | + | + |
| Bovine mastitis | + | + | + | + | − | + |
| Bovine brucellosis | + | + | + | + | − | − |
| Bovine salmonellosis | + | + | − | − | − | − |
| ETCB (scours) | + | + | + | + | + | + |
| Porcine atrophic rhinitis (*Bordetella bronchiseptica*) | + | + | + | + | − | + |
| Porcine atrophic rhinitis (*Pasteurella multocida*) | − | − | − | − | − | + |
| Bovine pneumonic pasteurellosis | + | + | − | + | − | − |
| CLA (cheesy gland) | + | + | − | − | − | + |

[a] Refer to Table 8.1 for details of diseases. +, assessed; −, not assessed.

manipulated bacteria as live vaccine vectors to carry the genes of protective antigens is considered.

## A. Bacterins, Live Cells, and Cell Surface Components

Bacterins refer to vaccines made from whole killed cells or cell lysates and live vaccines are wild-type or natural variants of parental strains. Early vaccines were formulated as bacterins or live cells because specific protective antigens were unknown, preparation was relatively inexpensive, and, most significantly, frequently they worked. Apart from the obvious importance of providing a vaccine of some efficacy, bacterin or live-cell vaccination trials have been critical to vaccine development because they have often revealed the specific antigens that elicit host protective immunity. Such antigens have included pili, toxins (including proteases), outer membrane proteins, and capsular material. Examples of each category will be discussed.

Footrot, pinkeye, and scours are examples of sheep, cattle, and pig diseases, respectively (Table 8.1) where bacterin vaccination revealed that piliated bacteria were necessary to achieve protection. The early bacterin vaccines for each of these diseases (footrot: 182; pinkeye: 161, 164; and scours: 143) failed to provide adequate levels of host protection. For footrot this was because first vaccines contained only one or two of the 8 possible serotypes of *Bacteroides nodosus* and that vaccine cultures were poorly piliated (167,182,184). The importance of *Moraxella bovis*

**Table 8.3.** Potential recombinant DNA approaches to veterinary vaccination.[a]

| Disease | Possible antigen preparation | | |
|---|---|---|---|
| | Pili | Toxin | Rationally attenuated bacteria |
| Ovine footrot | + | Protease | − |
| IBK (pinkeye) | + | Protease (cytotoxin) | − |
| Bovine mastitis | − | $\alpha$-Toxin | $\alpha$-Toxin |
| Bovine brucellosis | − | − | − |
| Bovine salmonellosis | − | − | aroA, galE, asd, PhoP[c] cya, crp, purA, Omp[R] |
| ETCB (scours) | + | Enterotoxin | − |
| Porcine atrophic rhinitis (*Bordetella bronchiseptica*) | − | DNT[b] | DNT[c] |
| Atrophic rhinitis (*Pasteurella multocida*) | − | DNT | DNT[c] |
| Bovine pneumonic pasteurellosis | − | Leukotoxin | Leukotoxin[c] |
| CLA (cheesy gland) | − | PLD[b] | PLD[−] |

[a] Refer to Table 8.1 for details of diseases; +, discussed; −, not discussed in this chapter.
[b] DNT, dermonecrotic toxin; PLD, phospholipase D.
[c] Genes have not yet been deleted from host chromosome but their inactivation is expected to be attenuating.

and *Escherichia coli* pili antigens in providing protective immunity against pinkeye (116,160) and scours (137), respectively, has also been established. Current, efficacious footrot and scours bacterin vaccines now contain 8 *B. nodosus* (90,121,167) and 12 *E. coli* (179) strains, respectively, thus in each case representing all of the known serotypes.

It has not been possible to formulate a similar bacterin vaccine for pinkeye. For this disease the quantity of pilus antigen required to elicit protective immunity against each of the 7 known serotypes (A–G, L.J. Moore and A.W.D. Lepper, personal communication) would require a very large total cell mass per dose of bacterin vaccine. The fact that piliated forms of *M. bovis* are not easily produced in liquid culture and vaccination with a large antigenic mass elicits an unacceptably high inflammatory response has cast doubt upon the practicality of formulating a bacterin vaccine containing more than two serotypes (A.W.D. Lepper, personal communication). Since a multiserotype pinkeye bacterin vaccine is not practical and vaccines prepared from two heavily piliated strains of *M. bovis* fail to provide any cross-protection against different serotypes (10,177), alternative antigens or forms of pili antigen will need to be identified.

Bacterin preparations of *M. bovis* containing both pili and cornea-degrading protease antigens have elicited significantly better protection against heterologous challenge than vaccines without protease enzyme

activity (74). Although the *M. bovis* cornea-degrading protease may be a candidate cross-protective antigen it has not been shown to be efficacious against some strains in field trials (177). Although we know that it is possible to formulate an efficacious, multiserotype bacterin footrot vaccine (90,121), *B. nodosus* is similar to *M. bovis* in that it is difficult to culture piliated cells in the liquid media preferred by industry. Thus a less complex vaccine preparation would also be desirable. Protection across serotypes against some *B. nodosus* strains was obtained when sheep were vaccinated using strain 198, and this was attributed to an outermembrane complex that contained both pili and a protease fraction (186,187). The *B. nodosus* protease is currently being examined for its potential for use in a subunit vaccine (see Sections 2C and 3B).

It has also proved desirable to identify factors, in addition to pili, that may be formulated into a vaccine against scours. Since enterotoxins are a major virulence factor of enterotoxinogenic *E. coli* (ETEC; Table 8.1) it was suggested that they may be a valuable vaccine antigen (173). At present, most commercial pig scours vaccines are whole-cell bacterins containing various pili antigens (representing different serotypes) along with heat labile toxin (143).

Although there is good evidence to suggest that live ETEC vaccines can elicit protective immunity (29,108,137), live vaccines are not favored commercially because of concerns about introducing viable ETEC into herds and their environment (109). Other examples of diseases where bacterin vaccination has revealed that toxins are important protective antigens are summarized in Table 8.2. Bacterin vaccination of sheep against caseous lymphadenitis (CLA, Table 8.1), for example, has had mixed success. This could be attributed to initial problems in reproducing the challenge disease artificially. It is likely that early *Corynebacterium pseudotuberculosis* bacterin vaccination trials (21) failed to protect sheep against CLA since the challenge at $5 \times 10^8$ iv was too harsh. When bacterin consisting of whole formalin killed cells was used to vaccinate sheep and challenge was designed to simulate more closely the field situation ($1.2 \times 10^7$ into a subcutaneous wound) significant protection of lambs was observed (115). Now the method of choice for controlled experiments is challenge by infection in the hind foot (18,98), which provides a reproducible and clear means of assessing vaccine efficacy since, should infection arise, it is largely restricted to the draining popliteal lymph node.

In contrast to bacterin preparations, there have been few uses of live *C. pseudotuberculosis* vaccines. Live vaccination has served mainly to determine the type of immune response elicited by *C. pseudotuberculosis*. Results indicate that live *C. pseudotuberculosis* stimulate predominantly cell-mediated immunity (73,98). Immunization of mice using an uncharacterized attenuated strain of *C. pseudotuberculosis* resulted in the development of resistance to infection with a virulent strain (85). This

result suggests that live attenuated *C. pseudotuberculosis* may be useful as a vaccine against CLA. Potential for using rationally attenuated strains of *C. pseudotuberculosis* for vaccination will be discussed in Section 3C.

Both bacterin and live-cell vaccines have been evaluated for use against bovine mastitis (Table 8.1). For this disease it was found that killed *Staphylococcus aureus* vaccines, administered into the mammary gland, induced substantial levels of immunity from *Staphylococcus* infections, one of the major causes of mastitis (81,168). However, the high doses of *S. aureus* bacterin required to stimulate protective immunity can result in loss of milk production during subsequent lactation (203). Intramammary vaccination is therefore not a feasible option for commercial application. Unlike the situation with killed material, systemic vaccination of cows with live *S. aureus* stimulates protective immune responses as only live cells growing *in vivo* produce immunogenic quantities of capsular and toxin antigens, the components thought to be important in eliciting protective immunity (34,204). Recently it was found that if *S. aureus* was grown *in vitro* in the presence of ruminant milk whey they produced a pseudocapsule containing antigens in common with staphylococci grown *in vivo* (206). Sheep vaccinated with killed pseudoencapsulated *S. aureus* provided protection against homologous and heterologous challenge (205). The fact that *in vivo* cultured *S. aureus* produces a pseudocapsule capable of stimulating protective levels of antibody provides the potential for commercial vaccine development.

Capsule and toxin antigens have also been identified as being important in stimulating protective immunity in cattle against bovine pneumonic pasteurellosis (shipping fever; Table 8.1). Initial studies showed that *Pasteurella haemolytica* bacterins and formalin-killed whole cells were of limited value in eliciting a protective response against shipping fever (36,37). Although good serum responses were detected, they did not induce protection against challenge with *P. haemolytica* (37). In contrast, vaccination of calves with live *P. haemolytica* significantly reduced lung lesion score resulting from homologous challenge (37,151). The concentration of cytotoxin neutralizing antibodies was higher in calves immunized with live bacteria than in those vaccinated using the bacterin preparation. As the live vaccination was more effective, this suggested that cytotoxin antigens may be important in stimulating protective immunity. In addition, aerosol immunization of calves with live encapsulated *P. haemolytica* provided better protection against experimental challenge than using nonencapsulated organisms (37).

Another example of an animal disease where bacterin vaccination has revealed the importance of a toxin as stimulator of protective immunity is provided by *Bordetella bronchiseptica*, the principal pathogen in atrophic rhinitis of pigs (75,78). Vaccination of pigs with inactivated, whole-cell *B. bronchiseptica* bacterin has been capable only of reducing the incidence of atrophic rhinitis in herds since bacterin antigens are not highly

immunogenic (75). This problem was addressed by investigating the specific immunogens of *B. bronchiseptica*, in particular the dermonecrotic toxin (DNT). DNT appears to be a major *B. bronchiseptica* virulence factor since (a) on its own, it is capable of inducing nasal turbine atrophy (78,170) and (b) *B. bronchiseptica* lacking DNT-producing ability does not produce atrophic rhinitis when injected into guinea pigs (144). DNT is regarded as a strong candidate vaccine antigen (Sections 2C and 3B).

Other *B. bronchiseptica* bacterin vaccination studies revealed that vaccine preparations most effective in protecting piglets from nasal atrophy also stimulated the highest antibody titers to a 68-kDa outer-membrane protein (147). In addition, a wild-type *B. bronchiseptica* strain lacking the 68-kDa antigen was unable to induce the disease state in pigs or act as a vaccine (146). The 68-kDa protein was found to have adenylate cyclase activity (ADC) (146) and was also capable of protecting mice against aerosol challenge by *B. bronchiseptica* (136). Recently, however, it was reported that the 68 kDa protein is not ADC but is related to the 69-kDa outer-membrane protein from *Bordatella pertussis* (25). Nevertheless, the 68-kDa *B. bronchiseptica* outer-membrane protein would appear to have potential in formulating a rDNA subunit vaccine for atrophic rhinitis.

The last two diseases that we will consider here are bovine brucellosis and salmonellosis (Table 8.1). These are examples of diseases where the bacterial pathogen possesses neither pili nor toxin virulence factors (Table 8.2). Nonetheless, information from bacterin and live vaccination regimes has been useful in further development of vaccines against these diseases.

Brucellosis is a disease of domestic animals and man caused by *Brucella* bacteria. The consequences of the disease are fetal death and abortion in infected pregnant females and epididymitis in the male (12). The organisms have been divided into various species based on preferred host. Thus, for example, *B. abortus* principally causes bovine brucellosis but can infect sheep, goats, and man; *B. melitensis* infects mainly sheep and goats but can spread to cattle and man; *B. suis* is mainly a pig pathogen but is highly contagious to man (4). *B. ovis* is specific for sheep, but differs from the other strains in having a rough, as opposed to smooth colony morphology. Because of the DNA identity shared between *Brucella* species, it was proposed that only one species (*B. melitensis*) be formally recognised for *Brucella* (201). Current species names would then be used to define different *B. melitensis* biovars (4). Our discussion in this chapter will be generally confined to bovine brucellosis caused by *B. abortus*. The live attenuated *B. abortus* strain 19 is presently the preferred vaccine against bovine brucellosis (3,4,15) although other live vaccines such as *B. suis* S2 and *B. melitensis* M5 have been extensively used for the control of brucellosis in China (156,209). Killed *B. abortus* strain 45/20 vaccine has been used effectively in some countries (4),

but has variable immunogenic activities (157). Strain 19 was originally isolated in the early 1920s (17) and as a vaccine is inexpensive and simple to use (157). Disadvantages of using strain 19 to vaccinate against brucellosis are that it is pathogenic for humans and a small percentage of vaccinated animals develop persistent infections and shed the organism. This problem with the traditional vaccine has stimulated studies aimed at identifying specific protective antigens (52). Using a lemming model for bovine brucellosis, three strain 19 salt extractable proteins (30, 20, and 12 kDa) have been shown to provide protection against *Brucella* infection. Vaccination using the 30-kDa antigen reduced splenic counts of *Brucella* by 4.5–5 logs, which was better than that achieved using either the 20- or 12-kDa proteins (195). Consequently, the gene encoding the 30-kDa protein (renamed 31 kDa) was cloned, expressed in *E. coli* (133), and the antigenicity of the product has been evaluated (15).

In contrast to *Brucella* vaccine development, isolation of specific protective antigens from *Salmonella* has not been a priority. Vaccination of calves with live virulent *Salmonella* (84) and with a killed whole-cell bacterin (134) has provided protection against oral *Salmonella* challenge but attenuated live bacteria (Table 8.3) are most often considered as vaccines against salmonellosis (see Section 3D).

It is clear from the preceding discussion that bacterin and live vaccination has aided the identification of specific antigens important in eliciting protective immune responses. Some examples have been pili, proteases, toxins, outer-membrane proteins, and capsular materials. Utilization of these cell components has enabled developments in vaccine technology. How this has been approached and some of the results gained will be discussed in forthcoming sections.

## B. Pili

As discussed in Section 2A whole cell bacterin vaccination experiments suggested that the efficacy of footrot (184), pinkeye (116), and scours (137) vaccines was related to the degree of bacterial cell piliation. Further that cross-protection among the various *B. nodosus* strains for footrot and ETEC strains for scours could be attained by incorporating the different respective serotypes into a whole-cell vaccine (footrot: 90,121; scours: 179). One approach to improving the 8 strain *B. nodosus*, 2 strain *M. bovis*, and 12 strain ETEC vaccines has been to evaluate in each case the possibility of producing vaccines based on purified pili. This approach has the potential to allow (a) formulation of a multiserotype vaccine consequently providing cross-protective immunity, (b) reduction in the total amount of bacterial antigen and thus associated vaccinal inflammation, and (c) easier regulation of the quantity of protective antigen per vaccine dose (64).

Toward achieving these goals, purified pili vaccines were prepared and shown to provide protection against footrot, pinkeye, and scours after homologous, but not heterologous, challenge with *B. nodosus* (64,185,187,188), *M. bovis* (116,117,162,163), and ETEC (99,138), respectively. In footrot, the fact that less highly purified pili preparations were able to protect sheep from heterologous challenge suggested that an immunogen distinct from pili was also inducing a protective response. A 78-kDa antigen found to be associated with the protective immune response was thought to be either an outer-membrane protein or a protein that anchors the pili to the cell wall (185,188). Cross-protection achieved using an alum-oil adjuvanted purified pili vaccine was attributed to potentiation of the antibody response to the small quantities of contaminating protein present in the pili preparation (186). Recent studies have suggested that the contaminating protein is likely to be outer-membrane rather than pili associated (S.J. Billington and J. Rood, personal communication).

Due to the difficulties in culturing highly piliated *B. nodosus* and *M. bovis*, multivalent pili preparations have not been used in vaccination trials. This, however, has not been the case for pig scours. Scours vaccine improvement has involved incorporating a mixture of the major protective pilus antigens: K88, K99, 987P, and F41 (143) into a single vaccine. Such multivalent pili vaccines appear to be efficacious against heterologous challenge (199) and vaccines containing multiple purified pili components are available commercially (50,199). Some problems associated with scours pili vaccines, however, have included an antigenic drift in the pili population, for example, K88ab, K88ac, and K88ad variants of the K88 antigen. These new serotypes are believed to have arisen by a process of natural selection for antigens that are not inactivated by antibody specific to the original pilus vaccine antigen (83). Another problem associated with the production of scours pili vaccines is that yields of pili from cultures have been influenced by phase variation in which culture conditions determine whether pili are produced. In general, aerobic cultures are nonpiliated whereas growth under oxygen limitation selects for the piliated phase (100). As a result, pili yields can differ considerably between fermentations, thus adversely impacting on the cost efficiency of industrial vaccine production (80).

There is a clear potential for use of purified pili vaccines against footrot, pinkeye, and scours. It has been established for these diseases that mixed-strain, piliated, killed whole-cell bacterin, and purified pili vaccines can provide immunity to homologous challenge (see Section 2A). As observed for scours (199) development in vaccinology for footrot and pinkeye would be to mix pure pili of each serotype to produce a subunit cross-protective vaccine. Because of the production costs, however, it is unlikely that economically viable commercial vaccines would arise from preparations requiring purification and mixture of pili from all

serotypes of these fastidious organisms. Production of pili using recombinant DNA technologies may provide a solution to the problem of antigen production. A complicating factor could be the phenomenon of antigenic evolution observed for scours, where environmental factors, such as the immune system or pili-specific phage (132) select for resistant variants within the bacterial population. This could potentially become a problem in footrot, pinkeye, or other purified pili vaccines once they become used widely.

## C. Toxins

It became clear from vaccinating animals against a number of diseases that the toxin components in bacterin preparations were capable of eliciting protective immunity (see Section 2A). Many of these toxins (Table 8.2) have now been purified and used as vaccines. Generally, toxin preparations have an advantage over serotype specific vaccines such as those using pili in that they are capable of providing cross protective immunity. Purified, extracellular protease from *B. nodosus*, for example, provides similar protection against homologous challenge in the field as that obtained using either purified pili or whole cell vaccines and performance improves if protease mixtures are used (183). In addition, protease vaccination has induced substantial heterologous protection against lesions of severe footrot (190). For pinkeye vaccination, Gerber et al. (74) showed that cornea-degrading enzyme (possibly a protease also referred to as exotoxin) antigens when administered with piliated cell bacterin may be important in stimulating protective immunity against *M. bovis* infections. However, the cross-protective efficacy of the protease fraction did not seem to extend to all strains encountered in the field (177). Details of both the *B. nodosus* protease (Stewart and Kortt, Australian Patent 51387/85) and recently an *M. bovis* (Gerber, US patent 560780 and Australian patent 56594) vaccine preparation have been patented. Furthermore there is a patent pending for a semipurified cytotoxin specific for bovine neutrophil leukocytes (George and Kagonyera, International Patent WO 90/07525) suggesting that these preparations are potentially of commercial significance.

Enterotoxinogenic *E. coli* (ETEC) produce heat-stable (ST) and heat-labile (LT) toxins that act as virulence factors (143). The toxins have been purified and characterized (83). Initial experiments showed that purified LT but not ST could act as a protective antigen in *E. coli* vaccines (47,48). In addition, vaccination of pregnant sows with pure LT significantly reduced the mortality rates and percent of piglets with diarrhea following challenge with both homologous and heterologous live ETEC (50). Exploitation of the ETEC heat-labile toxin as a vaccine antigen using rDNA techniques will be discussed in Section 3B.

One of the causative agents of bovine mastitis, *S. aureus* (Table 8.1), has many potential virulence factors, including 5 toxins: $\alpha$-, $\beta$-, $\gamma$-, and $\delta$-hemolysins and a leukocidin (67). Of the toxins, only the $\alpha$ toxin is believed to have an important role in the pathogenesis of mastitis (68). Some level of protection against mastitis was observed when rabbits were immunized with purified $\alpha$ but not $\beta$ toxin (1). In addition, an $\alpha$-hemolysin negative mutant strain of *S. aureus* showed a drastic decline in virulence, inducing only very mild changes in mouse mammary gland tissue (105). More recent work, however, suggests that the virulence of *S. aureus* appears to be multifactorial (see Section 3C), making it unlikely that a successful mastitis vaccine for cattle will be based on a single toxin.

In other veterinary diseases, vaccines based on single toxin factors can be efficacious. While cell surface lipids have been implicated in the pathogenicity and virulence of *C. pseudotuberculosis* (103) in caseous lymphadenitis (Table 8.1), the importance of the phospholipase D (PLD) exotoxin has been very clearly established (18,19). Vaccine preparations of culture supernatants from *C. pseudotuberculosis* fermentations that contained mostly PLD with some soluble cellular components provide high levels of protective immunity (19). The PLD protein is a good candidate for subunit vaccine development although toxoided, crude culture supernatants are themselves a subunit vaccine. A commercial toxoid vaccine (Glandvac, Commonwealth Serum Laboratories, Melbourne, Australia) derived from culture supernatant is used in Australia to immunize sheep against CLA. Successful vaccination against CLA using a PLD toxoid vaccine suggests that the *C. pseudotuberculosis* PLD is an important virulence factor. This has implications for vaccine improvement using rDNA technology (Section 3B).

Dermonecrotic toxin (DNT) is a virulence factor for *B. bronchiseptica* and *P. multocida*, which both cause atrophic rhinitis in pigs (see Section 2C), and for the whooping cough bacterium *Bordetella pertussis*. It has been demonstrated that the *B. pertussis* DNT can be toxoided by formalin treatment (like PLD for the CLA vaccine; Burrell, 19) to produce a vaccine capable of protecting animals against the toxin (142). An atrophic rhinitis vaccine based on formalin-treated DNT from *B. bronchiseptica* (170), *P. multocida* or both may also prove efficacious. Both the DNT from *B. bronchiseptica* (96,110) and *P. multocida* (145) have been purified, and rDNA technology is being used to study the structure and function of these important proteins (see Section 3B).

Bovine pneumonic pasteurellosis or shipping fever in feedlot cattle is our last example of a disease that may be controlled using a toxin-based vaccine and is a good example of how an approach to vaccination can progress from the conventional to the contemporary (see Section 3B for discussion). Early vaccination trials suggested that protective immunity was associated with anticytotoxic immunity (37). The toxin has been purified and characterized and is known to have cytocidal effects on

bovine alveolar macrophages and mononuclear leukocytes (8,24,89,140). However, there are discrepancies in the reported molecular weights and dissociation products of the various cytotoxic protein preparations suggesting that there may have been difficulties associated with obtaining pure leukotoxin. Demonstration of the ability of purified leukotoxin to provide protective immunity in cattle would confirm its role as an important vaccine antigen.

## 3. Contemporary Vaccines

Traditional approaches toward animal vaccination (Table 8.2) have identified many protective antigens that could be used to formulate subunit vaccines (see Section 2). Recombinant DNA (rDNA) technology is being used in at least two major ways to provide improved vaccines: (a) genes encoding protective antigens are being cloned and expressed to provide pure protein for vaccination studies and (b) genes encoding virulence determinants are being deleted to produce genetically attenuated bacteria for use as live vaccines. Examples of how these contemporary approaches have been applied to vaccine development for a number of bacterial veterinary diseases (Tables 8.1 and 8.3) will be discussed below. In addition, an important development in vaccinology that has arisen from the ability to attenuate bacterial pathogens is the use of these bacteria as live delivery vehicles for heterologous antigens. This form of contemporary vaccine will be discussed in detail in Section 4.

### A. Pili

Pili vaccines have been shown to be effective in controlling footrot in sheep (*B. nodosus*), pinkeye in cattle (*M. bovis*), and scours in pigs (enterotoxinogenic *E. coli*, ETEC) (see Section 2B for discussion). For each of these diseases, vaccines containing a mixture of pili antigens were required to account for the antigenic variation among strains of *B. nodosus*, *M. bovis*, and ETEC, respectively. A problem common to these three examples of pili vaccines is that it is not economically feasible to obtain pili directly from the native bacteria since it is difficult to culture the appropriate piliated forms of bacteria consistently (see Section 2B). rDNA technology is currently being applied to footrot and pinkeye vaccines to produce pili antigens in alternate bacterial host organisms that piliate more readily in liquid media. Genetic analyses have confirmed that pilins of *B. nodosus*, *M. bovis*, *Pseudomonas aeruginosa*, *Vibrio cholerae*, and *Neisseria* sp. all contain conserved amino-terminal regions of the molecule and belong to the mePhe (Type 4) group (reviewed by Elleman, 61). This has enabled a common approach to the improvement of pili vaccines for footrot and pinkeye.

*B. nodosus* strains are represented by at least 9 serogroups (A–I). The pili genes from representatives of all of the serogroups have been sequenced (reviewed by Mattick, 130), including subtypes H2 (97) and B1, B2, B3, and B4 (63). Pilin genes from strain 198 (serogroup A, 55) and strain 265 (serogroup H1, 57) were the first to be sequenced and expressed in heterologous hosts therefore are the prototype studies for the other genes.

*E. coli* expressing the strain 198 pili gene produced large quantities of pilin but mature pili were not assembled (5,56). In addition, vaccination of sheep with pilin derived from *E. coli* was not protective against homologous challenge (56). Lack of pilin assembly in *E. coli* was attributed to the dissimilarity between the pili produced by *E. coli* and *B. nodosus*. Since *P. aeruginosa* pili share amino acid sequence homology with those made by *B. nodosus*, *Pseudomonas* was chosen as a host for *B. nodosus* pili gene expression. Recombinant *P. aeruginosa* produced cell-surface-assembled strain 198 (58,131) and strain 265 pili (60).

Vindicating the choice of host further, pili purified from *P. aeruginosa* stimulated a protective immune response in sheep against homologous challenge (53,58,189). In addition to providing protection against homologous challenge, the pili vaccine formulated from recombinant *P. aeruginosa* was also capable of curing footrot (189). These results demonstrate the potential for improvements to vaccines using rDNA. Since a single pili vaccine is not cross-protective, however, multivalent pili vaccines need to be formulated. To this end a *Pseudomonas* expressing *B. nodosus* strain 198 and 265 pili genes as a single transcriptional unit was constructed, and this produced serologically distinct pili populations on the cell surface (59,60). Pili isolated from the recombinant bacteria protected sheep from footrot after challenge with either 198 or 265 (60). In addition, sheep vaccinated with a recombinant pili vaccine containing subtype B2 protected sheep challenged by strains of *B. nodosus* from B1, B3, and B4 subtypes (63). These results hold promise that a recombinant approach to vaccinating sheep using pili preparations may be successful.

The level of success attained with footrot pili, and the fact that *B. nodosus* and *M. bovis* pili are related (61) prompted a parallel rDNA approach toward the development of a *M. bovis* pili vaccine. *M. bovis* strains are represented by at least 7 serotypes A–G, formally I–VII (L.J. Moore and A.W.D. Lepper, personal communication). Only *M. bovis* strains Epp63 (serotype F) and Dalton 2d (serotype C) have so far been studied genetically. Epp63 produces two different pili types, the pili types originally defined as α and β, which are transcribed from discrete genes (172). It has been suggested that β-pilin be redesignated as Q-pilin (quick) and α-pilin as I (intermediate) on the basis of their respective speeds of migration in polyacrylamide gels. A third category, γ-pilin (188), has been renamed S (slow) pilin (69). Production of two pili types in *M. bovis* strains is believed to be characteristic of the species (172).

*M. bovis* strain Dalton 2d produces only a single pili type (Q) but may still fit the dogma since it likely possesses a second, but defective, pilin gene (62). The Epp63 Q-pilin (127) and Dalton 2d (62) genes have been cloned and sequenced and the Epp63 gene expressed in *E. coli*. As observed with *B. nodosus* pilin gene expression in *E. coli*, pilus assembly did not occur (127). When expressed in *P. aeruginosa*, however, Q-pilin of Epp63 (11) and Dalton 2d (62) were assembled into extracellular pili. Vaccination data are not yet available, but it is hoped that results will be similar to those reported for *B. nodosus*. More of the pilin genes from *M. bovis* and *B. nodosus* will have to be expressed in *P. aeruginosa*, or another suitable host, and undergo vaccination trials before the value of recombinant pili vaccines for pinkeye and footrot can be fully assessed.

Many advantages have also been recognized for a rDNA approach to producing a scours pili vaccine: genes expressed on multicopy plasmids would both provide larger quantities of antigen and avoid yield losses due to phase variation; one *E. coli* strain could produce multiple pili types and a host (e.g., K12) less immunologically reactogenic than ETEC, could be selected for gene expression (reviewed by Dougan and Morrissey, 50). Cloning and sequencing the genes for the major pilin antigens K88ab (70), K99 (171), 987P (45), and F41 (6) have enabled an evaluation of the rDNA approach. A vaccine containing equal quantities of K88ac, K88ab, K99, and 987P pili produced from cloned genes in *E. coli* K12, protected piglets from scours under both controlled and field challenge trials. Not only did the recombinant vaccine preparation provide substantial protection against pig scours but the recombinant *E. coli* had reliable pili expression in large-scale fermentations (80).

Notwithstanding this success, continued efforts to increase the quantity of foreign protein produced is a desirable goal since it offers the potential to decrease manufacturing costs. A common approach to achieving this is to incorporate a strong inducible promoter upstream of the gene of interest. This has been applied to the ETEC genes. For example, when expressed as a fusion with the *trp* or Tac promoters, the ETEC K99 pilin gene produced 10 times more protein than the wild-type. Overexpression of genes, however, is not without its problems. Plasmid stability is often poor when genes on plasmids are overexpressed leading to loss of the plasmid from the bacteria (7). In addition, host cell growth was inhibited when the K88ac gene was expressed from a derepressed *trp* promoter. Repressing the *trp* promoter restored cellular growth and resulted in the production of approx 2% total foreign cell protein, an amount thought to be close to the maximal level for K88ac pilin (107). This result shows that the strongest promoters are not always the best suited for optimal yield if the host imposes a maximum limit on the amount of foreign product produced. Substitution of natural promoters may be essential, however,

to attain reliable quantities of gene products. An example is the K99 ETEC pilin gene, where expression is subject to alanine suppression and is temperature sensitive, leading to problems in consistent expression (46). Environmental regulation of pili gene expression, however, did not appear to be a problem for Greenwood et al. (80), who expressed their recombinant K88ac, k88ab, K99, and 987P genes from their own pro- moters and reported reliable gene expression in large-scale fermentations.

Selecting the most appropriate promoter for pilin gene expression is only one of the problems associated with producing a rDNA pili vaccine. Phase variation is a phenomenon that can occur as a consequence of gene expression. ETEC, for example, undergo phases of nonpiliation and *M. bovis*, in addition to this, is capable of switching production to pili of a different serotype. The *M. bovis* strain Epp63 phase switches between I and Q pili, although this may not be a common phenmonenon in other strains. This is due to the inversion of a 2-kb region of DNA containing 5′ portions of the $\alpha$- and $\beta$-pilin genes, including the promoter (69,128). Low-frequency genetic inversion occurs in the ETEC K99 gene, initiating at the 5′ end between the promoter and the pilus subunit gene and preventing pili formation (7). *Neisseria gonorrhoeae*, which produces mePhe pili like *M. bovis* and *B. nodosus*, has multiple pilin gene loci and phase variation is thought to arise from recombination events between expressed and silent gene variants (128). Interestingly, as previously stated, *M. bovis* Dalton 2d appears to contain at least one silent pilin gene (62), raising the possibility that it may be feasible for *M. bovis* to undergo phase variation both by its own mechanism or possibly by that seen in *N. gonorrhoeae*. This is also a formal possibility for class II *B. nodosus* strains that contain a duplicate, potentially redundant fimbrial (pili) subunit gene (*fim*Z; 130). Mechanisms resulting in greater variation within pili populations could confound attempts to provide consistently effective vaccines due to the potentiation of antigenic drift.

The success of complex antigenic mixtures such as multivalent recom- binant pili vaccines will depend, in part, upon the ability of the host immune system to recognize each component sufficiently to provide protection against an homologous challenge. In addition, forces such as phase variation and random mutation will be capable of creating pools of antigenically variable pili populations. Environmental selective pressures such as the host immune system, pili-specific phage (see Section 2B) or enhanced virulence (e.g., Q-piliated *M. bovis* Epp63 are more infectious than I-variants; 172) could select certain phenotypes, thus causing anti- genic drift. An example of this has been cited for the ETEC K88 antigen (Section 2B), which has evolved at least three major antigenic variants (83). Multivalent pili vaccines will need to counter antigenic drift in order to remain efficacious. Given these potential problems, consideration of alternative or additional antigens should be beneficial for a vaccine devel- opment program.

## B. Toxins

Since bacterial toxins are often immunologically conserved within bacterial species, they provide a strong possibility for formulating cross-protective vaccines. In the preceding sections we showed that there is either circumstantial or direct evidence that a number of toxins are virulence factors and as vaccines can provide protective immunity. rDNA technology has been applied to a variety of toxin virulence factors (Table 8.3) initially to provide sufficient quantities of pure material both to confirm their role in virulence and their ability to elicit the protective immune response. In addition, isolation of toxin genes can allow genetic (rational) toxoiding of the proteins, formulation of recombinant products as either mono- or multivalent vaccines, rational attenuation of the parent host strain, and incorporation of manipulated genes into live delivery systems. The aim of this section is to describe some examples of how rDNA techniques have been applied to develop vaccines based upon toxin virulence factors, particularly in relation to the pathogenic bacteria discussed in the previous sections (Tables 8.1 and 8.3). The use of recombinant toxins to generate rationally attenuated bacterial pathogens and the potential for their incorporation into live bacterial delivery systems will be the subject of discussion in Sections 3C and 4, respectively.

The gene for the novel protease from *B. nodosus* strain 198 has been cloned and expressed in *E. coli*. Purified recombinant protease vaccine was found to elicit substantial protection against both homologous and heterologous challenge (190). The *B. nodosus* protease represents a strong candidate for developing an rDNA vaccine against ovine footrot. Further studies will reveal whether the protease has a commercial application.

Although there is good evidence that the *B. nodosus* protease may be useful in developing a cross protective vaccine for footrot, detailed evidence for the value of the *M. bovis* protease (74) for a pinkeye vaccine is lacking (see Section 2C). A bovine neutrophil-specific, partially purified cytotoxin from *M. bovis* culture supernatants reportedly can act as a protective antigen (Gerber, US patent 560780 and Australian patent 56594). Cloning of the cytotoxin gene and evaluation of purified protein will be required to confirm the potential for developing a new pinkeye vaccine.

The heat-labile toxin (LT) from ETEC can protect pigs from scours (see Section 2C). The toxin gene has been sequenced and found to be structurally, functionally, and immunologically related to the cholera toxin (CT) (44). It is in the A–B group of toxins that, in addition to CT, includes the pertussis (124) and diphtheria toxins (76). A–B toxins consist of two functionally distinct parts, an A component that is active enzymatically and a B component that binds to surface receptors to enable the A component to enter the cell where it acts (196). Since the

nontoxic B subunits are highly immunogenic either alone or in combination with A, *E. coli* strains expressing the B subunits alone have been constructed thereby producing a LT toxoid molecule (50,83). LT toxoid has been overproduced in recombinant *E. coli* (26) but information from pig vaccination trials has not been forthcoming, possibly for commercial reasons. This is a strong example of where rDNA technology has provided the means of rationally toxoiding an active molecule for the purpose of vaccine development.

The DNT from *P. multocida* plays an important role in the establishment of atrophic rhinitis and the gene (*tox*A) has been cloned and expressed from its own promoter in *E. coli* (114,154). The recombinant protein is approximately 143 kDa in size and has structural and functional identity with the native *P. multocida* DNT (154). DNA sequence analysis of the *tox*A gene revealed an open reading frame capable of encoding a protein of 146.3 kDa (20). Recombinant DNT accounted for up to 15% of total cell protein in *E. coli*. When toxin purified from recombinant *E. coli* was injected into pigs it reduced weight gain without affecting food consumption (114). This provides strong evidence that the *P. multocida* DNT, in addition to causing turbinate atrophy, contributes to the lowered economic performance of infected pigs. A recombinant vaccine containing toxoid analogues of both the *B. bronchiseptica* and *P. multocida* DNTs is likely to be valuable.

Another *Pasteurella* species, *P. hemolytica*, is the major causative agent of shipping fever in cattle and produces a leukotoxin virulence factor (see Section 2C). Studies of the leukotoxin provide another good example of how determination of the genetic structure of a toxin gene has the potential of assisting in the production a recombinant toxoid vaccine. The *P. hemolytica* leukotoxin gene has been cloned and sequenced (122,123). Sequence analysis revealed that generation of active toxin requires two gene products (*lkt*C and *lkt*A) and that the structure of the leukotoxin gene and protein are analogous to an *E. coli* product, α-hemolysin (123). Thus, as seen with the *E. coli* α-hemolysin, it is thought that the *lkt*C gene product (LktC) is required to activate the leukotoxic phenotype of LktA. Mutations of the leukotoxin gene in the *lkt*A domain, leaving *lkt*C intact (plktCAd1), expressed in *E. coli*, produced a protein capable of binding to bovine lymphoma cells and thus protecting them from lysis by native leukotoxin (39). These data indicate that the secondary structure in the binding domain of the mutated protein remained intact. Consequently, if antibodies were raised against plktCAd1 they should recognize the binding domain on native toxin proteins, preventing them from binding to and lysing the cell. This could be tested in *in vitro* assays (passive protection of bovine lymphoma cells) and *in vivo*, by conducting vaccination trials using the recombinant toxoid. This information, together with a recent analysis of the genes involved in secretion of *P. hemolytica* leukotoxin (88,193), will prove very useful in the production

and assessment of a recombinant, secreted toxoid leukotoxin for vaccine formulation.

It was noted in the previous section that there is evidence to suggest the *S. aureus* α-toxin is a major virulence factor in bovine mastitis (67). Some of the this evidence has been generated from studies based on chemically induced α-toxin mutations, therefore leaving open the possibility for simultaneous mutation of several genes (105). Site-specific mutagenesis enables the effect of single gene mutations to be examined, and thus avoids the problem described above. This technique has been applied to analyses of the *S. aureus* α-toxin, β-toxin (14,150), and protein A genes (152). Mutants of *S. aureus* α-toxin (150) and β-toxin (14) were generated by site-specific mutagenesis. Experiments using these strains in infection experiments in a mouse model for mastitis indicated that neither toxin contributed significantly to bacterial virulence suggesting other factors may play a more crucial role in virulence than either of the toxins (14).

In contrast, a toxin such as the *C. pseudotuberculosis* PLD has a critical role in pathogenesis (Tables 8.1 and 8.3). The fact that partially purified PLD vaccines are capable of stimulating high levels of protection in sheep against cheesy gland (CLA) indicates that the PLD toxin vaccine would be a strong candidate for vaccine development using rDNA technologies. The PLD gene has been sequenced and expressed in *E. coli* (91) and recently we have deleted the PLD gene from the *C. pseudotuberculosis* chromosome (Hodgson *et al.*, Infect. Immun. July 1992, in press) using a site-specific mutagenesis protocol similar to that used to mutate the *S. aureus* α-toxin gene (150). Preliminary sheep infection experiments, using the PLD⁻ *C. pseudotuberculosis* strain, suggest that the phospholipase D is a major virulence factor (Hodgson *et al.*, Infect. Immun. July 1992, in press). Further studies will be required to realize the full potential of a recombinant PLD vaccine for CLA.

The *S. aureus* and *C. pseudotuberculosis* site-specific mutagenesis and the ETEC toxin studies illustrate how rDNA technology can be used to (a) define the role of bacterial toxin virulence determinants and (b) produce genetically toxoided molecules. Bacterial toxins (toxoids) are likely to be invaluable components for many new generation recombinant veterinary vaccines. In addition, deletion of toxin determinants can attenuate pathogens and render them useful as live vaccine delivery vehicles (see Section 4 for examples).

## C. Rational Attenuation

There is no doubt that live attenuated vaccines have been the most effective produced, particularly for viral diseases. Attenuated bacterial pathogens have, however, been less successful. In veterinary medicine for example, attenuated vaccines are available only for the prevention of brucellosis (see Section 2A and Table 8.1) and in *Pasteurella* and

*Erysipelothrix* infections. For these vaccines, classical methods such as passage in nonhost animals or *in vitro* culture has been used to attenuate *Brucella abortus* strain 19 (17), *Brucella suis* S2 (209), and swine erysipelas strain 31 (113). Streptomycin-dependent strains of *Brucella melitensis* Rev 1 (54), *Pasteurella multocida*, and *Pasteurella haemolytica* (27,28,106,207) also appear to be attenuated.

Contrasted to classical attenuation is the more recent idea of *rational attenuation*. The distinction between the two methods is that in rational attenuation a known mutation is introduced, and then attenuation assessed, whereas in the classical process the order of activities is reversed. Randomly mutated strains are checked for attenuation, following which the exact nature of the mutation may be determined. Some attenuated strains of pathogen have been temperature-sensitive strains, a logical attenuation for a mammalian pathogen, but not fitting the rational definition as the nature of the mutation conferring temperature sensitivity is unknown.

Perhaps the most obvious rational attenuation method made available by recombinant DNA technology is the elimination of toxin genes from pathogenic bacteria. Genetic elimination of the toxin gene from, for example, the human pathogen *Vibrio cholerae* has created an effective live vaccine, albeit with some side effects (120,153). As discussed in Section 3B, a major virulence factor of the atrophic rhinitis agent *Bordetella bronchiseptica* (Table 8.1) is a dermonecrotic toxin (DNT). Recently a strain deficient in production of DNT has been shown to have reduced virulence and significant protective activity. Deletion of the DNT gene could ensure the complete absence of toxin activity. However, the DNT negative character of the mutant appeared to be stable both *in vivo* and *in vitro* suggesting deletion of the DNT gene may not be required (144). In programs for vaccine development, however, genetic deletion has the benefit of allowing the expression of a genetically toxoided toxin gene, without the risk of a recombination event that could restore virulence. This approach could be applied to the deletion of the *Pasteurella multocida* DNT and the *P. haemolytica* leukotoxin genes (Tables 8.1 and 8.3), thus generating rationally attenuated organisms for potential live vaccination. Such vaccines should have exceptionally effective protective capacities.

As discussed in Section 3B, another veterinary pathogen where toxin elimination is potentially the path to a live attenuated vaccine is *Corynebacterium pseudotuberculosis*, the causative agent of caseous lymphadenitis (CLA) in sheep (Table 8.1). We know that toxoided PLD isolated from *C. pseudotuberculosis* provides effective protection against this disease (18,19), suggesting that the action of PLD is necessary for virulence. After genetically deleting the PLD gene we found the organism to be significantly attenuated, although still capable of stimulating

serological and T cell mediated immune responses (Hodgson *et al*. Infect Immun: July 1992, in press). As strains of *C. pseudotuberculosis* that are low producers of PLD can vaccinate against CLA (73,141), the deleted strain should prove an effective single shot vaccine.

Mutations introduced in rational attenuation can either strike directly at a virulence mechanism, as in toxin deletion, or alternatively, lower the ability of the bacteria to survive in the intracellular environment. Mutants of *Salmonella* carrying *aroA* are archetypes of the latter, reducing the persistence of the organism within the vaccinated host (95). The *aroA* mutation blocks the capacity of the *Salmonella* to synthesize *p*-aminobenzoic acid (PABA), dihydroxybenzoate (DHB), and aromatic amino acids, making them dependent on exogenous sources for their aromatic amino acid requirements. It is the inability to synthesize PABA that appears to be the effective attenuating characteristic, as a mutation solely destroying this capacity is equally effective as *aroA* in attenuating *Salmonella* (191). Stocker (191) has speculated that this attenuation manifests itself by blocking folic acid synthesis, and although this may have several general effects, most of these can be complemented by compounds available within the host animal. An effect that cannot be alleviated is the elimination of fMet-tRNA$_f^{met}$ synthesis, a molecule required by bacteria for the initiation of new protein chains and not available within the host animal. It is postulated that it is exhaustion of this compound that is the limiting factor in the persistence of the *aroA* strains *in vivo*.

There are other mechanisms used for lowering the virulence of *Salmonella*. The *cya*, *crp* mutations down-regulate the transcription of a group of genes involved in amino acid and carbohydrate metabolism (41,42,43) generally lowering the metabolism of the bacteria but also possibly altering the expression of specific virulence genes. *PhoP$^C$* and *Omp$^R$* mutations may have a similar effect, affecting expression of gene groups (119,135,155). *GalE* mutants are phenotypically rough when grown in the absence of galactose, but the efficacy of the *galE* phenotype is debatable, as it has been found that the *galE* phenotype engineered into *S. typhi* does not produce an avirulent strain (93), suggesting alternative attenuating mutations in the *galE* strains used. The recent observations that some *aroA Salmonella* strains complemented with a functional aromatic synthetic pathway are still attenuated does not imply that *aroA* mutations lack attenuating ability (125), but it does serve as a reminder of the attenuating ability of the classical technique of *in vitro* culture.

Much recent interest has revolved around making *aroA* mutations in other species of bacterial pathogen. *Shigella* (202), *Aeromonas salmonicida* (200), *Yersinia enterocolotica* (149), and *Bordatella pertussis* (169) *aroA* strains have been constructed, and all are attenuated. Production of the

attenuated *aroA B. pertussis* vaccine suggests a similar strategy may be effective for the atrophic rhinitis agent *B. bronchiseptica*.

The *aroA* genes of *Mycobacterium tuberculosis* have been cloned (72) and consequently an *aroA* version of *M. tuberculosis* may be close to hand. If this follows the trend of other bacteria and is attenuated, as *M. tuberculosis* is nearly identical to *M. bovis* it would provide the potential for developing an alternative vaccine against bovine TB. What works for *M. tuberculosis* will almost certainly work for *M. bovis*. Results using *M. bovis* BCG to vaccinate against bovine TB have been equivocal (77,159). Another intractable mycobacterial problem is Johnes disease, for which no really satisfactory vaccine exists. Rational attenuation of *Mycobacterium paratuberculosis* via *aroA* may be the answer, although considering the extraordinarily slow growth rate of this organism and the years required for challenge experiments this is definitely a long-term prospect.

## 4. Live Delivery of rDNA Vaccines in Bacterial Vectors

Live delivery of antigens by their production from cloned genes within attenuated vaccine vectors holds several attractive features for veterinary vaccines. Possibilities include reduction in vaccine cost, lower animal handling costs using simple, single-dose oral vaccination, multivalent vaccination, and hopefully improved efficacy. Live delivery is likely to be particularly beneficial in those situations where cell-mediated immune responses are critical to protection.

Both viral and bacterial vectors are possibilities, respectively suited to the delivery of protective viral and bacterial antigens. Although bacteria have been used to deliver viral epitopes, prokaryotes do not have a good record in the production of conformationally correct viral antigens, and of course lack the eukaryotic ability to glycosylate proteins. These effects are reciprocal and it is likely that bacterial vectors will prove most useful for the delivery of bacterial antigens. Parasitic antigens may possibly be delivered by either system, depending on the nature of the protective epitopes, but the role for bacterial vectors is demonstrated by the repeated finding that parasite antigens purified from genetically engineered bacteria can stimulate effective vaccine responses (23,92,102).

There are points in favor of using bacteria as live veterinary vaccine vectors. Bacterial vectors may be more suitable for the construction of multivalent vaccines. Viral genomes are constrained by capsid size, and have only limited capacity for the introduction of extra genes, a factor not applicable to bacteria, which are capable of accepting large amounts of DNA. Bacteria do have constraints in the total amount of exotic protein they can synthesise, and the metabolic load of expressing large amounts

of protein can select against maintenance of highly expressed genes (see Section 3A, *E. coli* recombinant K99 pilin gene expression for example). Additionally, bacteria are susceptible to antibiotic therapy, allowing an extra measure of safety not available for viral systems if, by mischance, the vaccine developed a persistent infection in an immunocompromised animal.

Potential bacterial vectors have the ability to survive within the host animal macrophage and are resistant to immediate killing by lysosomal mechanisms. Persistence of the vector at this site permits the continual release of antigen at a locality most suited for antigen processing for presentation to T and B cells. The presence and persistence of the vector within the macrophage could well stimulate release of cytokines by the macrophage, enhancing the immune response and the development of cell-mediated immunity. Further, the bacterial vectors themselves contain commonly recognized antigens and this may improve reactions to the foreign antigen cloned within the vector by a hapten carrier effect. Most animals will have been exposed to bacteria that are antigenically related to the bacterial vectors proposed, as they are representatives of widespread genera. It is known that chemical cross-linkage of antigens to reactive T cell epitopes enhances the serological response of an inoculated animal to the antigen. This is a well studied immunological phenomena, but has been shown to be particularly effective when cross-linking of protein antigens or peptides is with tuberculin extracts, and *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG) vaccinated animals are inoculated (111,139), pertinent because BCG is a proposed vector. Such evidence suggests that the recognition by memory T cells of commonly reactive antigens presented on the macrophage enhances the response to the linked novel antigen, probably mediated through lymphokine release by the sensitized T cells. When using a bacterial vaccine vector the cloned antigen is linked to the vector antigens in that the macrophage acquires the bacterial vector along with the foreign antigen it is producing, and thus there is potential for this carrier effect.

Possible veterinary bacterial vectors could include any intracellular pathogen for which an attenuated strain is available or can be made, and examples of these have been discussed in Section 3C. In practice, only a limited number of species have been examined. Although there is no theoretical reason to discount other enteric bacterial species, the *Salmonella* have a leading position as prospective vectors, with well developed genetic systems and a variety of attenuated strains available. Less well understood genetically, but with a long track record in human use and immunological experimentation, BCG has recently emerged as a potential bacterial vector. When considering either host as a vaccine vector it is obvious that the pilin antigens referred to in previous sections are unlikely to be used, as the correct assembly of pili is improbable within these strains of bacteria.

## A. *Salmonella*

The genus *Salmonella* has been dominant in the field of live vaccine vectors. Examples where it has been used are listed in Table 8.4. Attenuated *Salmonella* vaccines can be delivered orally to both ruminant and nonruminant animals (30,104,112) and some strains are effective in chickens (9,38). Ingested infectious *Salmonella* persist in the intestinal tract, progressing into the Peyers patches of the gut and proceeding from there into the spleen and liver (22,35). In attenuated strains, this is the limit of infection and generally only small numbers of bacteria pass to the spleen. This is apparently sufficient to stimulate cell-mediated immunity, and often humoral and secretory antibody responses (Table 8.4). Oral administration and the stimulation of mucosal immune responses are attractive features for the development of vaccines against intestinal parasites, a persistent and economically damaging problem in many animal species.

Although *aroA* strains have been used for the delivery of cloned antigens (Table 8.4), using a strain crippled in its capacity to synthesize protein may be a less than optimal approach. *In vivo* production of the cloned antigen must be subject to limitation in *aroA* mutants, and this may be a reason for reports of plasmid instability in these strains (43,148,175,198,208) although plasmids have been found to be unstable in *Salmonella* attenuated through other mechanisms (31). While the antigen genes may be on unstable plasmids there is ample evidence that there is still sufficient antigen produced to establish immunogenicity. What is not clear is how much of the immunizing antigen is that material produced in the bacterial vector during culture prior to inoculation, and if this is enough material to immunize the animal effectively. Evidence would suggest that *in vivo* production of antigen is unnecessary if sufficient antigen is produced *in vitro* prior to inoculation (33,148). Many antigens have been expressed and found to be effective using the *tac* promoter, which is inducible only in culture. Fairweather et al. (65,66) have demonstrated that *Salmonella* carrying the tetanus toxin C fragment gene driven by the uninduced form of this promoter was totally useless in stimulating immune reactions, although a constitutively expressed form was very effective. This suggests there is no *in vivo* expression of the antigen from this promoter, and previous successes using this promoter have depended on antigen produced in *in vitro* culture.

The problem of plasmid stability is lesser using *Salmonella cya crp* strains, and stability has been further increased by the use of strains carrying an additional *asd* mutation. This is a lethal mutation if the bacteria is not supplied with diaminopimelic acid, but the mutation can be complemented by an *asd* gene on the plasmid. Loss of the plasmid is thus lethal to the host (40,71). Other lethal complementation systems are also available (158).

**Table 8.4.** Examples of responses to *Salmonella* expressing recombinant antigens.

| Strain | Antigen | Promoter[a] | Inoculation route no. doses[b] | Responses[c] Humoral | Cellular | Protection[d] | Reference |
|---|---|---|---|---|---|---|---|
| *S. typhimurium* SL3261 *aroA* | Rotavirus VP7 | LD[e] | (I/V)2 | − | NT[k] | NT | (175) |
| SL3261 | Galactosidase | I | (I/V)1 | + | + | NT | (16) |
| SL3261 | *E. coli* K88 | C | (I/V)1 | LOW | NT | + | (51) |
| | | | (Oral)1 | LOW | NT | LD[e] | |
| SL3261 | Leishmania gp63 | C | (Oral)2 | LD | + | + | (208) |
| SL3261 | Influenza nucleoprotein | LD | (I/V)1 | + | LD | NT | (198) |
| | | | (S/C)1 | + | + | NT | |
| | | | (Oral)1 | + | + | − | |
| SL3261 | Dengue 4 env. | I | (Oral) | LD | − | − | (33) |
| SL3261 | Tetanus toxin (C) fragment | I and C | (Oral)2 | + | NT | + | (66) |
| | | | (I/V)2 | +[f] | + | | |
| SL3261 | Hepatitis B, polio VPT | I and C | (I/V)2 | + | NT | NT | (148) |
| *S. dublin* SL5928 *aroA* | Hepatitis B[g] | C | (Oral)4[h] | + | NT | NT | (191) |
| | | | (I/M)5 | + | NT | NT | |
| | Cholera toxin[g] | C | (Oral)3 | − | NT | NT | |
| | | | (I/M)3[i] | + | NT | NT | |
| | *Streptococcus pyogenes* M. protein[g] | C | (Oral)3 | − | NT | NT | |
| | | | (I/M)3 | + | NT | NT | |
| | Hiv gl160[g] | C | (I/M)3[j] | + | NT | NT | |
| | | | (Oral)5 | − | NT | NT | |
| *S. dublin aroA* | *E. coli* enterotoxin | I | (Oral)2 | + | NT | NT | (32) |
| *S. typhimurium* X4064 *cya crp* | Brucella 31 Ks | C | (Oral)1 | + | + | NT | (180) |

(*continued*)

**Table 8.4.** *Continued*

| Strain | Antigen | Promoter[a] | Inoculation route no. doses[b] | Responses[c] | | | Reference |
| | | | | Humoral | Cellular | Protection[d] | |
|---|---|---|---|---|---|---|---|
| S. typhimurium X4072 cya crp | Influenza HA | I | (Oral)2 | – | NT | + | (155) |
| S. typhimurium X4072 cya crp | Francisella tularensis 17 Ks | LD | (Oral)2 | + | LD | + | (176) |
| S. typhimurium WR4024 | Plasmodium berghei CS | C | (Oral)3 | – | + | + | (2) |
| S. typhimurium galE | E. coli K88 | C | (Oral)3 (I/P)3 | + + | NT NT | NT NT | (181) |
| S. typhimurium WR4017 | Plasmodium berghei CS | C | (S/C)1 (Oral)1 | Low – | – + | + + | (174) |

[a] All genes on plasmids. Promoter constitutive (C) or inducible (I).
[b] S/C, subcutaneous; I/M, intramuscular; I/V, intravenous; I/P, intraperitoneal.
[c] In mice, unless otherwise stated.
[d] Any protective response is designated as positive.
[e] Limited data.
[f] Higher titers obtained with I/V inoculation.
[g] Epitopes of the antigen in a flagellin gene.
[h] Rabbits, guinea pigs, and mice.
[i] Rabbits and mice.
[j] Rabbits only.
[k] NT, not tested.

Various other solutions to the problem of plasmid stability have been tried. One is to decrease the copy number of the plasmid, lowering the number of cloned genes and level of foreign gene expression, thus cutting the metabolic load on the bacteria, reduces the selective effect against the plasmid (175). Another possibility is to locate the foreign gene on the chromosome, which would lower the gene dosage further and reduces the possibility of gene loss via plasmid segregation. This has been found to be effective in *Salmonella* (32,94,194), and a similar strategy has been devised for chromosomal location of exotic genes in mycobacteria (192).

There are several examples where *Salmonella* strains, principally *S. typhimurium*, expressing cloned antigens have been used to inoculate animals (Table 8.4). Although *Salmonella* carrying foreign genes have been found to stimulate antibody responses (Table 8.4), this is not a consistent finding, especially if oral dosing is used, and booster inoculations are often required. Protection has been demonstrated in the absence of antigen-specific antibody, and is attributed to cell-mediated activities (2,174). Multiple dosing is not satisfactory in a vaccine vector for veterinary purposes, and the lack of antibody responses commonly seen with oral dosing is not encouraging. It remains to be seen whether the more stable expression systems may overcome these problems.

## B. *Mycobacterium bovis* BCG

Use of the tuberculosis vaccine BCG as a vaccine vector for human use was first suggested by Barry Bloom (13), BCG being an attractive vector both for its proven human safety record and for its immunogenic properties. As it can safely immunise a number of other animal species, it also has potential in the veterinary field. Deriving from *in vitro* attenuation of *M. bovis*, the attenuating mechanism in BCG is unknown, and although BCG strains show variation (82) there is no evidence of reversion to pathogenicity.

When the possibility of BCG as a vector was first raised very little was known of mycobacterial genetics. Consequently the development of mycobacterial vaccine vectors differed from the genetically well studied *Salmonella* strains in that there were no plasmid and bacteriophage vectors capable of stable expression of foreign genes or methods for mycobacterial transformation and selection. Advances have been rapid over the past 4 years. First progress in this area was by Jacobs et al. (101) with the construction of an *E. coli–Mycobacterium* shuttle phasmid, an amalgamation of a mycobacteriophage and an *E. coli* cosmid vector. Following from this a mycobacterial plasmid, pAL5000, was cloned into *E. coli* plasmid vectors, DNA sequenced, and essential mycobacterial replication genes established (165,166). This allowed the creation of small shuttle vectors, and a promoter from the 65-kDa common mycobacterial antigen has been used to construct expression vectors (192). Con-

comitantly, electroporation conditions for transformation of mycobacteria with plasmid have been established (126), and *M. smegmatis* strains capable of highly efficient transformation selected (178).

Various genes, including HIV antigens and the *Schistosomiasis japonicum* antigen have been expressed in BCG (192), but there are no reports of antigens of veterinary relevance being expressed in this vector. Most of the expression has been from plasmid vectors, but Hatful et al. (87) have reported the isolation of the genes responsible for the chromosomal integration of the lysogenic *M. smegmatis* bacteriophage L5, and have created a plasmid vector carrying these genes that is capable of high frequency integration into the BCG chromosome. This is of obvious relevance to the creation of stable vaccine vectors, eliminating the need for antibiotic or other selection for the maintenance of antigen expressing genes on a plasmid within the BCG vector. The discovery of myco-bacterial insertion sequences (79,197) and a functional transposon (129) allows alternative potential for chromosomally locating foreign antigen genes. At the time of writing no protection had yet been shown using a mycobacterial vector, but vaccinated animals have shown antibody and cell-mediated responses to antigens expressed in BCG (192).

## 5. Conclusions

Conventional bacterin and whole-cell vaccine preparations have proved invaluable in the development of contemporary veterinary vaccines. Cell components such as pili, proteases, toxins, outer-membrane proteins, and capsular antigens have been identified as stimulators of protective immunity. Veterinary vaccine development has progressed from the use of simple bacterin preparations to mixtures of specific cellular components, and then to cloned protective antigen genes.

Recombinant DNA technology is playing a vital role in veterinary vaccine development and has thus far enabled the following major advances in bacterial vaccinology: (a) cloned protective antigen genes have been expressed to provide a ready supply of pure antigen for vaccine formulation, (b) cloned virulence genes have been used to delete their counterparts from the bacterial chromosome thereby effecting a rational attenuation of the pathogen, and (c) genetically attenuated pathogens have been used for live delivery of recombinant antigens, which can include genetically toxoided toxins.

The potential for live delivery is perhaps the most significant major development for veterinary vaccination since it provides a means of achieving lasting protection from a single dose. We anticipate that the role of rDNA technology in veterinary vaccination, particularly for live delivery, will continue to increase, and that recombinant vaccines will eventually be used widely in animal medicine.

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CHAPTER 9

# Conventional and Biotechnologically Engineered Bovine Vaccines

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## 1. Introduction

Immunization against infectious agents has proven to be one of the most cost-effective methods of reducing economic loss in animals. However, even though immunization to prevent infections has been practiced for nearly 200 years, animals still continue to suffer from a large number of infectious diseases. Thus even though we have been able to prevent devastating disease outbreaks with vaccination, we have not eliminated the scourge of these diseases. The reasons for this are multifold and are related to management conditions, the age when animals get exposed to the pathogens, the patterns of pathogenesis of the respective organisms, as well as possible ineffectiveness of conventional vaccines. For example, many of the conventional vaccines are administered by the intramuscular route. These vaccines do not provide complete protection at the mucosal surface, the route of entry of the pathogen. Therefore, implementation of effective immunization regimes requires information regarding the specific immune responses involved in providing protection as well as the specific antigens that elicit the appropriate response. For example, if the virulence factors of a bacterium include an extracellular toxin that is crucial to pathogenesis it will be inappropriate to use bacterins to protect against this particular infection. In many cases a local immune response or a cellular immune response is crucial for both preventing infection as well as aiding in recovery. If we hope to elicit the correct immune response to the specific organism, a considerable amount of basic microbiological and immunological knowledge is required concerning the actual antigens and the types of immune responses involved. Unfortunately, many viral and bacterial infections encountered in veterinary medicine have not been adequately characterized at the molecular level nor have the host immune responses to these agents been characterized. If this was achieved then it may be possible to engineer better vaccines to control diseases for which we already have at least partially

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effective vaccines as well as for those where no effective immunization regimes are presently available. In the present review we will attempt to discuss the various types of vaccines used for controlling the major bovine diseases, and some of the practical problems regarding immunization that are most relevant to bovine medicine. We will also discuss the potential for improving present vaccines by modern biotechnological methods. However, even with all the tools of producing new vaccines, totally effective vaccines will not be forthcoming until we gain a considerable amount of fundamental information regarding the specific antigens involved in eliciting protective immunity and the host's response to these antigens. In veterinary medicine one must always take into consideration the economics of producing these vaccines. Thus even if it was possible to use some of the more sophisticated methods to produce effective vaccines, there is no guarantee that they can be produced economically to ensure widespread use. These factors will all be discussed in this review.

## A. Practical Problems of Implementing Effective Vaccination Programs in Cattle

For practical purposes we have divided this review into respiratory, enteric, systemic, and other infections. In cattle, there are unique problems in controlling respiratory and enteric infections. The most economically important enteric infections generally occur within the first few weeks of life. Examples of these infections include *Escherichia coli*, rotavirus, coronavirus, and a myriad of other viral, bacterial, and parasitic infections. The age of susceptibility as well as the site of infection provide major impediments to effective immunization. Unfortunately, it is impossible to induce immunity within 2–3 days after immunization, yet that is how rapidly some of these infections occur after birth. If one expects to induce active immunity the most effective vaccine should be one that induces local immunity to prevent infection of the gastrointestinal tract. However, the presence of passive antibody, acquired from the dam during suckling, may interfere with oral immunization. In the case of bovine respiratory disease, management systems differ around the world thereby making a universal vaccine very difficult to produce. In North America bovine respiratory disease occurs following weaning and movement of animals to large feedlots. The process of mixing, stress, and exposure to different pathogens upon entry to the feedlot ensures that animals are exposed to multiple pathogens within a very short time period. In many cases these pathogens can act synergistically to increase the severity of respiratory infections. It would be ideal to immunize animals prior to entry into feedlots (preconditioning) but unfortunately animal husbandry practices prevent this

type of approach from being implemented. As described for enteric infection, immunization upon entry into the feedlot does not provide sufficient time for immunity to develop to prevent infection. As a result animals suffer from what is called "shipping fever." Another consideration for vaccine efficacy is the route of vaccine administration. Differences in immune responses occur between vaccine administered intramuscularly, subcutaneously, or intradermally. In the development of vaccines, knowledge about the pathogenesis of the disease and immune responses involved in inducing protection must be considered for judicious designing of vaccination protocols. Thus, if the pathogen is strictly localized to mucosal surfaces, systemic immunity may be of limited value. However, if the virus is both local and systemic then parenteral administration will be effective in preventing systemic spread. Unfortunately, in vaccine design, this is not always the primary concern. Often the primary concern is ease of administration. If effective vaccines are to be developed all these factors must be taken into consideration.

## B.  Types of Vaccines

At present the majority of veterinary vaccines are produced by conventional methods similar to those implemented by Jenner or Pasteur. These include conventional live vaccines or killed vaccines. Both of these types of vaccines have proven to be effective in at least partially reducing the clinical manifestations following exposure to virulent field strains of the pathogen. In the case of live vaccines one of the major impediments to their development is to ensure that the organism is attenuated sufficiently not to cause disease but still replicate to a sufficient level to induce the appropriate immune response. To date many of the vaccines produced by attenuation have been produced emperically with no true understanding of the genes that have been altered or the nature of the alterations. As a result of this serendipitous attenuation, live vaccines carry the remote risk that they may revert to a virulent state. As will be described later, a better understanding of virulence mechanisms is allowing us to directly alter the virulence genes or delete them completely to ensure complete attenuation. Similarly, with killed vaccines it is well known that only certain proteins of the pathogen are important in inducing protective immunity and that other proteins may suppress immunity to the protective ones. With techniques available to identify these proteins and produce the protective ones in large quantities, it is envisioned that more effective killed vaccines will be generated in the near future. It must be emphasized that developments in vaccine delivery and adjuvants will need to be conducted in parallel with vaccine development to make these new vaccines as efficacious as possible (see Section 6).

## 2.  New Technologies for Vaccine Development

Improvements in conventional biochemistry, recombinant DNA tech-
nology, peptide synthesis, molecular genetics, and protein purification has
laid the foundation for the development of new vaccines which should
be more efficacious, cost effective and lead to fewer side effects. In
this section we will briefly review what we perceive to be the vaccines
of the future. In some instances, these new vaccines will be used to
"spike" conventional vaccines to improve immunogenicity to selective
components whereas in other cases there new vaccines will comprise the
total vaccine.

### A.  Subunit Vaccines

Subunit vaccines can be defined as those that contain one or more pure or
semipure antigens. The potential advantages of using such a product are
numerous, including increased safety, less antigenic competition due to
the presence of less relevant components, ability to target the vaccine
to the site where immunity is required (mucosal sites), and the ability
to differentiate vaccinated from infected animals. This latter feature is
extremely important for pathogens which persist for long periods of time
(latent) in the animal and where countries are trying to eliminate the
specific disease. Similar types of diagnostic methods can also be used in
conjunction with "marked" live vaccines (see later). Although subunit
vaccines can be produced by conventional technology, the economics of
purification are generally not cost effective due to the low quantities of
protective antigens produced by the organism. Also, since the organism is
grown *in vitro*, some of the relevant antigens may not be present. An
example of this would be bacterial proteins required for the scavenging
of nutrients such as iron or carbohydrate. These impediments can be
overcome by using recombinant DNA or synthetic peptide technology.

    The development of recombinant DNA technology in the 1970s com-
bined with advances in gene expression during the past decade has made
it possible to produce large quantities of proteins in heterologous cells.
Methods involved have been described elsewhere and will not be dealt
with here except to state that they involve the following steps (12). First,
it is imperative that the protective antigens be identified. Usually one or
at most only a few proteins of the organism are involved in inducing
protective immunity. To identify the protective antigens a considerable
degree of knowledge concerning the pathogen itself, the pathogenesis of
the organism, and the host responses to that organism is required. Once
the protective antigens are known, the gene coding for the protein needs
to be identified, cloned and expressed.

    A number of expression systems are being used to produce large
quantities of these subunit vaccines. They include: (a) prokaryotic

systems and (2) eukaryotic systems including yeasts, mammalian cells, insect cells, algae, and filamentous fungi. The expression system chosen will often depend on the source of the gene being expressed. For example, prokaryotic systems are very attractive for production of subunit vaccines from bacteria. Yields as high as 2–5 g/liter of the desired product can be easily achieved in these systems (57). In contrast, insect and mammalian cell expression systems only yield 100 mg/liter of culture. Unfortunately, it is not always possible to express genes from viruses in prokaryotic systems and have them function in the same way as they would if they were produced in eukaryotic systems. This is related to the post-translational processing and folding of viral proteins in bacterial systems.

For the production of bacterial vaccines, bacterial expression systems are ideal. Depending on the yield and downstream processing capabilities and requirements, it is possible to engineer the bacterium in such a way as to have the product targeted to the periplasm, outer membrane, secreted into the medium or retained intracellularly (83). If the product is retained, the protein is often present as an insoluble protein mass (inclusion bodies), which requires solubilization and refolding into the native state (163). If the product is secreted, it may be possible to harvest the product directly from the media and use the culture fluid as a vaccine without any further downstream processing.

While *E. coli* and other Gram-negative organisms have been engineered to secrete some proteins into the growth medium, Gram-positive bacteria such as *Bacillus subtilis* and *Staphylococcus aureus* are potentially better systems (70,126). *Bacillus* sp. have been used for many years for industrial scale fermentations and, therefore, the logistics of scale-up from laboratory to commercial production levels are not as difficult to achieve as with other organisms. *B. subtilis* produces extracellular proteases that can affect the stability of secreted proteins but mutants lacking proteolytic activity are availabe (70,185). A number of secretion vectors have been constructed and can function as plasmids or integrated into the chromosome. While yields are not as high as with intracellular expression systems, the products are soluble and downstream processing costs are reduced since they do not have to be denatured and refolded. Effective secretion systems for *S. aureus* also have been developed based on the protein A gene. These have been shown to function in both *E. coli* and *S. aureus* and are suitable for peptides or small proteins (126).

In the case of viral subunit vaccines, prokaryotic expression systems are generally of limited use due to the fact that prokaryotic cells do not posttranslationally modify viral proteins correctly. As a result, a considerable amount of interest has been generated in the development of eukaryotic expression systems. These include yeast, mammalian cells, filamentous fungi, blue-green algae, and insect cells (16,68,85,97,182, 186). The primary advantage of yeast as an expression system is that extensive industrial experience is available with the yeast *Saccharomyces*

*cerevisiae*. This organism is not pathogenic for animals and, more importantly, the expense of removing all the yeast proteins from the vaccine does not appear to be required. This will greatly reduce the cost of the final product. Unfortunately in some cases yeast may overglycosylate proteins, which may influence immune responses to the specific subunit protein being produced. It is well known that the degree of glycosylation can influence immunogenicity of a variety of proteins. Although there are no vaccines licensed for use in veterinary medicine that have been produced in yeast, the first licensed human recombinant DNA vaccine, for hepatitis B, is still produced in *S. cerevisae* (186). Other varieties of yeast such as *Pichia pastoris* are also being developed for use as hosts for gene expression. Using these newer systems, the level of production of foreign protein has been increased to approximately 400 mg/liter.

A second expression system that has received considerable attention is the baculovirus, *Autographa californica* nuclear polyhydrous virus (AcNPV), grown in insect cells (85,106). The principle behind the use of baculovirus/insect cultures to produce foreign products is that some genes such as that coding for the polyhedron of the AcNPV virus are dispensable for virus replication. If one uses the high efficiency promoter of the polyhedron gene to drive the transcription of a foreign gene, which replaced the polyhedron gene, large quantities of protein can be produced. Furthermore depending upon the construction, the protein can either remain within the cell or be secreted into the culture medium. The development of synthetic media requiring no serum should dramatically reduce the cost of culturing cells as well as remove the potential problem of introducing extraneous protein and agents such as bovine virus diarrhea virus (BVD) into the vaccine (88,107). One possible disadvantage of the insect viral expression system is that insect cells do not posttranslationally modify the protein in a fashion identical to that occuring in mammalian cells. Thus, as with yeast, some loss of immunogenicity may occur for some proteins if they are not glycosylated appropriately.

The ideal subunit expression system would be to use mammalian cells for production of viral proteins or glycoproteins. To achieve this goal, one can use mammalian viruses such as vaccinia, herpes, adenoviruses, picornaviruses, and Sindbis virus for expression of the desired protein (45,66,68,119). Similarly it is possible to directly engineer a mammalian cell to express fully functional processed proteins (16,182). Unfortunately in many of these cases the yields of the expressed protein are still generally low for their potential production of vaccines for veterinary use.

In many cases it would be advantageous to immunize an animal with a vaccine containing a number of protective proteins from different organisms. This can be achieved by purifying individual proteins from different expression systems. An even more attractive approach is the possibility of developing chimeric proteins containing the protective epitopes from various organisms as one protein. This requires the iden-

tification of the protective components from two different organisms, constructing one chimeric gene containing the protective proteins from the different organisms and expressing them in the expression system of choice. This would dramatically reduce production costs. An attractive possibility might be to identify epitopes on rotavirus and coronavirus and express them on the surface of *E. coli*. Thus it might be possible to immunize calves against *E. coli* K99 expressing linear epitopes of rotavirus and coronavirus as one vaccine. This appears to be at least feasible for rotavirus since the important protective epitopes on this virus have been identified and are known to be linear epitopes (73). Unfortunately at present the majority of the important epitopes identified on coronavirus are conformational (40). As stated above, *E. coli* would not be the expression system of choice for these epitopes. Although there are only two recombinant products that have been approved for use in food animals, a number of relevant genes from pathogens of cattle have been cloned and expressed. It is anticipated that over the next 2–5 years field testing and commercialization of a number of these recombinant subunit products will occur.

## B. Peptides

Subunit vaccines can be produced by chemical synthesis of short polypeptides. Although the technology is not new, improvements made during the past decade in solid phase peptide synthesis have increased the efficiency of the process and lowered the cost. Developments in two complementary areas during the 1970s have made the application of synthetic peptides to vaccine production possible. These are the availability of monoclonal antibodies to define protective epitopes on antigens and the ability to accurately sequence DNA in order to localize these epitopes. While there are no commercially available peptides vaccine for the veterinary market, a number of experimental vaccines have been developed.

One of the first viruses used to investigate the feasibility of using synthetic peptides as a vaccine was foot-and-mouth disease virus (FMDV) (17,20). Unfortunately, in many viruses, including FMDV, antigenic variation can occur. Thus, one needs to find crucial conserved epitopes on the virus before a single broad spectrum synthetic peptide vaccine can be developed. Conserved regions on a number of viruses have been identified. For example, in the case of bovine rotavirus, VP4 contains a proteolytic cleavage site that is conserved on all rotavirus serotypes (44). This protein must be cleaved for virus infectivity. Animals immunized with a peptide corresponding to the cleavage site are protected from infection by a number of rotavirus serotypes (73). Based on these findings we feel that peptide vaccines may be part of the armamentarium of the

vaccinologist in the future. However, once again effective delivery of these peptides, in combination with carriers, adjuvants or appropriate vectors will need to be considered before a truly effective peptide vaccine can be developed.

## C. Live Vaccines

One of the most exciting areas of vaccine research over the past 5 years has been the development of genetically attenuated viral and bacterial vaccines. In principle, a properly delivered live vaccine should be more effective than the corresponding subunit product since all of the relevant antigens are present to stimulate both cellular and humoral immunity, at the appropriate site (e.g., mucosal). The main problem with live vaccines in the past has been the methods used to reduce virulence of the organism. These have included passage *in vitro*, passage through alternate host cells (in the case of viruses), chemical modification, heat inactivation, and the use of conditional mutations (e.g., streptomycin dependence, temperature-sensitive mutants, etc.). In many cases the genetic basis for attenuation was not clear and, therefore, reversion to virulence during commercial scale production or following administration into the animal remained an ever present threat. Perhaps a larger problem is that exposure of an animal to live, albeit attenuated, organism can result in immunosuppression and predisposition to other infections. These problems highlight the need for more rational and defined methods of attenuation and delivery of live organisms as vaccines.

The establishment of a successful bacterial infection requires the expression of a large number of genes specifying classical virulence determinants such as fimbriae and toxins, and components needed for physiological functions of the cell. Strains can be attenuated by creating defined mutations in genes involved in either class of function. For example, *Bacillus anthracis* mutants, which do not produce capsule, are avirulent, although they are still capable of replication in the host (76). Anthrax strains that are defective in aromatic amino acid biosynthesis are less virulent than the nonencapsulated strains and yet provide protection in experimental disease models (75). Thus, the impairment of specific physiological functions that cannot be complemented by the host can be effective in the reduction of virulence. Although we have used *B. anthracis* as an example, above, most of the work has been carried out with enteric pathogens, mainly *Salmonella* sp. (28). Examples of some bacterial attenuating mutations are listed in Table 9.1. Each of these mutations can result in different levels of attenuation as measured by the ability of the organism to persist in the host. For example, *pur* mutants of *Salmonella typhimurium* are able to establish only a transient infection whereas *aro* mutants of the same organism establish infections that are

**Table 9.1.** Bacterial mutations which reduce virulence.[a]

| Gene | Function | Species |
|------|----------|---------|
| *aroA, C, D* | Aromatic amino acid biosynthesis | *Salmonella, Bordetella, Bacillus, Yersinia* |
| *cya, crp* | Adenylate cyclase | *Salmonella, Bordetella* |
| *purA, E* | Purine metabolism | *Salmonella, Yersinia* |
| *ompR* | Porin regulation | *Salmonella* |
| *phoP, Q* | Acid phosphatase | *Salmonella* |
| *galE* | Galactose epimerase | *Salmonella* |

[a] In addition to the above, mutations to rifampicin-resistance, streptomycin-dependence, and various temperature-sensitive mutations have been shown to reduce bacterial virulence, but these have not been well characterized. Mutations in classical virulence determinants are not included.

more persistent albeit subclinical (28). Therefore, it is in theory possible to attenuate organisms to varying degrees depending upon the mutations that are introduced. This technology can be applied to virtually any species of bacteria although it is easier to accomplish in organisms that are genetically characterized. Ideally, at least 2 deletion mutations should be introduced into a host organism in order to ensure safety. For example, Munson et al. (120) developed *cya/crp* double mutants of *Salmonella typhimurium* that when used as a vaccine resulted in protection against challenge by both *Salmonella* and *E. coli* infection in poultry (120). Similar mutations could be introduced into bacterial pathogens of cattle.

Once a vaccine strain has been selected, it is possible to introduce foreign genes into the strain in order to produce a multivalent vaccine. In this case the attenuated host strain would act as a delivery vehicle for heterologous antigens. Table 9.2 lists some representative examples of heterologous genes that have been delivered in attenuated *Salmonella* strains. Generally speaking, high-level expression of the heterologous

**Table 9.2.** List of representative antigens delivered in attenuated *Salmonella* strains.

| Source | Antigen | Attenuating mutation |
|--------|---------|---------------------|
| *E. coli* | LT, B-subunit | *galE, aroA* |
| *E. coli* | K88 pilin genes | *aroA, galE* |
| *E. coli* | K1 capsular polysaccharide | *aroA* |
| *C. tetani* | tetanus toxin, c-fragment | *aroA, aroC* |
| *Str. sobrinus* | spaA | *cya crp* |
| *Str. pyogenes* | m5 protein | *aroA* |
| *S. sonnei* | form 1 antigen | *galE* |

gene product is not required since large numbers of bacteria colonize the host. Therefore, strong promoters used for subunit vaccine production such as *tac* or Lamda-pL are not required. Rather, natural systems using outer-membrane protein promoters and signal sequences, or similar sequences from other surface structures are the most useful. For example, oligonucleotides coding for protective epitopes can be placed in the gene coding for the flagellin protein and expressed on the surface of *S. typhimurium* (95). In cases where recombinant protein production interferes with the growth of attenuated bacteria, it is possible to construct regulated expression systems that synthesize protein only in response to signals encountered in the target animal. For example, promoters that are activated in the absence of free iron or the presence of specific carbohydrates could be used (123). Thus, the bacteria could be easily grown in the laboratory or production facility by limiting the production of otherwise lethal components while maintaining the efficacy of the product.

Organisms attenuated by specific genetic manipulation are not the only potentially useful bacterial systems for the delivery of foreign antigens. Recently, *Mycobacterium bovis* BCG has been adapted for use as a delivery vehicle (77,112). *M. bovis* has been used extensively for vaccination and has excellent adjuvant properties capable of stimulating both cell-mediated and humoral immune responses. While this system will likely find application mainly in human medicine, it is conceivable that it could be adapted for use in the veterinary market if companion diagnostic tests were developed to distinguish vaccinated from infected animals.

Viruses can be used to produce proteins from other viruses. These proteins can then be used either as subunit vaccines or as a live vectored vaccine. Vaccinia virus was one of the first viruses into which foreign viral genes were inserted and shown to be capable of producing the foreign protein and inducing immunity to that foreign antigen (47,119). Since those early experiments, vaccinia has been used extensively to express genes from almost every conceivable virus. In addition, cytokine genes have also been incorporated into vaccinia virus to reduce its virulence and possibly modulate immunity to any coexpressed proteins (48). Vaccinia appears to be extremely attractive for foreign gene insertion, since its genome is very large and it is possible to delete large quantities of DNA and still maintain a viable virus. Based on this observation it should theoretically be possible to insert multiple genes into the vaccinia genome and thereby produce a multivalent vaccine. With increased knowledge of vaccinia virus genetics and promotors that are capable of producing high quantities of proteins, it is proving to be extremely attractive as a carrier for cattle vaccines. The recent introduction of genes coding for Rinderpest proteins into vaccinia and the demonstration of its efficacy in preventing Rinderpest virus infections make it an attractive vehicle for

producing vaccines for Third World countries where thermal stability and cost of the vaccine are primary concerns (198).

The discovery that other viruses, such as herpesvirus and adenoviruses, also have regions within their genome that are nonessential for either *in vitro* or *in vivo* replication has prompted investigators to test these viruses as potential viral vectors. For example, bovine herpesviruses have a number of genes that can be deleted (TK,gIII) without significantly altering replication *in vitro* (90). Such deletions can dramatically change the virulence characteristics of the virus *in vivo* as well as the ability of virus to induce latency (89). Since animals immunized with these herpesviruses deletion mutants will not produce antibody against the protein produced by the deleted gene, these vaccines can be used in conjunction with a diagnostic test to differentiate between animals that are potential carriers of latent field strains of virus and those that have been immunized with the vaccine. This approach is very attractive for immunizing animals in countries or regions where elimination of a specific virus infection is a target (108). European countries that are moving toward bovine herpesvirus and pseudorabies virus eradication programs appear to be embarking on a course to eliminate conventional herpesvirus vaccines. The "marked" vaccines can either be used as live vaccines or killed conventional vaccines.

Adenovirus has also been shown to be an acceptable vector for producing immunity to a variety of antigens inserted into deletable regions of the human and canine adenovirus genome (66,140). Similar studies are now proceeding to identify homologous regions of the bovine adenovirus that can be deleted and used as insertion sites for foreign genes. Since both herpes and adenoviruses are considerably smaller than vaccinia, there is less potential for inserting as many genes into them. However, these viruses may offer practical advantages such as delivery of the virus into the oral or respiratory tract as well as political advantages since countries are still reluctant to reintroduce vaccinia immunization. The introduction of vaccinia recombinants into wildlife populations to control wildlife rabies obviously is overcoming some of the concerns about using vaccinia as a vehicle for controlling infections (132).

In addition to using DNA viruses as vectors recent studies have shown that RNA viruses such as Sindbis and polio can also serve as vectors for vaccine antigens. In the case of polio virus, sequences coding for epitopes from a variety of viral and bacterial pathogens have been incorporated into the virus (21,45). Exposure of animals to the engineered virus resulted in development of immunity not only to polio virus but also to the foreign epitope. Since cattle can be infected with a number of picornaviruses, the ease of constructing the chimeric viruses and efficiency of replication *in vitro* makes this approach easy to test in cattle. One impediment to this approach is the restriction on the size of the genetic material that can be incorporated into picornaviruses.

## 3. Bovine Respiratory Disease

Bovine respiratory disease is the leading cause of morbidity and mortality in cattle around the world. This syndrome can be divided into two major classes depending on the age of the animal and management practices that lead to this disease complex. For example, enzootic pneumonia normally occurs in calves reared under a variety of different management systems. In contrast, "shipping fever" occurs in calves that have been weaned and transported to feedlots for final finishing. Other management conditions will obviously result in manifestations of different types of respiratory disease. In spite of many years of investigation into the pathogenesis and etiology of the various types of bovine respiratory diseases we do not fully understand all the interactions between the host and the pathogen that lead to respiratory disease. What does appear clear is that within this complex, whether it be enzootic pneumonia, shipping fever, or other manifestations of this disease syndrome, no one factor is solely responsible for precipitating the disease. Most people will agree that management (inclement weather, stress, mixing of animals, weaning, crowding, poor nutrition, or immunity) and viral infections (infectious bovine rhinotracheitis, bovine respiratory syncytical virus, parainfluenza 3, bovine viral diarrhea virus, and adenovirus) interact to create an environment that favors colonization and growth of the bacterial agents (*P. haemolytica*, *P. multocida*, *H. somnus*, *Mycoplasma* spp., and *Chlamydia* spp.) in the lung. This does not mean that an animal needs to be exposed to all of these infectious agents and environmental insults at the same time. However, it appears that as the number of insults accumulate the animal has a greater chance of succumbing to infection and severe respiratory disease. In some cases one specific predisposing factor is the major culprit in precipitating the disease, where as in other cases another factor is more important. As a result of these multifactorial interactions an animal suffers from infection and must be treated. In spite of treatment and vaccination, some animals die or develop chronic respiratory disease. Treatment costs, death losses, and reduced performance all lead to tremendous economical losses to the cattle producer and indirectly to the consumer and the economy of each country involved in raising cattle. Unfortunately, even with the best vaccines these losses would not be eliminated without altering management practices. Furthermore, since there are such a large number of pathogens involved in inducing or precipitating the disease it is probably unrealistic to imagine that even with the best vaccines for the most common causes of bovine respiratory disease that this syndrome will be eliminated. In this section we will describe the vaccines available for some of those pathogens perceived to be the most important in this disease complex and how we envisage that these products could be improved through modern vaccine technology.

## A. Bacterial Infections

The principal bacteria associated with bovine respiratory disease are *Pasteurella haemolytica*, *Haemophilus somnus*, *Pasteurella multocida*, *Mycoplasma* spp., and *Chlamydia* spp. However, other bacteria can, under appropriate conditions, cause respiratory infections in cattle. Vaccines for at least some of these organisms have been commercially available for a number of years. These vaccines include bacterins, extracts, and live cells.

### i. Pasteurella haemolytica

*Pasteurella haemolytica* has traditionally been the organism most commonly isolated from the lungs of pneumonic animals in North American feedlots. There are at least 15 serotypes and 2 biotypes of *P. haemolytica*, with biotype A, serotype 1 being the most common isolate found in respiratory disease. However, in many cases it is very difficult to experimentally reproduce the disease by exposure of animals to an aerosol of *P. haemolytica* itself. Second, it is often possible to isolate *P. haemolytica* from the upper respiratory tract and tonsillar crypts of healthy animals. These factors all support the contention that *P. haemolytica* is an opportunistic pathogen. Although the mechanism of pathogenesis by *P. haemolytica* is not completely clear, a number of potential virulence determinants have been identified. These include a secreted leukotoxin, proteases, neuraminidase, capsular polysaccharide, endotoxin, outer-membrane components, and fimbriae (4,13,39,117, 129,137,167). While each of these antigens may provide some level of immunity it appears likely that a combination of secreted and cellular components will be necessary to provide maximal resistance to *P. haemolytica* infections (170).

In experimental trials, vaccination with several products including extracts, live bacteria, and bacterins have provided some degree of protection (25,33,111,169,197). Unfortunately, data obtained from field trials were not as convincing (15,34,110). This is not surprising, since vaccination often occurs at entry into feedlots and there is insufficient time for protective immunity to develop. Second, many of the extracts or bacterins do not contain sufficient quantities of the extracellular leukotoxin, a component recognized to be crucial in preventing damage to the leukocytes that are responsible for aiding in clearing the Pasteurella from the lungs (54,168). Third, although extract vaccines are probably more efficacious than bacterins, there are problems associated with antigenic competition between protective and nonprotective components and immunosuppression due to some as yet undefined bacterial components (34).

The live *P. haemolytica* vaccines include streptomycin-dependent, chemically altered, and attenuated live strains of *P. haemolytica* (18,

84,148). Intradermal vaccination of calves with the modified live vaccine has given mixed results. In some conditions it appeared to be protective where as in other cases there was no decrease in morbidity or mortality (148,173). One can only speculate as to the reasons for these inconsistent results but they could be related to the route of administration and the level of replication of the bacterium in the animal. Many producers treat animals with antibiotics upon entry into feedlots to reduce bovine respiratory disease. Obviously, if animals are treated either through medicated feed or direct injection of antibiotics this will have a impact on the degree of replication of the bacterium and as a result the level of immunity induced by the vaccine. Regardless of the reasons for mixed responses, under field conditions, this type of vaccine has not made a significant impact on reducing bovine respiratory disease.

As a result of the problems encountered with live and killed bacterial cell products, a number of investigators have focused on identifying the important protective components of *P. haemolytica* and producing vaccines containing predominantly those components (34). At present an extract vaccine supplemented with culture supernatant containing leukotoxin is showing some benefit in high risk calves under field conditions (80). With a greater understanding of the protective components present in the bacterins and the extracts as well as the ability to produce these components economically by recombinant DNA techniques it is envisaged that the new and improved future vaccines will be subunit products containing only a few protective components. Candidate antigens for these vaccines include leukotoxin and one or more outer surface proteins. The gene coding for leukotoxin has been cloned and expressed in *E. coli* and experimental plus field data indicate that it is protective on its own but increased protection can be attained by combining it with other *P. haemolytica* antigens (101,138). The leukotoxin has been shown to be structurally similar to the *E. coli* α-hemolysin (102), as well as several other toxins produced by Gram-negative pathogens (93,179,193). Therefore, the potential exists to develop cross protective vaccines based on leukotoxin by constructing chimeric genes which code for neutralizing epitopes specific for each protein. Genes for a serotype 1 specific antigen plus other outer membrane proteins have been cloned and expressed (60). Preliminary data indicate that at least some of these outer membrane proteins, when combined with leukotoxin, provide enhanced protection against *P. haemolytica* infection (Potter et al., unpublished results) and we anticipate that fully recombinant subunit vaccines will be available in the very near future.

### ii. Haemophilus somnus

*H. somnus* is associated with a number of disease syndromes of cattle, including ITEME, pneumonia, myocarditis, arthritis, and reproductive

disorders (67). The incidence of *H. somnus* in bovine respiratory disease has increased in recent years, perhaps due to more reliable detection of the organism. Both upper and lower respiratory tract infections are encountered in the field, including laryngitis, tracheitis, and suppurative bronchopneumonia. There are reports that under experimental conditions, exposure of calves to respiratory viruses results in increased susceptibility to *H. somnus* pneumonia (136). All commercially available vaccines are killed bacterins. Both experimental and field efficacy has been demonstrated for only one of these products (62,150,162,178), and two vaccinations are required for adequate protection, a practise not often followed in the field. An experimental acellular vaccine consisting of the anionic fraction of a surface component extract was shown to be effective in the prevention of ITEME, but this has not been field tested and is not commercially available (177). However, the results demonstrate that vaccination with the appropriate subunit antigens can be effective. Gogolewski et al. (56) demonstrated that antibody directed against a 40,000 MW outer membrane protein (OMP) was capable of providing passive immunity, while similar experiments with a 78,000 MW OMP did not result in protection. Therefore, the 40,000 MW OMP appears to be a good candidate antigen for a subunit vaccine. Other potential virulence determinants include fimbriae, Fc receptor, and surface components necessary for nutrient scavenging and transport (Pontarollo and Potter, unpublished observations, 194,196). Czuprinski and Hamilton (36) have shown that *H. somnus* is able to survive in phagocytic cells and also to impair neutrophil function (96). The latter is due to secreted adenine, guanine, and guanidine monophosphate plus high-molecular-weight components (29). It would be a great advantage for any subunit vaccine to block both neutrophil suppression and the ability of the organism to survive in phagocytic cells, although more work has to be done to identify the mechanism involved before such a vaccine can be developed. As with *P. haemolytica*, it is likely that at least two subunit antigens will be necessary for protection, and the choice of antigens may vary depending upon the particular disease syndrome being targeted. Combination *H. somnus–P. haemolytica* subunit vaccines might also include shared antigens such as the 78,000 MW antigen recently described by Kania et al. (86). This *H. somnus* outer membrane protein was present on all strains examined, reacted strongly with convalescent serum, and cross-reacted with proteins of similar molecular weights from *P. multocida*, *P. haemolytica*, *Actinobacillus lignieresii*, *A. equuli*, *Enterobacter cloacae*, *H. influenzae*, and *H. agni* (86).

### iii. Mycoplasma

The principal mycoplasmas involved with bovine respiratory disease are *M. bovis*, *M. dispar*, and *M. mycoides*. The latter is the causative agent of contagious bovine pleuropneumonia (CBPP). Attenuated vaccines

have been available for a number of years and are generally effective, but vaccination is controlled by local legislation in parts of the world. Vaccination with inactivated *M. bovis* has been shown to prevent pneumonia and mastitis, but killed *M. dispar* vaccines are not effective. When compared to the other bacterial agents associated with bovine respiratory disease, mycoplasma virulence is poorly understood. Adhesion to the respiratory epithelium is an important step in pathogensis, but the nature of the adhesin has not yet been shown, although ruthenium red-staining material may be involved (153). *M. bovis* and *M. dispar* both impair the phagocytic capacity of bovine neutrophils and *M. mycoides* can induce leukopenia (153). This may be one mechanism by which mycoplasma can predispose high risk cattle to infection with other bacterial agents. Prior to the development of new vaccines it is clear that we must have a greater understanding of these virulence mechanisms of the organisms and their interaction with the host.

## B. Viruses Causing Bovine Respiratory Disease

As stated above, bovine respiratory disease is often a complex where in any one of a number of viruses can either cause infections individually or collectively. The viruses incriminated in bovine respiratory disease include bovine herpesvirus-1 (BHV-1) also referred to as infectious bovine rhinotracheitis, parainfluenza-3 (PI-3), bovine respiratory syncytial virus (BRSV), and adenoviruses (154). Although bovine viral diarrhea (BVD) virus has also been incriminated in bovine respiratory disease it will be discussed in Section 5B. Of these viruses, bovine adenoviruses are probably of the least significance in causing severe clinical infections. In addition to causing respiratory infections, adenoviruses of cattle can spread systemically and cause a variety of other often self-limiting diseases, with the severity possibly increasing following specific stressors. Although a number of vaccines have been tested to control bovine adenovirus infections, their general mild nature and the large number of serotypes (nine) present, it seems unlikely that vaccination will ever play a predominant role in the control of adenovirus infections globally. Vaccines against bovine adenovirus containing various combinations of serotype 1, 3, and 5 have shown some ability to prevent infection against the homologous serotype in Europe. At least 2–4 doses are required to confer protection. Such vaccines are not licensed in North America (22). The observation that it is possible to insert genes into a number of nonessential regions of adenoviruses has prompted a number of investigators to speculate that adenoviruses may be a useful vector as a live delivery method for delivering vaccines to mucosal areas. If this proves to be correct then one could immunize against the foreign antigen being produced by adenovirus as well as against adenovirus itself. These possibilities are highly likely in the future.

The most widely used virus vaccines to control bovine respiratory disease include a combination of BHV-1, PI-3, and BRSV. Many vaccine companies combine all 3 or a minimum of 2 of these pathogens either as live attenuated vaccines or killed vaccines. Although all of these vaccines can induce some immunity following either single or double vaccinations the actual value of the vaccines for controlling respiratory diseases in cattle has been questioned. There are a number of reasons for this possible lack of efficacy. (a) To ensure that the viruses themselves do not cause infections they need to be attenuated sufficiently to reduce the level of replication *in vivo*. If this is achieved they may not replicate sufficiently in the animal to produce a sufficient antigenic mass to stimulate a high enough level of immunity. (b) In the case of killed virus vaccines, the quantity of the individual protective components may not be sufficient to stimulate the immune response. (c) Probably the most important reason for the lack of efficacy is in how they are used. Animals are often weaned and transported to sales barns, where they are mixed with other animals and their pathogens before being transported to feedlots where they are immunized. Thus some animals are probably infected even before vaccination and adequate immunity develops. If immunization with good vaccine occurred prior to weaning and transportation they should have a much better performance record.

Another possible reason why these vaccines do not significantly reduce bovine respiratory disease is that the disease is a complex, and that etiological agents other than the ones present in the vaccine could be inducing the disease. Thus, the development of vaccines for all of the agents, as well as immunization prior to movement and mixing of cattle should greatly reduce disease incidence. Whether this will ever become a reality remains to be determined. Recent evidence indicates that some of the live virus vaccines in the bovine respiratory disease complex may actually be immunosuppressive (72,155). Thus combining a myriad of viral and bacterial antigens in one vaccine may be contraindicated. To overcome this immune interference, considerable progress has been made at identifying the important protective proteins of all of the viruses involved in the respiratory disease complex. These include the gI, gIII, and gIV glycoproteins of BHV-1, the G and F proteins of BRSV, and the HN and F proteins of PI-3. In the case of BRSV it is possible that the F and G proteins from the two serotypes will be required for maximal protection. These proteins are being cloned in various expression systems and will hopefully provide excellent immunity against the viruses involved in this complex.

At present excellent models exist to test the efficacy of BHV-1 and PI-3 vaccines, therefore, it should be easy to prove the efficacy of the subunit vaccines for these two viruses (10). Unfortunately, there is not a good model available for testing efficacy of BRSV vaccines. This will probably delay the speed with which effective vaccines can be developed and

verified for BRSV. A second approach to developing new vaccines to the bovine respiratory disease complex is to use either adenovirus or bovine herpesvirus as a vehicle for delivering protective antigens from a number of the respiratory disease pathogens (see Section 2C). Bovine herpesvirus has at least 5 different genes that are considered to be nonessential for virus replication *in vitro*. These include TK, gIII, and gX. A number of these genes have been deleted from BHV-1 virus and substituted with genes coding for the protective proteins of other viruses involved in the disease complex (98). Although none of these chimeric virus vaccines has been licensed yet, it is envisaged that within the next 5 years we will witness a number of vaccines based on this technology on the market. An advantage of these chimeric viruses is that one can deliver the vaccine intranasally to provide protection at the site of initial infection. The economics of producing one vaccine that will protect against 3 or 4 different viruses should also be very attractive. However, regardless of how effective these future vaccines are in inducing immunity, it will be important to incorporate alterations in management systems to provide an adequate opportunity for the development of immunity to the pathogens prior to movement of animals into high risk environments.

## 4.  Enteric Pathogens

As in the case of the bovine respiratory disease complex, calf diarrhea is also a disease complex involving interactions between different viral and bacterial infectious agents, environmental factors, and the immunological status of the animal. The major viral causes of neonatal diarrhea include rotavirus and coronavirus. However, bovine viral diarrhea, bredavirus, and a few minor viruses such as calicivirus and astroviruses have also been incriminated as pathogens in calfhood diarrhea. Bacteria involved in inducing diarrhea include enterotoxigenic *E. coli, Clostridium perfringens* Type C, *Salmonella* spp., and *Campylobacter* spp. In each case the severity of diarrhea is related not only to the virulence of the specific pathogen but to the age of the animal at the time of infection as well as to the presence of other pathogens. It has been shown in a number of studies that only a minority of cases of diarrhea in cattle are caused by a single pathogen (11,160,175). Therefore, if two pathogens can coinfect an animal their combined effect may be much more severe than if they infected the animal individually. In addition to the interactions of various pathogens, a number of other factors such as climatic conditions, standard of housing, hygiene, population density, and nutritional and immunological status of the animal all influence the severity of diarrhea. These cofactors are outside the scope of this review and therefore will not be discussed further. However, one should not overlook the importance of these cofactors in determining the severity of diarrhea in calves. Since

most cases of diarrhea in newborn calves are clinically characterized by an acute perfuse watery diarrhea leading to progressive dehydration and acidosis, it is impossible to differentiate the causes of diarrhea based on clinical observations. If animals are not treated quickly, death can occur. As a consequence, clinicians must institute therapeutic and control strategies to ensure survival of the animal. In many cases this treatment involves the use of intensive fluid and electrolyte therapy to replace the water and electrolyte deficits and alkalizing agents such as sodium bicarbonate to reverse acidosis. Oral therapy is effective only if used before the animal becomes overly dehydrated and diarrheic. Additional treatments usually involve restriction of milk intake to prevent the osmotic movement of fluids into the lumen. If these management systems are implemented quickly mortality is often quite low.

## A. Bacterial Vaccines

### i. *E. coli*

Although enterotoxigenic *E. coli* is most often associated with bovine diarrhea during the first 3–5 days of life, it is not uncommon to see occurrences of the disease for an additional 2 weeks. Many serotypes have been found to cause disease, including O8, O9, O20, O64, and O101 (3,64). The main determinants of virulence associated with these strains are colonization factors, capsular polysaccharide, and toxins (3). Effective vaccines for the prevention of neonatal *E. coli* infections have been available for a number of years. These include formalin and heat-killed bacterins, live vaccines, fimbrial capsular extracts, and toxoids. Acres et al. (2) showed that protection correlated with anti-K99 fimbriae antibody levels, but not with antibody to capsular polysaccharide, although the latter component has been shown to induce protective immunity (65,121). All of these vaccines are administered to the dam 3 weeks prior to parturition and antibodies are transferred by colostrum and milk to the newborn. Since most infections in calves occur during the first 3–5 days postpartum, this method of vaccination is usually effective. In addition to vaccinating the dam, feeding calves colostrum with high levels of antibody or monoclonal antibody to K99 has proven to be very effective in controlling *E. coli* K99 induced diarrhea (166).

The initial stage in colonization of the intestine is mediated by fimbriae (52,116). K99 and/or F41 fimbriae are associated with virulence and both are effective immunogens, forming the basis of many effective vaccines presently available (1,2,64). Since type-1 fimbriae do not play a major role in colonization of the intestine they do not induce protection in cattle or humans. Capsular polysaccharide may play a role in colonization, perhaps as a secondary event to fimbrial-mediated binding. Antibody to

capsular polysaccharide has been shown to correlate with protection in some studies (65,121).

Enterotoxins have been shown to be virulence determinants. These toxins are members of either heat-labile (LT) or heat-stable (ST) families. Heat-labile toxins are antigenically related to cholera toxin and exert their effect by interfering with the regulation of adenylate cyclase activity (46,64). This is mediated by the A-1 subunit of the toxin. Heat-stable toxins can belong to one of two families, STa or STb. STa is unrelated to LT and exerts its effects by the stimulation of intestinal guanylate cyclase (46). STb, which is structurally distinct from STa, does not effect guanylate or adenylate cyclase.

The genes coding for fimbrial antigens plus LT, STa, and STb have all been cloned in *E. coli*, raising the possibility of using either recombinant subunits, or more likely, live attenuated vaccine stains producing these antigens. The principal advantage of using attenuated bacteria to deliver recombinant antigens is their ability to stimulate an effective mucosal immune reponse. The ability to manipulate these toxin genes has resulted in the production of a toxoid that retains its antigenic properties (64). Therefore, it is possible to construct attenuated *Salmonella* strains, for example, which carry K99 and/or F41 fimbrial genes and produce toxoids, which are specifically targetted to the gut (see above). Licensed swine vaccines based on recombinant strains that produce fimbriae are currently available in the United States and Europe.

### ii. *Salmonella*

*Salmonella* infections in animals can cause enteritis, abortion, septicemia, or a combinations of the above three diseases. The bovine enteric form is often caused by *Salmonella typhimurium* and *Salmonella dublin*. Both adult animals and calves are susceptible to the disease, but unlike *E. coli* diarrhea, it is usually not seen in calves less that 2–3 weeks of age. *Salmonella* strains produce a number of virulence determinants similar to those described for *E. coli*. Colonization and invasive factors have been described and several different toxins have been implicated in stimulating intestinal fluid production. The pathogenesis of *Salmonella* infections is different from enterotoxigenic *E. coli* in that the organism can replicate intracellularly in macrophages (30,32). Therefore, it is inaccessible to antibiotic therapy and this can lead to the development of chronic carriers. Although humoral antibody is likely important in resistance to disease, cell-mediated immunity is more important, as one would expect with an intracellular pathogen (100,152). Vaccination with attenuated strains can lead to protection without a humoral response, whereas vaccination with bacterins which elicit a good antibody response are not always effective (100,152,174). Attenuated vaccine strains (*aroA*) have been shown to elicit antibody, delayed-type hypersensitivity and cytotoxic T-lymphocyte

responses in mice (49,135,158). In a recent study, 7 out of 8 calves which were immunized orally with an *aroA aroD S. typhimurium* strain 7 days after birth were protected against experimental challenge 7 weeks after vaccination. These studies demonstrate the feasibility of using live *Salmonella* vaccines (81).

## B.  Viruses

As stated above, rotavirus and coronavirus appear to be the most common viral pathogens involved in gastroenteritis of neonatal calves. It is for these two viruses that vaccines have been developed. Since no vaccines are presently available for bredaviruses, astroviruses, caliciviruses, and other viruses seen periodically in diarrheaic calves they will not be discussed further. However, the principle of vaccination of neonatal calves described for rota and coronaviruses could be applied to controlling or developing vaccines against these latter viruses. Vaccination against rotavirus and coronaviruses has been directed at two basic modes of immunization: (a) active immunization of the calf and (b) passive immunization of the calf via hyperimmunizing the dam to transmit antibody to the calf during suckling. In both approaches vaccination appears to have limited effectiveness (180,190). The possible reason that active immunization is of limited value is directly related to the epidemiology of this infection. To provide adequate protection from infection, local immunity in the gastrointestinal tract is required (160,180). To provide local immunity a oral vaccine has been developed for calves. Since almost all cattle have antibody to rotavirus and coronavirus in their milk, antibodies in the milk quickly neutralize the vaccine virus and thereby prevent induction of immunity. Thus the vaccine must be administered at a time prior to suckling. This is often difficult to achieve and a delay in suckling may influence antibody transfer to the calf. A second reason for lack of possible activity in the field is that the present vaccines contain only one serotype (serotype 6). Recent studies indicate that calves can be infected with more than one serotype and immunization of calves with one serotype does not always protect against challenge with a heterologous serotype (180). A third reason why oral vaccines are of limited value is that calves are susceptible to infection early in life, very shortly after colostral antibodies decline. This occurs within 5–7 days postcalving. Therefore, the time interval between oral immunization and exposure to field strains of virus is insufficient to develop protective levels of immunity required to prevent infection in the neonatal calf. Thus, the epidemiology of this disease makes it very difficult to implement effective active immunization methods.

   The impediments to active immunization of the calf has led to the recent trend of hyperimmunizing the dam at mid-gestation and boosting at late gestation. This procedure results in much higher initial levels of

colostrum and milk antibodies. More importantly, even though milk antibodies drop after parturition they remain above a threshold level for the first few weeks after parturition. Under experimental conditions such an approach has resulted in protection from diarrhea. Unfortunately, under field conditions, the efficacy of the presently licensed vaccines has been questioned (159,180,190). The reason for the low level of effectiveness of these vaccines is probably related to the low levels of virus in the vaccine. Unfortunately, both of these viruses are relatively difficult to culture *in vitro* to high levels. However, improvements in growing these viruses during the past few years has increased the antigenic mass in the vaccines. To further increase the efficacy of these vaccines will require the use of genetic engineering techniques. Considerable progress has been made recently in this regard. For example, in rotaviruses, VP4 contains a proteolytic cleavage site that is conserved among all rotavirus serotypes. A synthetic peptide vaccine directed against the VP4 cleavage site has been shown to induce immunity against not only the bovine rotavirus serotype 6, from which the peptide was derived, but also against a variety of other serotypes from various species (73). Neutralizing antibodies have also been produced against a baculovirus produced VP4 protein (106). Since both synthetic peptides and recombinant proteins have shown to induce high levels of neutralizing antibody, it is envisaged that this protein or a portion of it could be produced in *E. coli* at sufficient levels that would make the vaccine economical. The ability to produce rotavirus proteins in *E. coli* makes it very attractive to insert the rotavirus gene into a *E. coli* K99 producing strain; thus one vaccine production system could result in a vaccine against both the *E. coli* and rotavirus. In the case of bovine coronaviruses, the proteins involved in inducing neutralizing antibodies have also been identified and cloned. Unfortunately, in this case the majority of the protective epitopes are conformational, thereby requiring recombinant subunit production in eukaryotic systems.

The final method of providing high levels of antibody in the lumen of the calf is by feeding monoclonal antibodies to the animal. Although this has proven to be very effective in preventing *E. coli* induced diarrhea (see above), it is not envisaged to be practical for viral induced neonatal diarrheas. The reason for this is that diarrhea in young calves can occur over an extended period of time. Thus it would not be economical to feed monoclonal antibodies to calves for a 3-week period. However, in a very severe outbreak it may be possible to prevent infection until management conditions are altered.

The final method of reducing enteric infections is by proper management. Since it is assumed that infection occurs as a result of virus shedding from adults in the environment, animals should not be crowded into contaminated areas. Movement of young calves into clean environments, away from other animals, will greatly reduce the rate of infection and economic loss (1,3).

## 5. Systemic Diseases

## A. Bacterial Infections

### i. Hemorrhagic Septicemia

*Pasteurella multocida* causes not only respiratory disease in cattle but also hemorrhagic septicemia in cattle and water buffalo. The disease can result in severe morbidity and mortality, primarily in tropical regions such as Asia, Africa, and South America. Two serotypes, B:2 and E:2, are associated with the disease, with the latter occurring primarily in Africa. Virulence determinants and protective antigens have not been studied in detail although the B:2 strains produce hyaluronidase, neuraminidase, and cell-associated protein antigens (27,42,69,141,142,143). Capsular polysaccharide is also produced and vaccines based on capsule have been shown to be protective in experimental challenge models (26,122,134). While *P. multocida* strains associated with other disease syndromes produce a dermonecrotic toxin, no relationship between toxin production and hemorrhagic septicemia has been observed.

Following exposure of animals to the organism, clinically healthy animals often carry the bacteria in the nasopharynx and tonsils. Shedding of the bacteria by such carriers can be induced by environmental stress. It is possible to passively protect animals from experimental challenge with serum from hyperimmune animals, indicating that a humoral response to the bacteria should be sufficient for protection in the field (26). In fact, oil-adjuvanted bacterins are generally effective in preventing disease. As with the pneumonic strains, live vaccines including streptomycin-dependent mutants have been used successfully in experimental vaccine challenge trials (192). Also subcutaneous vaccination with live B:3,4 strains, could protect against experimental challenge with B:2 strain. Since the disease occurs principally in isolated areas, the ideal vaccine would have to be stable, inexpensive, and easy to administer. The most appropriate type of vaccine would be a live attenuated strain that has defects in one or more "housekeeping" genes (see above) and could be delivered in drinking water or intranasally. While other products (bacterins and subunits) may be as stable as attenuated organisms and inexpensive to produce, intramuscular injection may not be as practical in rural areas of developing countries where animal density is low.

### ii. Anthrax

*Bacillus anthracis*, the causative agent of anthrax, is a Gram-positive, spore-forming organism found throughout the world. Spores can be found in environmental and tissue samples that have been exposed to the

atmosphere. Such spores can enter the host by ingestion, inhalation, or through skin lesions and the organism quickly enters the bloodstream where it establishes secondary sites of infection. Death is usually rapid, due to bacteremia and toxemia.

Two virulence determinants have been well characterized—the poly-D-glutamate capsule and the anthrax toxin (87,99,139). Genes coding for these virulence determinants are carried on two plasmids, pXO1 and pXO2 (115,184). Antibody against toxin is necessary for protection while antibody to capsule is not required (76). The currently used veterinary vaccine is based on this principle and contains spores from a toxigenic, noncapsulated strain. Vaccination results in protective immunity within 10 days, with annual boosters required. Current research is focussing on the development of attenuated *B. anthracis* strains and also subunit vaccines based on anthrax toxin. Vaccination with transposon-induced mutants defective in aromatic amino acid biosynthesis have been shown to be protective in a mouse model, as has recombinant *B. subtilis* carrying the genes for the protective antigen (PA) toxin component (75). Well-characterized, attenuated strains and delivery systems such as these will likely be the focus of a new generation of veterinary vaccines. Potential recombinant subunit vaccines are also being developed, based on the protective antigen. The protective antigen, one of the three components of anthrax toxin, binds to host receptors and is then proteolytically cleaved (172,188). This cleavage is necessary for interaction with the other toxin components. The gene coding for PA has been altered by site-directed mutagenesis to remove the cleavage site and the modified gene expressed in *B. subtilis* (172). Administration of this modified PA to mice blocked the lethal action of authentic PA, presumably by competition for receptors. The altered protein may be an effective subunit vaccine for humans, replacing the subunit PA vaccine now used. It may also form the basis of an effective subunit vaccine in cattle if the downstream processing of the antigen is minimized to reduce production costs to an economical level.

### iii. Brucellosis

Brucellosis is a disease of global importance affecting most species of livestock. *Brucella abortus* infection often occurs in pregnant cattle as a result of contact with aborted fetuses or placental tissue. The bacteria are then able to colonize and penetrate the mucosal epithelium. The organism is able to replicate intracellularly in phagocytic cells and this virulence trait may enable it to reach other tissues, especially regional lymph nodes, although this is certainly not a requirement for secondary infection. This process is slow, taking weeks to months and chronicity of the disease is aided by its survival in phagocytic cells. Attenuated *B. abortus* strain 19 has been used extensively as a veterinary vaccine and it

has been shown to protect 65–85% of calves vaccinated in the field (181). Strain 19 delivered orally also protects against experimental challenge (125). Inactivated adjuvanted virulent strains have also been used with similar efficacy rates. Since most countries still slaughter *Brucella* infected animals, it is important for one to be able to distinguish vaccinated from infected animals. Thus, either a subunit vaccine or subunit component delivered on a live attenuated carrier (e.g., *Salmonella*) is attractive. It is clear that any vaccine should stimulate not only a humoral response, but also cell-mediated immunity. In this respect, the appropriate antigen delivered with attenuated carriers such as *S. typhimurium*, *Francisella tularensis*, or BCG may form a very effective vaccine.

### iv. Vibriosis

Vibriosis is a bovine venereal disease caused by *Campylobacter fetus*. The organism is spread by sexual contact or contaminated semen resulting in infertility, delayed conception, and abortion. The disease is characterized by inflammation of the epithelial surfaces of the female genital tract. Virulence determinants of the organism have not been studied in detail, but it is clear that *C. fetus* is resistant to phagocytosis, possibly mediated by capsular polysaccharide. In the presence of opsonizing IgG, the organism is efficiently killed (35). Thus, commercially available bacterins appear to be effective due to their ability to stimulate a systemic IgG response. The predominant antibody isotype found in the genital tract is IgA, which cannot opsonize *C. fetus*, but will compete with IgG (35). Thus, stimulation of an efficient mucosal response to vaccination may not be desirable in this case. Since commercially available vaccines are effective in the prevention of vibriosis, it is unlikely that new products will appear in the near future. However, improved diagnostics are needed as companion products to vaccines for the screening of contaminated semen. Thus, species-specific reagents, including monoclonal antibodies to surface components and gene probes, would be useful companion products with traditional vaccines.

### v. Moraxella bovis

*Moraxella bovis* is the agent responsible for infectious bovine kerato-conjunctivitis (IBK), or pinkeye. Once established in a herd it can spread rapidly, infecting a large number of animals. Symptoms of the disease appear quickly and the infection is generally cleared within 1–2 months. A less severe form of IBK can also be caused by Mycoplasma infection and this can result in an increased susceptibility to colonization by *M. bovis* (147). However, this is not a prerequisite for *M. bovis* infection. The primary economic problem is weight loss in beef cattle or decreased milk production in dairy cows. *M. bovis* is a relatively well-characterized veterinary pathogen and a number of virulence determinants have been

identified. These include well-characterized fimbriae, thought to be involved in colonization, heat-labile hemolysin, hyaluronidase, and collagenase (8,133,161). Nonpiliated, nonhemolytic strains are encountered frequently in the field as part of the normal bovine flora. These can also occur spontaneously upon laboratory passage of virulent strains. Such variants are avirulent, and nonhemolytic strains have been shown to be effective live vaccines, probably due to the induction of localized humoral or cellular immunity (146). Conventional bacterins and extracts are not always effective as vaccines, whereas subunits composed of pili have been shown to be protective in experimental models (145). The gene coding for the fimbrial subunit has been cloned and expressed in *E. coli* and this may make the production of a subunit vaccine economically feasible for the veterinary market (109). The assembly of fimbrial subunits on the surface of a live delivery vehicle (see above) is also an attractive alternative since such a product should potentially stimulate a cell mediated immune response.

### vi. Clostridia and Leptospira

At least 7 different clostridial species are economically important pathogens of livestock. These include *Clostridium novyi*, *C. chauvoei*, *C. haemolyticum*, *C. septicum*, *C. perfringens*, *C. sordellii*, and *C. tetani*. All of these species produce one or more potent toxins that are central to the disease process eventhough their modes of action are different. A summary of the *Clostridial* species, disease syndromes, and virulence determinants is shown in Table 9.3.

Conventional vaccines against *Clostridial* diseases are composed of chemically inactivated, aluminium hydroxide-absorbed cultures with or

**Table 9.3.** Disease syndromes and toxins produced by clostridial species.

| Species | Disease syndrome | Toxin produced |
|---|---|---|
| *C. botulinum* | Botulism | Strains produce one of eight different neurotoxins ($C_2$ = inhibitor of protein synthesis); Types A, B, C, D are associated with cattle |
| *C. tetani* | Tetanus | Tetanus toxin; hemolysin (tetanolysin) |
| *C. chauvoei* | Blackleg, wound infection | $\alpha$-Toxin (necrotizing hemolysin); $\beta$-toxin (deoxyribonuclease); $\gamma$-toxin (hyaluronidase); $\delta$-toxin (hemolysin) |
| *C. septicum* | Wound infection | $\alpha$-, $\beta$-, $\gamma$-, $\delta$-Toxins ($\alpha$, $\beta$ serologically related to *C. chauvoei*), neuraminidase |
| *C. novyi* | Gas gangrene | Type A produces $\alpha$-, $\gamma$-, $\delta$-, $\varepsilon$-toxins; Type B produces above plus $\beta$-, $\zeta$-, $\varepsilon$-, $\theta$-toxins; Type C is nontoxigenic |
| *C. haemolyticum* | Bacillary hemoglobinuria | Produces *C. novyi* $\beta$- (phospholipase), $\varepsilon$-, and $\theta$-toxins |

without detoxified supernatants. In addition, vaccines composed of the toxoid alone are effective products and are available for some species. Vaccines are usually sold as 2-way, 7-way, or 8-way products, containing *C. chauvoei* and *C. septicum* (2-way), *C. chauvoei*, *C. septicum*, *C. novyi*, *C. perfringens* Type C and Type D, *C. sordellii* or *C. tetani* (7-way), and the 7-way product plus *C. haemolyticum* (8-way). Due to the effectiveness of these products, it is unlikely that they will be replaced in the near future. The toxins produced by *Clostridial* species have generated considerable interest for the human vaccine market. For example, genes coding for tetanus toxin, which are localized on a bacteriophage, have been cloned and expressed in *E. coli*, raising the possibility of producing a recombinant DNA subunit vaccine. It is clear from Table 9.3 that the production of recombinant subunit vaccines for all *Clostridial* species in cattle will be a major undertaking. Due to the number of antigens that would have to be produced the costs will be excessive when compared to conventional products. However, it may be feasible to supplement chemically inactivated cells with recombinant toxoids rather than the detoxified culture supernatants currently in use.

Leptospirosis in cattle can be caused by several serovars, including *L. canicula*, *L. hardjo*, *L. pomona*, *L. icterohaemorrhagiae*, and *L. grippotyphosa*. Upon infection, these organisms colonize the liver and then spread to other tissues especially the kidney. The most common clinical signs of disease include fever, depression, anorexia, abortion, stillbirth, and decreased milk production in dairy cattle. Frequently, the mode of transmission is via organisms that are shed in the urine. Leptospiras are also capable of passing to the placenta causing fetal infection and abortion. Although very little is known about the basic physiology or pathogenesis of these organisms, this has not hindered development of effective vaccines. Inactivated bacterins have been used extensively for vaccination and this has met with a high degree of success in the field. Due to minimal cross-protection between serovars, five-way bacterins containing each serovar are needed. These conventional products are safe and effective and it is therefore unlikely that a focused effort will be made to apply the tools of modern biotechnology to improve their efficacy.

### vii. Mastitis

Bovine mastitis can be caused by infection with a wide range of Gram-negative and Gram-positive organisms, the most common being *E. coli*, *S. aureus*, and several environmental streptococcal species (*Streptococcus agalactiae*, *Str. dysgalactiae* and *Str. uberis* for example). It is the largest single factor contributing to economic losses caused by infectious disease to the dairy producers world wide. The mammary gland provides a rich environment for the growth and multiplication of bacteria that

enter through the teat canal. Chemotactic metabolites produced by the organisms result in an influx of PMNs, causing a severe inflammatory response (63). If the infection persists for an extended period, then the mammary gland exhibits clinical mastitis. The control of mastitis by immunization against specific pathogens, mostly *S. aureus* and *E. coli*, has been practised for a number of years. However, vaccination against one or two pathogens in a multifactorial disease syndrome cannot be expected to reduce the overall incidence of the disease.

The *E. coli* strains associated with mastitis are indistinguishable from fecal isolates and likely originate from contaminated bedding. These strains produce typical *E. coli* virulence determinants, such as capsule, fimbriae, endotoxin, and cytotoxin, with the latter two being the most important in eliciting a severe inflammatory response and damaging the teat end and mammary parenchymal tissue. Blockage of iron acquisition has been shown to be bacteriostatic and thus iron-regulated outer-membrane proteins produced for the transport of iron may be useful antigens for a subunit vaccine (183). Immunization with the *E. coli* mutant J5 has been shown to reduce the severity of experimental mastitis and field studies have shown that vaccination could increase annual income by $32.00/cow (58,59). Immunization with a commercially available *E. coli* scours vaccine also reduced the severity of the experimentally induced coliform mastitis (187). However, neither vaccine is efficacious in reducing the rate of new *E. coli* infections.

The principal Gram-positive organism associated with mastitis is *S. aureus*, a common inhabitant of the skin. The organism produces a wide range of enzymes and virulence determinants including coagulase, hyaluronidase, nucleases, lipases, proteases, and a number of toxins ($\alpha$-, $\beta$-, $\gamma$-, and $\delta$-leukocidin) (9). $\alpha$-Toxin and leukocidin affect PMNs and can lyse target cells before or after phagocytosis (i.e., from both extra- and intracellular environments) (189). However, the organism is often resistant to phagocytosis and this is possibly mediated by capsule, protein A or coagulase-mediated aggregation of cells (6). Not all strains produce capsule or protein A and the role of the former in virulence is unclear. Vaccination with $\alpha$-toxin or coagulase does not result in protection against experimental challenge, whereas vaccination with protein A can offer minimal protection (131). However, since not all strains produce each virulence determinant, a single subunit is unlikely to work in the field. A virulent isolate which was attenuated by *in vitro* passage until it became nonhemolytic was shown to be an effective experimental live vaccine (191). Inactivated bacterins which are currently lisenced for use are only partially effective. A mixed lysate of *S. aureus* strains containing polyvalent antigens is also commercially available in North America and can provide immunity to experimental challenge for up to one year.

The three streptococci, *Str. agalactiae* (Group B), *Str. dysgalactiae* (Group C), and *Str. uberis*, are not as well characterized as *S. aureus* and

*E. coli.* Streptococcal virulence determinants include hyaluronic acid capsule, produced largely by Groups A and C, fimbriae used for attachment, M protein, streptolysin O, streptolysin S, hyaluronidase, and streptokinase (14). Other extracellular products such as proteases and nucleases are also produced. Not all groups produce each virulence determinant and there can be considerable variation between strains within a group. The capsule produced by some strains of *Str. dysgalactiae* has antiphagocytic properties but is produced only by exponentially growing cells. Hyaluronidase, which is synthesized later in the growth phase, effectively removes the capsule. At the present time there are no effective vaccines for streptococcal mastitis.

Anderson (7) has pointed out one fundamental problem with the development of vaccines for mastitis. That is, the colonization of the mammary gland by Gram-negative or Gram-positive bacteria invariably results in an inflammatory response, which is in itself a definition of mastitis. Since the inflammatory response is the primary defense mechanism against bacterial colonization in the mammary gland, immunization is likely to enhance this mechanism and therefore the reaction of an immunized gland to infection will be mastitis. It is therefore desirable for a vaccine to induce essentially a subclinical case of mastitis, which is eliminated quickly. It is likely that one of the easiest way to achieve this will be vaccination with live attenuated strains of *E. coli*, *S. aureus*, and streptococcal species, with or without recombinant antigens. Development of such vaccines will likely take several years.

## B. Systemic Viral Infections

### i. Foot-and-Mouth Disease (FMD)

The most dreaded bovine virus disease in many countries is FMD. Although the disease in cattle rarely leads to death, production losses can be high. For countries that have FMDV, losses due to trade embargoes on export of farm products are the major economic loss. Thus in addition to being a economically important disease in its own right it is also a politically important disease. In epidemic areas such as Africa, Asia, and South America, vaccination is the method of control. Although both live attenuated and inactivated vaccines have been used, the majority of the vaccines are prepared by inactivation of tissue culture grown virus. It is estimated that over 1.5 billion doses of virus are administered annually. This makes FMDV vaccines the most frequently used products to control any animal disease in the world. In North America, Australia, and Europe, where the disease does not normally occur, vaccination is not used. The method of control in these areas is an embargo on cattle and unprocessed cattle products. These embargoes are supported by

legislation and local veterinarians are required to notify the appropriate authorities upon the initial observation of vesicular disease.

Due to the economic importance of FMD, extensive research has been conducted on methods of immunization and a better understanding of the molecular biology of this virus in particular. As a result of these activities the molecular structure and antigenic components involved in inducing protective immunity have been dissected. It has clearly been shown that there are a number of serotypes: O, A, C, SAT1, SAT2, SAT3, and ASIA 1. In addition to these 7 serotypes, a considerable amount of antigenic variability occurs within these serotypes. As a result of this antigenic variability, it is important to ensure that vaccination occurs with the specific serotypes that are circulating within the country where control is being attempted.

Molecular studies on the antigenic structure of FMDV has indicated that the major immunogenic site is located on the VP1 protein of the virus. As a result of this localization, FMDV VP1 was one of the first proteins expressed in *E. coli* and tested as a vaccine in the early 1980s (91). In addition to serving as a model for understanding the problems of protein folding it also demonstrated that a vaccine produced by genetic engineering can protect animals from a disease caused by a virus. Molecular analysis of the VP1 protein indicated the location of the protective epitopes as well as the basis for antigenic variation responsible for evasion of immune responses. This virus protein has served as a model for synthetic peptide vaccines and has helped elucidate how a change in a single amino acid may influence the immunogenicity of proteins (17,20). Thus by substituting one specific amino acid within a 20 amino acid peptide one could broaden the neutralizing capacity such that the peptide would now induce protective immunity to heterologous virus within the same serotype as well as the homologous virus strain. Based on these observations, Dr. Brown concluded that it should be possible to tailor peptide vaccines that have a broad antigenic range of protection. Whether these vaccines will ever replace the current cheaper vaccines remains to be determined.

### ii. Bovine Viral Diarrhea

Since animals infected with bovine virus diarrhea (BVD) can manifest a variety of clinical signs ranging from enteric to respiratory and systemic infection, BVD virus is considered in this section of generalized infections rather than being allocated to any one of the specific entities. Originally it was thought that BVD and mucosal disease were two different viral infections. However, it is now clear that both of these are just different manifestations of the same virus. This disease occurs worldwide and can cause morbidity and mortality in its own right but as a result of its ability to cause immunosuppression it also plays an important role

in predisposing animals to secondary infections (155). In most cases, infection of seronegative animals results in a transient subclinical infection. However, there are reports that the virus can cause clinical disease in healthy seronegative animals. The most important feature of bovine virus diarrhea is its ability to cause fetal infections. Depending on the virus strains, the time of gestation that a pregnant animal is infected and its serological status will determine the eventual outcome of the disease. *In utero* infection with noncytopathic BVD virus occurring prior to 120 days of gestation leads to immunotolerance and persistence of the virus, possibly for the life of the animal. If the immunotolerant animal is later exposed to an antigenically related cytopathic BVD virus, it cannot mount an effective immune response and the result is uncontrolled replication and severe mucosal disease (19). Thus, to control severe mucosal disease caused by BVD it is important to immunize cattle prior to breeding. This can be achieved by using either live or inactivated vaccines. However, there is no assurance that immunization with any vaccine will prevent fetal infection. Whether this is due to the multiple variants of the BVD virus or its ability to effect leukocytes and spread to the fetus, is not fully understood but it is clear that there is a need for vaccines that are safe and efficacious against all field strains of BVD virus. Whether it will be possible to identify conserved epitopes within the various strains of BVD virus, produce them by genetic engineering methods, and provide fetal protection remains to be determined. Due to the immunosuppressive nature of live BVD vaccines it is not recommended that animals entering the feedlots or other high risk areas be immunized (155).

Monoclonal antibodies that neutralize virus *in vitro* have been developed (41). Whether these antibodies can recognize all variants of BVD remains to be determined. Recently, considerable progress has been made at localizing the genes coding for the specific proteins recognized by neutralizing monoclonal antibodies (31). As a result of these developments it is hoped that large quantities of the proteins will be produced and tested for their ability to reduce or prevent BVD infections. Whether any of these vaccines will be able to prevent fetal (*in utero*) infection remains to be determined.

### iii. Rinderpest

Rinderpest is a member of the morbillivirus genus of the Paramyxovirus family. This virus can cause acute systemic disease in ruminants resulting in erosion of the mucosal epithelium in the respiratory and digestive tract. If introduced into seronegative herds, the disease can be extremely explosive and result in large economic losses. Fortunately this virus has been eliminated from a number of countries and now occurs only in Africa and Asia. In countries free of rinderpest, control measures are

designed to prevent introduction of the virus. These control measures are similar to those described above for foot-and-mouth disease virus. In countries where rinderpest is enzootic, or where the disease has a high probability of being introduced, vaccination is the method of control (144,164,165). Although inactivated viral vaccines have been used, immunity induced by these vaccines was often temporary, requiring repeated annual revaccination. The development of live attenuated vaccines has dramatically improved the level and duration of immunity. In fact, it has been stated that the live attenuated tissue culture vaccines are among the best available for any bovine disease. The basis for this statement is that the vaccines induce life long immunity and are cheap. One of the problems with this vaccine is its thermal stability. To maintain vaccine efficacy it is mandatory to maintain an adequate cold chain from manufacturing to administration. Unfortunately in many of the countries in Africa and Asia, where rinderpest is a problem, maintenance of the cold chain may not always be as effective as one desires. In an attempt to overcome this problem a heat-resistant strain has been developed. In addition other methods of producing the vaccine are being investigated. The most recent approach, using recombinant DNA technology, involves incorporation of the F and HN protein of rinderpest into vaccinia virus (198). Animals immunized with the recombinant vaccinia virus, carrying the genes coding for rinderpest proteins, developed immunity to rinderpest and were resistant to challenge with virulent virus. Whether this vaccine will be licensed for use in controlling rinderpest and will eventually replace the highly effective live attenuated rinderpest vaccine remains to be determined.

### iv. Minor Bovine Viruses

A number of other viruses that can cause infections of cattle include Akabane disease, caused by a mosquito-borne virus in the Bunyavirus family. This virus is restricted to areas of Japan, Australia, some countries in the Pacific Southwest, East and South Africa, and some Middle Eastern countries. Inactivated viral vaccines have been shown to be effective at preventing abortions and congenital abnormalities in cattle infected with the virus (92). Annual boosters are required. Recently a live attenuated vaccine has been licensed in Japan (74).

Although bluetongue is generally considered to be a major problem in sheep it can cause infection in cattle as well (23). In general cattle suffer milder infections than sheep and have a low mortality rate. Attenuated vaccines have shown to be successful in preventing clinical bluetongue or congenital abnormalities. However, it must be emphasized that immunization of pregnant cattle should not be practised since the vaccine virus is not sufficiently attenuated to prevent in from causing congenital abnormalities. Since there are 24 different immunological serotypes, it is

important to design the vaccines to contain the constellation of serotypes endemic in the specific area. Recently a number of the bluetongue virus genes involved in inducing neutralizing antibody have been cloned and expressed in a variety of expression systems. One of the more interesting developments is the observation that coexpression of a number of individual genes in baculovirus can result in assembly of virus-like particles (50,103). It may be possible that this totally recombinant self assembled virus-like particle may prove to be an excellent vaccine against bluetongue virus. Whether this will indeed prove to be a new approach to immunization remains to be determined (156).

Rift valley fever virus can infect cattle as well as sheep, goats, and humans. In areas where the virus in endemic an attenuated vaccine is used (171). As is the case with many viruses that can cause abortions or congenital infections it is not recommended that pregnant animals be immunized with the live attenuated vaccines. Whether the new minute plaque or highly mutagenized candidate vaccines could be used in pregnant animals awaits further investigation. Formalin-inactivated vaccines are safe but need to be given at least twice before effective immunity develops. These vaccines are not very stable since they are provided in a liquid form. They are also more expensive than the live attenuated vaccines. In all cases annual booster immunization is recommended to maintain protection (195).

### v. Viruses Causing Skin Infections

Several viruses have the ability to produce either localized or systemic infections of the skin. These include members of the Poxvirus, Herpesviruses, and Papilloma virus families. Within the poxviruses a number of members can cause skin infections in cattle. These include vaccinia virus, cowpox, and pseudocowpox (130). These infections are generally rare in North America but are more common in other parts of the world. Variants of vaccinia virus can also cause infections in water buffalo in various areas of India and Indonesia. All of the members of the poxvirus family induce similar types of lesions, which initially start out as small papules eventually developing into larger lesions. In the case of lumpy skin disease, lesions may develop as plaques and then ulcerate. In the majority of pox infections mortality is generally very low but economic losses may be high due to loss of milk production and in the case of lumpy skin disease damage to hides. Since these viruses do not cause severe economic losses, vaccines are generally not in use. However, based on the observation that these viruses induce excellent immunity it should be possible to develop vaccines against them if a vaccine was needed.

Bovine herpesvirus-2 can induce either generalized skin lesions throughout the body or more localized lesions of the mammary gland

(55). For this reason the virus is often called bovine mammalitis virus. In contrast to the lesions caused by the poxviruses, BHV-2 produces much more obvious ulcerative lesions. Upon introduction of the virus into a totally susceptible herd the frequency of infection in very high with virus being spread from one cow to another by mechanical methods during milking. However, the mortality rate is very low. The major economic losses are due to loss of milk production or complications resulting from mastitis. No commercial vaccines are presently available for BHV-2 and it is unlikely that vaccines will be developed for this disease even though experimental vaccines have been shown to be effective; the infection occurs worldwide and only one serotype of the virus is responsible for infection (157).

Warts are a common infection of cattle. Although animals of all ages can be affected, the incidence is highest in calves and yearlings, especially if they are held in close proximity. This indicates the infectious nature of the disease. The disease is often self-limiting, thus it is often unnecessary to implement control measures. However, autongenous vaccines produced by formalin inactivation of homogenous warts are often used to expedite the regression of warts (176). These vaccines are given either intradermally or subcutaneously. The value of such a vaccine is often questioned, since many warts regress spontaneously even without such treatment. However, surgical removal of the wart and reintroduction of antigens in the form of formalized autongenous vaccines can ensure cure. An experimental recombinant vaccinia papilloma virus vaccine has been developed (114). However, since protection appears to be serotype specific, immunization will need to be carried out with the appropriate serotype (78,79).

# 6. Delivery/Adjuvants

In many instances where killed vaccines are used to elicit immune responses, it is important to include adjuvants to nonspecifically stimulate the immune response toward the injected antigens. This is becoming even more crucial with the subunit vaccines produced by recombinant DNA technology or synthetic peptides since the purified proteins are poorly immunogenic on their own. In the present review we will not discuss adjuvants in detail. However, we feel that a brief introduction is warranted since the efficacy of the presently licensed conventional vaccines and the future new generation of vaccines can be influenced by the type of adjuvant used. For more detail the reader is referred to an excellent review by Allison and Byers (5) regarding the different classes of adjuvants. The requirements for good adjuvants include the ability to enhance cell-mediated immunity, an adequate level of humoral immunity of the correct isotype, as well as the ability to elicit both B and T cell

memory. In addition, the adjuvant should not result in tissue damage (granulomas) at the site of injection and it should not induce pyrexia or autoimmune responses. Unfortunately, many of the agents that stimulate high levels of immunity also elicit some of the undesired side effects. One of the best adjuvants, Freund's complete adjuvant, is not suitable for use in food-producing animals (51). Furthermore, its adverse side effects are so dramatic that they should not be used in any animal. However, it has laid the foundation for our understanding of the requirements of effective adjuvants. Some of this work was pioneered by Ribi Immunochemical Research Incorporation which resulted in switching from a water in oil mixture to an oil in water mixture (151). The concentration of oil has been reduced to 1–2% of the vaccine with minimal occurrence of granulomas and abscesses. Other organizations are developing a wide range of adjuvants, which undoubtedly will be added to their repertoire of vaccines. These include vehicles or slow release formulations, cytokines (interleukins and interferons), immunostimulatory complexes (ISCOMS), liposomes or variations thereof (virosomes, immunosomes), purified bacterial components, surface active components (saponin), and quaternary amines (avridine and dimethyl dioctadecyl ammonium bromide) (5,37,43,53,61,71,82,94,104,113,118). One common feature of all these agents is that they enhance some aspect of the immune response. Since it is well known that protection for some organisms is mediated by one or the other arm of the immune response (humoral or cellular), it is important to choose the adjuvant that will stimulate the most appropriate immune response. Furthermore, the route of administration may influence whether cellular or humoral immunity is preferentially enhanced. Thus in vaccine design all of these factors must be considered. Finally the ease of administration in the field must not be overlooked.

In addition to improving adjuvants it is often important to link subunit antigens, especially peptides, to larger carrier molecules to improve immunogenicity or to target the antigen to antigen-presenting cells. The recent development of viral-based particles (hepatitis B, tobacco mosaic virus, and yeast TY-VLPs) has increased the immunogenicity of subunit vaccines (38,68,124). Another recent development, which may further improve the immunogenicity of these subunit vaccines, is to actually target the antigen to antigen-presenting cells. Such targeting has recently been demonstrated using antigens linked to anti-MHC Class 2 antibodies or solid matrix particles (24,149). Finally the increase in our knowledge of immune regulation and the interactions and roles of cytokines in immune responses should provide us with very effective methods to enhance immunity to many of the newer vaccines. However, parallel advances will need to be made with regard to slowed delivery and targeting of specific cytokines and the antigens for maximal immune responses (127,128).

**Table 9.4.** Summary of organisms where potential future vaccines could be developed by recombinant DNA technology.

|  | Bacterial | Viral |
|---|---|---|
| Respiratory | *Pasteurella haemolytica* | Bovine herpesvirus-1 (BHV) |
|  | *Haemophilus somnus* | Parainfluenza-3 (PI-3) |
|  | *Mycoplasma* sp. | Bovine respiratory syncytial virus (BRSV) |
|  | *Pasteurella multocida* | Adenovirus |
| Enteric | *Escherichia coli* | Rotavirus |
|  | *Salmonella* sp. | Coronavirus |
| Systemic and other | *Pasteurella multocida* | Foot-and-mouth disease virus (FMDV) |
|  | *Bacillus anthracis* | Bovine virus diarrhea (BVD) |
|  | *Brucella abortus* | Rinderpest |
|  | *Moraxella bovis* | Bluetongue |
|  | *Staphylococcus aureus* | Papilloma |
|  | Streptococcal sp. |  |

## 7. Summary and Future

As is evident from the material presented in this review there are a number of viral and bacterial diseases of cattle for which improved vaccines would make a significant impact on the economics of livestock production. However, it is also evident that the recent technology available for identifying important antigens involved in inducing protective immunity combined with methodologies to characterize the specific immunological responses involved in recovery from infections provides a great potential for improving vaccines used in veterinary medicine. It is envisaged that within the next decade many of the conventionally produced vaccines will either be supplemented with recombinant components or be totally produced by recombinant DNA technology either as live or subunit vaccines (Table 9.4). However, even with these new vaccines, factors other than the antigen itself must be considered in immunization strategies to improve the animals resistance to infection. These include more appropriate delivery systems and their combination with immunomodulators or adjuvants to increase the immune response to these vaccines. There are, however, a number of conventionally produced vaccines that are safe, effective, and economical and therefore will continue to be used. Many academic institutions as well as companies are devoting a considerable amount of effort toward these areas of investigation. However, regardless of all the scientific knowledge that is available, vaccine strategies must also consider the practical problems of animal husbandry in various parts of the world. In some instances the best scientific approach is not always practical under field conditions and a compromise often has to be reached. Fortunately, many of the vaccine companies recognize these diverse animal husbandry practices and, therefore, design their vaccines to accommodate them. Unfortunately, such compromises may result in reduced efficacy of vaccines.

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CHAPTER 10

# Vaccines for the Skin and Mammary Gland of Ruminants

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## 1. Introduction

For many infectious diseases of ruminants vaccination is a feasible and
desirable alternative to therapy. Vaccines that result in elevated herd
immunity and disease prevention have important advantages in terms of
cost effectiveness, animal welfare, and reduction of the environmental
contamination that occurs when animals are treated with drugs and
chemicals. Currently on the market are many efficacious and successful
vaccines for ruminant diseases, but there are very few vaccines available
for diseases of the mammary gland and skin. Undoubtedly the diseases
that are easiest to control by vaccination have already been conquered; it
is equally true that at the time such vaccines were developed the process
was *not* easy! In the 1990s we have at our disposal new technologies such
as molecular engineering and peptide synthesis. These technologies have
dramatically improved our ability to synthesize usable quantities of
antigens, yet many economically important diseases of the skin and
mammary gland remain intractable. In this chapter we review progress
that has been made with vaccines against such diseases and try to identify
areas that may be profitable for future research endeavours.

## 2. Vaccines for Ectoparasites of Skin

### A. Introduction

The skin of mammals provides an efficient physical barrier to assault by
pathogens. There are, however, a variety of organisms that can breach
this barrier and gain access to less-well protected tissues. Of these, a few
have proved to be of major economic importance in ruminants.

Microorganisms that exhibit a tropism for epithelial tissues such as
foot-and-mouth disease virus (6), and bacteria such as *Bacteroides nodosus*,
the causative agent of footrot in sheep (25), are examples of pathogens

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that are extremely important commercially. These agents have been the focus of intense investigation that, in the case of footrot, has led to development of a successful vaccine (56).

The other major group of economically important skin pathogens are the arthropods. The skin is host to many parasitic arthropods including ticks, fleas, mites, lice, and flies. Each has developed mechanisms such as prestomal teeth, hypodermic probosci, or extracorporeal enzymes to damage or penetrate the skin. Even against some ectoparasitic arthropods, the skin is still an effective defense. For example, secondary strike flies such as screw worm fly can establish only on open wounds. Ectoparasites are also important as transmitters of diseases such as babesiosis, theileriosis, trypanosomiasis, and summer mastitis.

## B. Economic Impact of Parasite Control

The justification for research on ectoparasites of ruminants is the very significant impact that these parasites have on animal industries. One of the most important examples of ectoparasites with a major economic impact is *Boophilus microplus*, the cattle tick. This parasite and associated tick-borne diseases cost approximately $8 billion annually in production losses and costs of control world wide (34). In Australia, cattle ticks and related diseases cost at least $150 million annually (177). In a similar manner, the buffalo fly, *Haematobia irritans exigua*, has been estimated to cost the cattle industry about $200 million per year (H. Standfast, personal communication). In sheep, the most important parasite of skin is the larvae of *Lucilia cuprina*, the sheep blowfly, which are involved in up to 90% of all cases of cutaneous myiasis (blowfly strike) in Australia. This parasite may cost producers more than $200 million annually (22).

In dollar terms, there is already sufficient justification for considerable expenditure on research to control ectoparasite infestation. This is made imperative, however, by the steadily increasing levels of resistance shown by most of these parasites to the chemicals currently used to control them (10,109,135). Indeed the outlook for continued control of arthropod ectoparasites by chemicals is poor (41), and alternatives such as vaccination must be found soon.

## C. Immune Mechanisms in Skin

The induction of immune responses requires the presentation of antigens to sensitized lymphocytes, which can then mature along one of several effector pathways. Normal skin is richly endowed with antigen-presenting cells and immunocompetent cells that are continually replenished by immigrants from blood (73,139). Preeminent among antigen-presenting cells are Langerhans cells, which are found in the lower epidermis (144).

**Table 10.1.** T lymphocyte phenotypes in normal sheep skin.

| Source | $n$ | T4 (%) | T8 (%) | T19 (%) | Immunoreactive cells/5 fields 200× magnification |
|---|---|---|---|---|---|
| Site | | | | | |
| Breech | 10 | 17.0 ± 4.2 | 33.1 ± 5.9 | 49.9 ± 6.4 | 132.6 ± 27.8 |
| | | ns | ns | ns | ns |
| Mid-flank | 11 | 18.7 ± 2.3 | 29.5 ± 4.0 | 51.9 ± 4.9 | 191.7 ± 25.4 |
| Gender | | | | | |
| Wether | 11 | 16.9 ± 3.6 | 33.0 ± 5.8 | 50.1 ± 6.2 | 109.8 ± 30.6 |
| | | ns | ns | ns | ns |
| Ewe | 10 | 18.9 ± 3.0 | 29.3 ± 3.7 | 51.8 ± 4.9 | 169.1 ± 32.2 |

Several hours after application of antigens to the skin surface, lymphocytes are found to be clustered around Langerhans cells (139). Antigen is also detected on Langerhans cells within dermal lymphatics draining to the regional lymph node where systemic sensitisation to the antigen may occur (139). A unique population of lymphocytes recirculates from blood through skin into afferent lymphatics draining toward the regional node (73). In sheep and cattle, T lymphocytes bearing the $\gamma\delta$ T cell receptor predominate among lymphocytes in skin (95) and may represent a population of memory cells (96). Thus skin is efficiently provided with mechanisms for induction of new immune responses and subsequent ongoing immunological surveillance. The prevalence of T cell phenotypes in normal sheep skin determined in our laboratory is presented in Table 10.1.

During bacterial infection and external parasitism of skin, disruption of the epidermis leads to an acute inflammatory response. Inflammation provides the first arm of the host defense mechanism and helps deliver the mediators and effector cells for immunological defence of skin. Five important phases in this defense process are recruitment of leucocytes, adhesion reactions, production of cytokines by keratinocytes, expression of MHC antigens, and expression of immunity. These topics will be dealt with in turn.

### i. Recruitment of leukocytes

As noted above, lymphocytes, macrophages, Langerhans cells, and other resident dermal leukocytes such as mast cells migrate constitutively from blood into the dermis and epidermis. During inflammatory reactions this traffic is up-regulated, and in addition neutrophils, eosinophils, and basophils may be recruited into skin. The chemotactic agonists contributing to the enhanced migration of leukocytes into skin are summarized in Table 10.2. Two important mediators of skin inflammation are interleukin-1 (IL-1) and interleukin-8 (IL-8). IL-1 is stored preformed in

**Table 10.2.** Endogenous inflammatory mediators inducing leukocyte accumulation in skin.

| Mediator | Target | Source | Reference |
|---|---|---|---|
| Activated complement | N,E,M | Plasma | 74 |
| LTB$_4$ | N,E,M | Cell membrane | 106 |
| PAF | N,E,M | Cell membrane | 43 |
| IL-8 | N | M,K,F,En | 47,46 |
|  | L |  | 88 |
| Il-1 | N,E,M | M,K,F,N | 50,100 |
| TNF-$\alpha$ | N,E,M,L | M,K,L | 67 |
| IFN-$\gamma$ | L | L | 72 |

keratinocytes (38,58) and may be released during epidermal trauma. IL-8 is synthesized by several cell types found in skin including endothelial cells, fibroblasts, macrophages, and keratinocytes (11). IL-8 appears to play a central role in inflammatory responses in skin and may act as a keratinocyte mitogen (120) as well as a chemotactic agonist for neutrophils and perhaps lymphocytes (11). Tumor necrosis factor-$\alpha$ (TNF-$\alpha$) is the most potent inducer of lymphocyte accumulation in skin so far tested (67). The inflammatory activity of chemotactic agonists and cytokines in skin has recently been reviewed (42).

## ii. Adhesion

The function of leukocytes is greatly influenced by their capacity to bind to cells within the tissue matrix and to targets. It has recently been found that several cytokines induce the expression of intercellular adhesion molecule-1 (ICAM-1) by keratinocytes. Included among these are interferon-$\gamma$ (IFN-$\gamma$) and TNF-$\alpha$, whereas IL-1$\alpha$, IL-5, IL-6, granulocyte–macrophage colony-stimulating factor (GM-CSF), IFN-$\alpha$, and transforming growth factor-$\beta$ (TGF-$\beta$) were inactive (13,87). ICAM-1 is a ligand for the leukocyte integrin LFA-1 and influences a broad range of leukocyte functions including T-helper and B-lymphocyte responses, natural killer cell activity, and antibody-dependent cytotoxicity mediated by monocytes and granulocytes (142). Thus expression of ICAM-1 by keratinocytes may play a key role in focusing immune defense reactions at this site.

## iii. Cytokine Production by Keratinocytes

A vast range of peptide mediators with autocrine and paracrine activity influencing immune and inflammatory responses have been identified in recent years. Keratinocytes are a very rich source of these cytokine mediators and produce IL-1, IL-3, IL-6, IL-8, GM-CSF, granulocyte-CSF, macrophage-CSF, TGF-$\alpha$, TGF-$\beta$, TNF-$\alpha$, platelet-derived growth

factor, and monocyte chemotactic factor (9,117). Of these mediators only IL-1 is produced constitutively but it is not usually released from cells (37). Production of these cytokines endows keratinocytes with the capacity to recruit leukocytes into skin and activate their effector functions. The production of cytokines by keratinocytes has recently been reviewed (87).

### iv. Expression of MHC Antigens

The induction of immune responses requires the presentation of antigens to T lymphocytes in the context of MHC class II. Expression of MHC class II is usually restricted to cells of the monocyte/macrophage lineage including Langerhans cells, however, following stimulation with cytokines, epithelial cells frequently express MHC class II. This is the case for keratinocytes which express class II molecules following stimulation with IFN-$\gamma$ (21).

### v. Expression of Immunity

From the above discussion it is evident that skin is well equipped to detect the incursion of new antigens and to mount immune responses against the invaders. Natural exposure to parasites frequently leads to hypersensitivity reactions, which often fall short of providing protective immunity. The challenge to immunoparasitologists now is to harness those components of naturally acquired immunity or utilize novel parasite antigens to selectively sensitize animals. Two stages are likely to be essential to attaining this goal. Firstly, host defense mechanisms must be identified that are able to deliver a lethal hit to the parasite. Second, immunological adjuvants and vaccination protocols will need to be devised to selectively sensitise the host for expression of protective immunity without sensitization of the host for adverse reactions such as allergic hypersensitivities.

## D. Naturally Acquired Immunity

The host–parasite relationship can be variously influenced by the host immune response to parasitism. The argument follows that acquired immunity deleterious to the parasite may be augmented by vaccination with appropriate parasite antigens.

As is the case with most parasites, parasites of skin interact with the host through secretory–excretory material produced to assist with feeding or maintaining position on the host, and through direct contact between host and parasite. With blood-feeding ectoparasites, contact with the host is almost entirely through salivary molecules, although there is evidence that ticks regurgitate gut-associated antigens into feeding sites (32). Even in the case of tissue-feeding parasites such as blowfly larvae, the majority

of antibody in parasitized hosts is directed against salivary antigens and other secretory products, with little against the larval cuticle (140). On this basis, salivary antigens should be high on a list of possible vaccine antigens.

One reason to place an emphasis on parasite saliva is a link with an essential role in feeding by the parasite on the host. For example, apyrases that catalyze hydrolysis of ATP and ADP have been found in salivary glands and saliva of a variety of hematophagous arthropods (121–124). ADP induces platelet aggregation (52,148). It was suggested that apyrases in arthropod saliva make plasma ADP unavailable to catalyze aggregation of platelets at sites of vessel penetration by feeding parasites (121,122,124). In other examples, aminopeptidase and acid phosphatase activities are secreted into feeding sites by *Hyalomma anatolicum* (61,151). Acid phosphatase may promote adhesion within the attachment cement (152), and aminopeptidase may be a digestive enzyme (65). Some studies suggest that the attachment cement of ticks itself stimulates a foreign-body reaction and immune response (61), although Moorhouse and Tatchell (105) believe it to be inert. In either case, both aminopeptidase and acid phosphatase from saliva of *H. anatolicum* have been shown to be antigenic and are thereby candidates for inclusion in vaccines to augment natural immunity. Esterases are secreted into the host by *B. microplus* (133) and *Rhipicephalus appendiculatus* (151). Esterase was found in mononuclear cells at the site of tick feeding (151) suggesting that the host immune system is presented with the molecule. Indeed, sensitized animals are allergic to an esterase isolated from *B. microplus* (175) and secreted esterase was shown to be rapidly removed from feeding sites on hosts that rejected ticks (147). The role of esterase may be to promote mast cell degranulation and thus vascular permeability at the site of feeding (60). This molecule may also be useful in a vaccine against ticks. Other activities in saliva of hematophagous arthropods that may assist with feeding and that should be considered for vaccine development are trypsin inhibitor (*B. microplus*) (179), anticoagulant (*Ornithodoros moubata*) (168), neutrophil inhibitor (*Ixodes dammini*) (125), immunosuppressant (*I. dammini*) (122), and glucosidases and galactosidases (*H. irritans*) (71).

Examination of the mechanisms associated with rejection of parasites by immune hosts is important in order to develop strategies to augment natural immunity. Immune hosts express immunity in a number of ways: parasite mortality, interference with feeding, prolongation of feeding time, low weight, low egg numbers, and decreased egg viability and hatchability (176). The predominant effect varies with host and parasite. Immunity to *B. microplus*, for example, is manifested by 30% lower weight of engorged female ticks, as well as minor effects on feeding time and fecundity (149,150). Interestingly, these ticks appear little damaged by an encounter with a resistant host (176) and will survive in an incubator for

an equal length of time as ticks fed previously on a nonimmune host (127). The results suggest that *B. microplus* on immune cattle are removed by grooming stimulated by the irritation caused by hypersensitivity to tick saliva (86,181). In other tick–host interactions, however, ticks have been shown to die on the host as a result of host immunity (176).

Acquired resistance to tick infestation is generally better correlated with antibody responses than to T cell-mediated immunity (176). Cell transfer experiments have not demonstrated that T cells alone are capable of mediating immunity to ticks, although T cell-enriched fractions of cells from immune animals are as effective as unfractionated cells (12,169,172). Willadsen (176) suggests that animals that develop poor immunity to ticks have low levels of reaginic IgE antibody. It is not known whether this is due to an inability to produce high levels of IgE, or a genetic incapacity to respond to tick antigens presented during natural exposure. The latter explanation is supported by a study linking certain class I major histocompatibility antigens (W6 and CA31) with susceptibility to tick infestation (143). Such considerations may be important when developing vaccines to augment natural immunity.

Examination of the lesion at feeding sites gives an insight into mechanisms leading to tick rejection from immune animals. The cellular response in naive guinea pigs to feeding by ticks consists predominantly of neutrophils and eosinophils (33). In sensitized animals, however, the response consists of an intense basophil infiltration and a smaller number of eosinophils (33). Elimination of basophils with antibasophil antisera abolishes acquired resistance (35), whereas antieosinophil antisera only partially diminishes tick rejection in immune animals (35). The idea that basophil degranulation, presumably mediated by IgE antibody, is important in tick resistance is supported by evidence that rejection can be blocked by histamine receptor antagonists (173), and that injection of histamine into feeding sites causes tick detachment (80). Vaccination protocols to mimic acquired immunity should, therefore, attempt to promote reaginic antibody responses.

Antigens from the salivary glands of *D. andersoni* were injected into guinea pigs and caused an almost 95% reduction in the number of larvae engorging on immunized hosts (173). This effect, however, was dependent on concentration of antigens, the type of adjuvant, and the route of administration, confirming that the quality of the immune response to tick salivary antigens was as important as the antigens that it was directed against. This result was supported by Wikel (171) and Brown and Askenase (34), who showed that salivary gland extracts from *D. andersoni* and *Amblyomma americanum*, respectively, were more efficacious when presented in Freund's incomplete adjuvant (FIA) than in Freund's complete adjuvant (FCA). Brown and Askenase (34) attributed this to a preferential induction of $IgG_1$ by FIA, which would promote a cutaneous basophil infiltration (68). In contrast, $IgG_2$, which

would be preferentially induced by FCA, would not induce an influx of basophils at the site of feeding by ticks.

Some molecules in saliva from ectoparasites may be immunosuppressive and thereby inhibit responses to vaccination with important salivary antigens. Ribeiro et al. (122) found that saliva from *I. dammini* inhibited T cell function *in vitro*. This activity was attributed to prostaglandin E2. In another study, Ribeiro et al. (125) showed that *I. dammini* saliva also contained factors that inhibit neutrophil functions including aggregation, degranulation, and phagocytosis. Wikel (174) found that saliva from *D. andersoni* contained inhibitors of T cell mitogenesis and IgM antibody production. Another less obvious problem with assessing the potential for salivary antigens to protect animals from parasite attack is that molecules that are crucial to tick survival *in vivo* may be of low immunogenicity as an evolutionary adaptation to tick survival. In this case, artificial stimulation of a response to these antigens would produce very effective protection from subsequent parasitism.

Work on ticks constitutes the major portion of the literature on acquired resistance to ectoparasites. There are, however, a few references to acquired immunity to other arthropods that are worth highlighting inasmuch as they differ from similar work with ticks.

Cattle exposed to the cattle grub *Hypoderma lineatum* develop marked immunity to reinfestation. Experiments have shown from 50 to 100% mortality of first instar larvae feeding on previously exposed hosts (62,63). In contrast to ticks, this resistance did not correlate well with humoral immunity, but was associated with cell-mediated immunity (114). Exposed animals developed immediate hypersensitivity to a number of fractions from *H. lineatum*, but only one of these fractions elicited a delayed-type hypersensitivity in cattle. This molecule would clearly be a good candidate to study for a vaccine to augment naturally acquired immunity.

Cattle infested with the parasitic mite *Psoroptes ovis* develop antibodies to *P. ovis* antigens (153). This was associated with an eosinophilia and development of immediate and delayed-type hypersensitivity reactions to these antigens (94). Similarly, work from our laboratory has shown the development of antibodies against buffalo fly (*H. irritans*) in exposed animals. These animals also had hypersensitivity reactions to buffalo fly antigens and an eosinophilia (Kerlin, R.L. and Allingham, P.G. manuscript in preparation). It is not known, however, whether, in either of these two cases, the responses to parasitism adversely affects the parasite.

A number of studies have shown that sheep struck by the blowfly *L. cuprina* develop antibody responses to *L. cuprina* antigens (57,111, 129,130,140). The most intense antibody response was directed against salivary antigens, although excretory–secretory products and midgut of larvae also reacted strongly (140). There is no consensus among workers, however, as to the effects of exposure and the immune response to

blowfly strike on larval survival. Indeed, one group suggested that struck animals exhibit a more severe myiasis than unexposed sheep (111). Others have shown that hypersensitivity to larval antigens correlated with protection from blowfly strike, but that this required exposure to large numbers of larvae on at least four and up to eight occasions (130). Later work has confirmed that at least *in vivo*, effects correlating with protection are observed only after prolonged exposure of animals to the larvae (57). On the basis of these studies of natural immunity to myiasis, it seems unlikely that augmenting the acquired response to secretory–excretory products of the larval blowfly would achieve success. Some workers, however, have persevered with this approach and report at least limited success in vaccinating sheep with proteases secreted by larvae establishing on sheep skin (128).

## E.  Experimental Vaccines

### i.  Warble Flies

The warble flies, *H. lineatum* and *Hypoderma bovis*, are widely distributed throughout the Northern Hemisphere. The larvae penetrate the skin and then migrate, *H. bovis* via the spinal cord and *H. lineatum* via the esophagus, through the tissues to the lumbar region. Here, the larvae feed and when ready to pupate, exit through a hole in the skin. Considerable natural immunity develops following exposure (63).

Early attempts at immunization reduced larval survival by as much as 81% (17,85,98). However, these studies were done with cattle with previous exposure to warble fly. Immunization of unexposed calves with soluble extracts of first instar larvae reduced larval survival by only 26% (115). This is similar to the levels of protection acquired through previous exposure. Inclusion of endotoxin from *Salmonella typhimurium* enhanced the effect of immunization, with only 4.9% of larvae surviving compared to 30.8% in control animals (16).

Immunization of calves with metabolic antigens from cultures of first instar *H. lineatum* reduced survival of *H. lineatum* by 30% but had no effect on *H. bovis* during a challenge infection (17,18). Vaccination of naive calves with a purified preparation containing predominantly the secreted serine protease, hypodermin A, resulted in a 90% mortality of larvae compared to 30% in control calves (116). Chabaudie et al. (39), however, observed only a 28% reduction in larval survival after vaccination with hypodermin A. The discrepancy between these two results may be associated with differences in the number of injections or the adjuvants used viz. Freund's complete compared to Freund's incomplete, aluminium phosphate, or no adjuvant. Vaccination with a combination of hypodermins A, B, and C combined with the immunostimulator monophosphoryl lipid A reduced larval survival by 85% (14).

The hypodermins are secreted from the midgut and aid both digestion and migration (19). They have also been implicated in inhibition of bovine C3 (an enzyme involved in initiation of the inflammatory response (15)) depleting complement, inhibiting coagulation, and inhibiting lymphocyte proliferation (40). Thus the mechanism of action of vaccination with hypodermins would seem to be inhibition of digestion and migration coupled with prevention of immune suppression. This hypothesis is supported by the reports that treatment with either the immunostimulator, monophosphoryl lipid A alone (14) or endotoxin from *S. typhimurium* alone (16) both reduced larval survival by 50% (14).

### ii. Blowflies

Blowfly strike is the predominant ectoparasitic disease of sheep in Australia. The myiasis produced can lead to toxemia and death (10). Over 90% of strikes are initiated by one species, *L. cuprina* (51,167). Little evidence of natural immunity exists and such immunity if present is limited and transient.

The first evidence that vaccination may be successful in controlling *L. cuprina* was when O'Donnell et al. (110) demonstrated impairment of larval development *in vitro* when larvae were fed on sera from sheep vaccinated with a soluble extract of third instar larvae. However, the vaccination had no effect on growth of larvae implanted on the vaccinated sheep's backs. Eisemann (personal communication) has also inhibited larval growth with extracts of second or third instar larvae but also report inconsistencies between results obtained *in vivo* and *in vitro*. The greater sensitivity of *in vitro* assays in measuring inhibition of larval development may be related to the greater intake of antibody from a synthetic diet *in vitro* (Eisemann, personal communication).

Vaccination with gut extracts also causes inhibition of larval growth *in vivo* [Broadmeadow, quoted in (128); Howells, personal communication]. Studies in this area are continuing and antigens derived from gut have induced up to 70% inhibition of larval growth (128; Eisemann, personal communication).

An alternative source of vaccine antigens are the secretory–excretory products of *L. cuprina*. Broadmeadow [quoted in (128)] successfully vaccinated sheep with salivary gland extracts. This was refined to vaccination with secretory products collected from *in vitro* cultured larvae and resulted in lower larval returns and inhibition of development (27,30,137). As described for *Hypoderma* spp. earlier, the secreted enzymes of *L. cuprina* can suppress the host's inflammatory response and coagulation (26). Isolation of individual enzymes is proceeding and at least one pure enzyme is capable of inhibiting larval development when used as a vaccine (128). The closely related *Lucilia serricata* can also cause myiasis in sheep. Shaw and Wynne-Jones (138) successfully vac-

cinated sheep against *L. serricata* with excretory/secretory products from cultured larvae, however, they were unable to extend the results to *L. cuprina* (Montague, personal communication).

A number of other antigens from *L. cuprina* including the developmental enzymes dopa-decarboxylase (Howells, personal communication) and phenyloxidase (20) have been tested unsuccessfully.

The screw worm flies, *Cochliomya hominovorax* and *Chrysomya bezziana* also cause myiasis by laying eggs in existing wounds of sheep and cattle. Eisemann (personal communication) was able to inhibit the *in vitro* development of *C. bezziana* larvae with sera from sheep vaccinated with whole larval extracts of *C. bezziana*. Cross-protection studies showed that *in vitro* development of *C. bezziana* larvae could also be inhibited with anti-*L. cuprina* serum.

### iii.  Hematophagous flies

The hematophagous flies of the muscid diptera are important not only for causing direct production losses but also as vectors of disease. The Tsetse fly, *Glossina mortisans*, transmits trypanosomiasis and the buffalo fly, *H. irritans exigua*, transmits the nematodes, *Stephanofilaria* spp., bovine herpes virus, and is implicated in the spread of pinkeye, *Moraxella bovis*.

Most studies have concentrated on Tsetse fly. Nogge (107) showed a small increase in mortality and decrease in fecundity when *G. mortisans* fed on rabbits immunized with whole flies from which the midgut-mycetome had been removed. Kaaya and Alemu (78) showed that females of *G. mortisans*, which were maintained for three generations on rabbits immunized with whole bodies, engorged guts, or gravid uteri, showed decreases in fecundity of 52, 59, and 70%, respectively. In addition, pupal mortality increased and pupal weight decreased. Smaller effects were observed after vaccination with salivary glands or unfed guts. Vaccination with trypsin from *G. mortisans* resulted in increased mortality, decreased fecundity, and lower pupal weights (79).

Alternative strategies for vaccination have been highly successfully with Tsetse. Feeding flies on rabbits vaccinated with symbiotic bacteria from the midgut resulted in 80–90% reductions in fecundity and flies feeding on some rabbits with high titres of antisymbiont antibodies were totally sterile (107). Host albumin is an essential dietary requirement of *G. mortisans* and is incorporated into insect tissues. Feeding flies on an antibody solution specific for a species of albumin previously fed to the flies leads to death of the flies within two hours (108).

Vaccination of rabbits against the stable fly, *Stomoxys calcitrans*, with extracts of various fly tissues resulted in increased mortalities and a range of physical abnormalities including paralysis of legs and wings and difficulties in probing (134).

The Buffalo fly, *H. irritans*, is a pest of cattle throughout Asia and Australia. It is closely related to the horn fly of North America. Until the mid-1970s, buffalo fly was confined to northern Australia south to Gladstone, by the practice of dipping cattle to control the cattle tick, *Boophilus microplus*. However, the introduction of amidine acaricides which have no effect on buffalo fly has contributed to the spread of the fly throughout the wet subtropical regions of Australia.

Recently, a program commenced at CSIRO Long Pocket Laboratories to develop a vaccine for the control of buffalo fly. Cattle have been immunized with extracts of various fly tissues and blood from the immunized cattle assessed for anti-fly activity in an *in vitro* feeding system. With some of these immunizations, mortality of flies is 5-fold greater than on control blood (East, unpublished results). Fractionation of these tissues to identify protective antigens is continuing.

**iv. Ticks**

Ticks of various species are prevalent in most cattle rearing regions of the world. There are many examples of effective vaccination of both cattle and laboratory animals with crude extracts including whole tick extracts (1,55,69,101,102), salivary gland extracts (31,36,77,171), and midgut extracts (4,76,112,170).

Vaccination with salivary glands has not, in general, induced protection superior to that obtained by natural exposure to ticks. Kemp and Willadsen (82) have argued that vaccination with salivary antigens is unlikely to be successful because natural exposure to these antigens during tick infestation is insufficient to promote a strong immunity. Vaccination with salivary glands has worked best in laboratory models and these may not reflect the true interaction between the tick and its natural host. Three proteins from *B. microplus* that induce immediate hypersensitivity reactions in cattle with naturally acquired resistance have been isolated and characterized, however, none of them was effective as a vaccine (176,177).

The concept of novel or concealed antigens was first suggested by Galun (59). The first practical demonstration of the idea was when Allen and Humphreys (4) vaccinated guinea pigs and cattle against *D. andersoni* with midgut and reproductive organs. The ability to vaccinate against ticks with midgut antigens and partially purified extracts of midgut has since been confirmed by many authors (76,178,182). Recently, Willadsen et al. (180) isolated and characterized a gut membrane glycoprotein, called Bm86, from *B. microplus*, which, when used to immunize cattle, reduced larval production by 92%. This antigen, which has homology with a putative protective antigen from *Plasmodium falciparum*

and with the epidermal growth factor precursor, has been cloned
and subsequently expressed in *E. coli* (119). The recombinant pro-
tein is as effective as the native protein when used as a vaccine (83),
and is now undergoing field trials (Willadsen, personal communica-
tion).

The mode of action of this vaccine has been well characterized. Inges-
tion of blood from vaccinated animals results in massive damage to the
tick gut (2). Antibodies to Bm86 bind to digestive cells lining the tick gut
and mediate complement dependent lysis of these cells (81). The actual
glycoprotein, Bm 86, appears to be involved with the endocytotic activity
of digestive cells. Antiserum to Bm86 inhibits endocytosis in gut cells sus-
pensions (180) and this inhibition occurred before cell membrane damage
was observed (81).

This protective antigen may well be appropriate as a target for vac-
cination against other species of ticks. The use of polymerase chain
reaction technology with primers designed from the known sequence of
Bm86 (119) would provide a rapid means to screen other arthropod
parasites for an analogous antigen.

Finally, patents have been lodged covering vaccination of cattle against
*R. appendiculatus* using a hemeglycolipoprotein from fully developed tick
eggs (7) and vaccination of cattle against *B. microplus* using extracts of
tick synganglion (8). However, no published information concerning the
efficacy of these antigens is available.

### v. Other Species

Mosquitoes, although not important pests of ruminants, can serve as a
model to provide additional data for vaccination against insects.

Alger and Cabrera (3) vaccinated rabbits against a range of extracts
from *Anopheles stephensi*. Immunization with dissected midguts increased
mortality but had no effect on fecundity. Hatfield (66) also showed
increased mortality after vaccinating rabbits with midguts of *Aedes
aegypti*, but again there was no effect on fecundity. In contrast, Sutherland
and Ewen (145) found that vaccination of rabbits with whole body ex-
tracts of *A. aegypti* reduced fecundity but had no effects on mortality.
The vaccination was species specific and had no effect when *Culex tarsalis*
fed on the immunized rabbits. Ramasamy et al. (118) also reported
reduced fecundity of *A. aegypti* after feeding on rabbits vaccinated with
extracts of midgut, abdomen, or head and thorax.

Ben-Yakir and Mumcuoglu (23) reduced fecundity and development of
the body louse, *Pediculus humanis*, by vaccinating with whole body or
midgut extracts. In addition, the midgut vaccination caused much higher
mortalities with many lice having massive damage to the gut. Vaccination
of rabbits against the skin bot, *Dermatobia hominis*, caused expulsion of
larvae from the host (93).

## F. Conclusions

The promise of vaccines to control ectoparasitic infections is much closer to reality than imagined only 5 years ago. As yet, only two vaccines, those against *Hypoderma* spp. and *B. microplus*, are fully defined both in the identification of the protective antigen and in their mode of action. Both are highly dependent on specific aspects of the particular host–parasite relationship and the physiology of the parasite.

Vaccination with midgut extracts, to mimic the tick results, have been successful with the louse, *P. humanis*, but much less successful with flies and mosquitoes. It is unlikely that analogs of the tick antigen, Bm86, will be found in species other than ticks. This antigen is intimately associated with the special digestive system found in ticks. Other parasitic arthropods such as *L. cuprina*, *H. irritans*, or *C. bezziana* have digestive systems where the meal is digested in the lumen of the gut and do not utilize pinocytosis. It would not be surprising, therefore, if an equivalent protein was not found in these species.

It is possible that secreted proteases similar to hypodermin A may provide effective vaccines for parasites with a tissue-invasive phase such as *L. cuprina*, *C. bezziana*, and *D. hominis*.

The search for effective vaccines for these parasites will continue. It is clear, however, that success will almost certainly be contingent upon research into the fine detail of host–parasite interactions and parasite physiology.

## 3. Vaccination against Mastitis

### A. Introduction

Mastitis in ruminants causes huge economic losses. In the United States the estimated loss to the economy is more than US$2 billion per annum (75) and the worldwide cost of the disease is reported to be US$35 billion per annum (99). Every case of clinical mastitis in a cow in California is estimated to cost at least US$200 (24).

Since the inception of vaccination as a means of controlling infectious diseases in livestock and humans, attempts have been made to vaccinate against mastitis. Generally these efforts have not met with great success for a variety of reasons (45):

1. Mastitis may be caused by a very wide range of microbes (mainly bacteria).
2. Many of the attempts to vaccinate were empirical in nature or naively followed protocols that had been successful with other diseases (systemic immunization with toxoids).

3. Until the 1960s little information was available on the immunology of the mammary gland.

During the past 20 years rapidly accumulating new information on bacterial pathogenesis, the inflammatory response, and expression of immunity on secretory epithelia has provided opportunities and impetus for the development of vaccines to control mastitis in ruminants. In the remainder of this chapter we shall briefly cover some key aspects of mammary gland immunology and describe a vaccine recently developed for control of staphylococcal mastitis in ruminants.

## B. Immunology of the Ruminant Mammary Gland

The ruminant mammary gland has evolved to fulfill two important functions for the suckling infant, namely, nutrition and the transfer of passive immunity from mother to young. There is no placental transfer of immunoglobulin from mother to foetus in ruminants (unlike primates and rodents), and the mammary gland is entirely responsible for transfer of maternal immunity (158). In the latter stages of pregnancy, during colostrogenesis, immunoglobulin is actively transported from blood into mammary secretion. This is a highly selective process in favor of the $IgG_1$ isotype (126). Thus, colostrum has concentrations of $IgG_1$ of around 60 mg/ml in sheep and 75 mg/ml in cattle (89). Selective transport of $IgG_1$ into milk occurs throughout lactation, albeit at greatly reduced levels compared to that which operates during colostrum formation (97). In ruminants, mammary secretions contain relatively little IgA, unless antigenic stimulation of the mammary gland has occurred (89), and in this respect ruminants are quite different from monogastric species. In humans, for example, IgA is quantitatively the major immunoglobulin isotype in colostrum, occurring at 20 times the concentration of IgG and IgM combined (28). These differences between species in mammary gland immunology are very important in establishing logical strategies for development of mastitis vaccines. In this connection the mouse mammary gland (with strong IgA orientation) is not a good model to use for developing mastitis vaccines intended for ruminants.

The various classes of leukocytes play important roles in defending the mammary gland from infection. In milk from normal, uninfected udders the most prevalent leukocytes (>80%) are macrophages (92). Mammary macrophages are important not only for their ability to phagocytose bacteria (54), but also for the role they play in antigen processing and presentation (113). T and B lymphocytes are present in mammary secretion in variable numbers depending on stage of lactation (48) and appear to be important in immunological surveillance. Following infectious mastitis or sterile antigenic stimulation, plasma cells, especially IgA-containing cells, may be found underlying the secretory epithelium (91,163).

Neutrophils arrive in mammary tissue and secretion within a few hours following bacterial infection of the udder and may reach numbers in excess of $10^7$ per ml in secretion. These cells play a crucial role in udder defense. It has been established that in cows made neutropenic by intravenous infusion of antineutrophil serum, low grade chronic bacterial mastitis rapidly converts to acute gangrenous mastitis (132). Furthermore, experimentally induced elevation of neutrophil numbers in mammary secretion (achieved by infusing small amounts of endotoxin into the gland) greatly increases resistance of the gland to experimental infection with viable pathogenic bacteria (131). Ruminant neutrophils have membrane receptors for $IgG_2$ (103,154), and cytophilic $IgG_2$ antibody plays an important role in opsonizing bacteria for neutrophil-mediated phagocytosis (155).

## C. Vaccination against Staphylococcal Mastitis

A feature of the scientific literature on staphylococcal mastitis vaccines is the relatively low efficacy achieved with bacterin and toxoid vaccines, used with or without adjuvants (5). In contrast, there are just a few reports of subcutaneous immunization with live *Staphylococcus aureus* vaccines and the data suggested that this approach to vaccination offered considerable promise (29,53). Studies carried out in Australia using sheep have confirmed that live *S. aureus* vaccines, given subcutaneously, provided significantly greater protection from experimental staphylococcal mastitis than did conventionally prepared killed *S. aureus* vaccines (162).

Several immunological and inflammatory mechanisms appear to be responsible for the immunity which is observed following vaccination with a live *S. aureus* vaccine. These are summarized below:

1. Immunizing ewes with live *S. aureus* vaccines results in mammary neutrophils from these animals having enhanced phagocytic capacity compared with neutrophils from nonimmunized ewes or ewes immunized with killed staphylococcal vaccines (156). This enhanced phagocytic capacity is specific for *S. aureus* and is attributable to cytophilic $IgG_2$ antibody on the neutrophil membrane (155). Live staphylococcal vaccines promote the synthesis of $IgG_2$ antibody whereas killed staphylococcal vaccines induce much greater synthesis of $IgG_1$ than $IgG_2$ (84,159).
2. Systemic immunization of ewes with live *S. aureus* results in an enhanced inflammatory response in the mammary gland following intramammary infection with staphylococci (44). Most of these early inflammatory cells are neutrophils and the identification of this mechanism has broad implications for mastitis vaccines as Hill (70) showed that the severity of experimental coliform mastitis in cows depended on the rate at which neutrophils were mobilized from blood into the gland, as well as the presence of opsonins in mammary

secretion. In the case of ewes immunised with a live *S. aureus* vaccine, the arrival of neutrophils at the infected focus occurs earlier, the neutrophil influx is of greater magnitude in the first 6 hrs postinfection, and these freshly recruited neutrophils carry cytophilic $IgG_2$ opsonins on their membrane.

3. It is now established that fresh clinical isolates of *S. aureus* possess a pseudocapsule (glycocalyx) that may be lost on subculture *in vitro* (49). *In vivo* growth induces expression of the pseudocapsule (141,164) and we have shown that *S. aureus* in milk collected from cows with naturally acquired, clinical, staphylococcal mastitis have a thick pseudocapsule shrouding the cell wall (166). *S. aureus* grown under *in vivo* cultural conditions (in dialysis sacs implanted in the peritoneal cavity of sheep) are more virulent than when grown under *in vitro* conditions (161). This increase in virulence/pathogenicity is associated with the expression of the pseudocapsule and key virulence antigens ("*in vivo*" antigens), which seem to be embedded in the pseudo-capsule. These antigens are antiphagocytic but animals immunized with a live *S. aureus* vaccine develop significant levels of $IgG_2$ antibody against them and are relatively resistant to experimental staphylo-coccal mastitis (162). The protective significance of $IgG_2$ antibody directed against antigens of the pseudocapsule is shown in Fig. 10.1.

4. Many of the early experimental *S. aureus* vaccines were simply toxoided culture filtrates/supernates (104) and there is no doubt that they provided some degree of protection from the more acute forms of the disease, presumably by neutralizing toxins in the udder, thereby limiting local tissue damage and reducing toxemia. Our studies have shown that an attenuated, live vaccine (produced by reducing hemolysin expression) provided significant protection against experi-mental challenge with *S. aureus* strains of low toxigenicity (157). However, the attenuated, live vaccine did not protect ewes which were challenged with strains of *S. aureus*, which produced high titers of exotoxins (D.L. Watson, unpublished data). These results suggest that antitoxic immunity is an important component in staphylococcal mastitis vaccines, especially as a means of limiting tissue damage when antibacterial mechanisms fail to eliminate all cocci.

Our more recent efforts in this laboratory have been concerned with developing a staphylococcal mastitis vaccine that would possess the desirable antigenic/immunogenic attributes of a live vaccine without the undesirable hazards of the latter. To this end, we have developed and patented an *S. aureus* vaccine comprised of killed bacteria bearing pseudocapsule, toxoided exotoxins, and a composite adjuvant (Australian Patent No 589185). The adjuvant dextran sulfate is particularly effective in stimulating an $IgG_2$ antibody response to antigens in the pseudo-capsule, and to this component a mineral oil is added to extend the
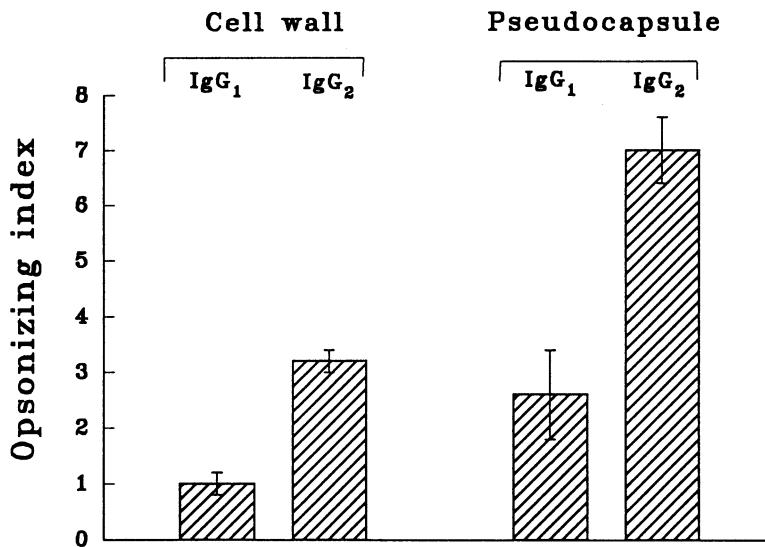
**Fig. 10.1.** Data showing the opsonizing efficiency of purified immunoglobulin preparations having different antibody specificities. Effector cells were mammary neutrophils. Values represent enhancement relative to nonopsonized control. Bars are standard errors.

duration of the protective immune response. This vaccine provides a significant degree of protection to lactating ewes (160) and cows (D.L. Watson, unpublished data) from intramammary challenge with a range of heterologous strains of *S. aureus*. Vaccination of heifers followed by intramammary challenge during the dry period resulted in significant reductions in new intramammary infections compared to unvaccinated controls (S.C. Nickerson and D.L. Watson, unpublished data).

The vaccine was used in a field trial carried out on five commercial dairies (total of 582 cows) in Australia (165). In each herd principle and placebo-treated cows were paired on the basis of age, milk production, infection status, and mastitis history. Two doses of vaccine were given in the dry period prior to calving; control cows received only the adjuvant as placebo. Various parameters of mastitis were assessed over a full lactation. There were four major, statistically significant benefits from vaccination:

1. Vaccinated animals had reduced attrition of functional quarters compared with controls.
2. There was a reduction of 50% in clinical mastitis in vaccinated cows. This resulted in a substantial reduction in usage of antibiotic drugs.

3. There was a reduction of 18% in subclinical infections in vaccinated cows. This was due to two factors. First, the quarters of vaccinated cows were more resistant to infection than those of control cows. Second, the spontaneous cure rate was greater in vaccinates than in controls.
4. The new infection rate, of previously uninfected quarters, for *S. aureus* was reduced by 25% in vaccinates.

These results suggest that this vaccine has substantial potential for use in mastitis control programs in commercial dairies, especially where staphylococcal mastitis remains a problem.

## D.  The Future for Mastitis Vaccines

Mastitis is a complex disease that may be caused by several different organisms. In addition to the research being conducted on staphylococcal mastitis vaccine (136,183), other experimental mastitis vaccines are currently under investigation. Of particular interest are the coliform vaccines that are being developed in the United States. Studies at Ohio State University have shown that iron-regulated outer-membrane proteins isolated from *Escherichia coli* and *Klebsiella pneumoniae* are leading candidate antigens for a subunit coliform vaccine (146). In addition, some very encouraging results have been obtained with bacterins prepared from the J5 mutant strain of *E. coli*. This is an epimerase-negative strain in which a terminal sugar is absent from the lipolysaccharide moiety of the cell wall, thus exposing the lipid A determinant. The J5 vaccine has been shown to reduce clinical coliform mastitis by up to 80% in trials in California and, importantly, it appears to have efficacy against a range of Gram-negative udder pathogens (64).

It is important to accept that vaccination *alone* cannot answer the economic and animal welfare problems posed by mastitis. Hygiene, management, and therapy all have important roles to play in controlling this disease. In contrast to previous eras, however, mastitis vaccines have now reached a level of efficacy that will make them an integral part of mastitis control.

## 4.  General Conclusions

Diseases of the skin and mammary gland of ruminants remain a challenge for scientists involved in immunology and vaccine development. Recombinant DNA technology and peptide synthesis have greatly improved our ability to synthesize antigens, so that for experimental purposes usable quantities of antigen are often not a limiting factor, as might have been the case in the past. In many respects the ability to produce antigens

has raced ahead of the means to use them effectively as vaccines. Our knowledge of disease pathogenesis and of basic immunology of skin and mammary gland has progressed significantly in the past decade or so. Unfortunately, the area of vaccine delivery and adjuvants has not received the attention it deserves and most experimental vaccines still involve empirical approaches in the choice of vehicle or adjuvant. To emphasize this point one has only to look at a catalogue of commercially available veterinary vaccines—overwhelmingly the adjuvants being used are aluminium salts and mineral oil. In this regard there has been little progress for 70 years! Recent exciting developments with ISCOMs and liposomes have shown that improvements can be made if sufficient research effort is invested, and the result has been some new, efficacious veterinary vaccines (e.g., ephemeral fever, equine influenza). Similar attention to the immunology of adjuvation will be required for the new vaccines against mastitis and parasitic diseases of skin of ruminants.

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CHAPTER 11

# Current and Future Vaccines against Theileriosis

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## 1. The Parasites and Their Life Cycles

Organisms of the genus *Theileria* are important tick-borne protozoan pathogens of domestic livestock occurring throughout much of the world. In many developing countries the diseases that they cause impose serious constraints on the improvement of livestock, particularly cattle. The two most important species infecting cattle are *Theileria annulata* and *T. parva*. *Theileria annulata* is transmitted by *Hyalomma* species ticks and occurs across a vast area extending from the Mediterranean to China. It infects domestic or swamp buffalo (*Bubalus bubalis*) and causes tropical theileriosis in cattle. *Theileria parva* is a parasite of African buffalo (*Syncerus caffer*) and cattle. It is transmitted by *Rhipicephalus* species ticks in eastern, central, and southern Africa. It causes diseases known variously as January disease, corridor disease and East Coast fever. The distribution of the two parasites does not overlap and Sudan is the only country in which both are known to occur (30).

The *Theileria* are obligate intercellular parasites and the life cycles of *T. annulata* and *T. parva* are very similar. Sporozoites are introduced in tick saliva during feeding and rapidly enter bovine cells. *Theileria annulata* parasitizes T and B lymphocytes and monocytes (89) while *T. parva* parasitizes T, B, and null lymphocytes (6). It is believed that the process of entry is initiated through a ligand–receptor interaction (37,101). The parasite is then rapidly internalized by a "zipper"-like interaction of host and parasite cell membranes. Subsequent dissolution of the host cell membrane allows the sporozoite access to the cytosol (38,87) and it develops through a trophozoite stage to a multinucleate schizont, which is closely associated with the Golgi apparatus (91). The infected cell is induced to divide synchronously with the parasite, the schizont being separated by the spindle apparatus during anaphase (46), and in this way infected cells undergo clonal expansion. Following a

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number of divisions some schizonts differentiate into merozoites in response to an unknown stimulus and these are released as the host cell ruptures, invading erythrocytes as piroplasms. Although division of *T. parva* piroplasms is limited (21,38), the piroplasms of *T. annulata* divide actively.

Piroplasms ingested by the tick in blood from infected cattle differentiate into gametes in the lumen of the gut and fuse to form zygotes. These enter gut epithelial cells and develop to mobile kinetes that migrate in the hemocoel to the salivary gland, invading E cells of type III acini around the time of moulting of larval and nymphal ticks (55). In the next instar they are activated by warmth and feeding (80,105), and develop by sporogony to a ramifying syncitium (37), which produces up to 50,000 sporozoites in each infected acinar cell. Transmission is transstadial and infected ticks cleanse themselves, although male ticks may infect more than one animal by interrupted feeding.

The diseases caused by the two parasites differ in some respects. Both give rise to lymphoproliferation in response to schizont infection followed by lymphodestruction due both to the parasite and the immune response of its host. Parasitized lymphocytes invade all tissues and tissue spaces, and clinically the disease presents as a generalized condition with fever, enlarged lymph nodes, panleucopenia, ocular discharge, depression, occasional diarrhea, and very frequently a progressive interstitial pneumonia. Lesions ranging from submucosal lymphocytic infiltration to erosion and ulceration are common in the abomasum and are occasionally seen in the intestines. Peyers patches are frequently infiltrated and may become necrotic. In longer standing cases lymphomata in the kidneys may be observed (7). The more pronounced merogony in the piroplasm stage of *T. annulata* (20) gives rise to anemia, which is rarely seen in *T. parva* infections.

Recovered animals remain carriers of infection, but the mechanisms by which parasites persist and evade the host immune response are not known. Replication of piroplasms may be responsible, and piroplasms must be present for transmission to occur. However, schizonts are also known to persist, probably in sites of low immunological surveillance (28). Recrudescence of parasites occasionally occurs in response to nutritional deficiencies, stress, or concurrent infection, and this may produce atypical disease (57). The most common atypical manifestation in *T. parva*-infected cattle is "turning sickness" where an intravascular and perivascular proliferation of parasitized lymphocytes occurs in the central nervous system (5). A marked natural resistance to theileriosis is found in endemic areas (58,92,102), but indigenous, cross-bred, or exotic animals are all susceptible when introduced into endemic areas.

## 2.  Current Methods of Control

Theileriosis has been controlled in eastern, central, and southern Africa since the early 1900s by the use of acaricides. This method has additional advantages in controlling tick burdens and other tick-borne diseases. However, it does maintain a population of cattle that is highly susceptible to tick-borne disease and if the system breaks down, huge losses may occur (51). In more recent years confidence in tick-borne disease control by acaricides has been reduced by the development of acaricide resistance, the high cost of the compounds, poor management and maintenance of dips, and civil unrest.

Chemotherapy has also been used effectively in the control of theileriosis, particularly with the more recent discovery of potent anti-theilerial drugs, halofuginone (85) and the naphthoquinones parvaquone and buparvaquone (53). *Theileria annulata* infection is particularly responsive to buparvaquone treatment (54) while *T. parva* is responsive to parvaquone, buparvaquone, and halofuginone (18,19,54).

### A.  Current Vaccines

Attempts have been made to immunize cattle against theileriosis since the early part of the century. Spreull (90) immunized 283,000 cattle against *T. parva* in South Africa using lymph node and spleen homogenates from diseased cattle. This method was abandoned due to the erratic availability of fresh immunizing material from sick cattle, the serious risk of transmission of other diseases, and the heavy losses to theileriosis following immunization in the absence of effective chemotherapy. *Theileria annulata*, unlike *T. parva*, can be transmitted using infected blood, and this was exploited as a means of immunization using strains of low virulence (14,73,86). Although this method did provide protection, it was marred by the risk of transmission of other diseases and the failure to provide a consistent infective dose.

The successful *in vitro* cultivation of *T. annulata* schizont-infected lymphocytes (98) was followed by the development of culture-attenuated cell lines that produced reliable immunizing infections (73). Some vaccine strains have been so effectively attenuated that they do not develop to piroplasms (72), although this is not always the case. This method has been applied with considerable success in large numbers of animals in Iran, Israel, India, USSR, and China. Between $10^4$ and $10^6$ cells of a passage attenuated cell line are inoculated with minimal clinical effects, and standardized cell culture vaccines are now produced commercially in India, China, and USSR. They are maintained as stabilates in liquid nitrogen and standards for their preparation and safety testing have been drawn up by OIE (70).

*In vitro* cultivation of *T. parva* schizonts was achieved unequivocally much later than was the case with *T. annulata* (52). However, immunization with *T. parva*-infected lymphocytes proved very much less successful. It was found that $10^8$ *T. parva*-infected cells were required to infect cattle and that the responses were unpredictable (14). Important biological differences exist between *T. parva* and *T. annulata* that influence the outcome of inoculation with parasitized lymphocytes. Unlike those of *T. annulata*, the schizonts of *T. parva* transfer very rarely from donor to recipient cells (13), and because of the histocompatibility barrier, donor cells do not survive within the recipient. Even if it were possible to produce sufficient numbers of cells to immunize animals successfully, it is likely that the antigenic complexity of *T. parva* would necessitate a mixture of stocks as cell lines to provide effective protection in the field. The use of vaccines based on *T. parva*-infected cell lines is therefore somewhat impractical (27).

Because it was known that susceptible cattle could in some instances recover from *T. parva*, attempts were made to provide low dose challenge with infected ticks (50,102 and others), but quantitation of infection in ticks was not reliable (102). However, the development of cryopreserved stabilates of ground-up infected ticks (23) provided the opportunity to explore reproducible infective doses. This led to the development of a method of immunization known as "infection and treatment" in which a potentially lethal "known" dose of stabilate is administered together with a long acting formulation of oxytetracycline (81,83). Although an unreliable therapeutic agent, oxytetracycline suppresses the development of the parasite and allows effective cell-mediated immune responses to be generated. Minimal disease reactions occur and the vaccinated animal has a long lasting immunity to homologous challenge (16).

As a result of laboratory cross-immunity studies using stabilates of parasites isolated in different areas it was quickly realized that unlike *T. annulata*, where one attenuated cell culture isolate was very broadly protective, *T. parva* was antigenically complex. However, a combination of three stocks did provide a broad but not universal protection, and a vaccine based upon these stocks is advocated for use throughout eastern and southern Africa (81). Because the vaccine is live and induces a carrier infection, an alternative strategy using local isolates from within countries or geographically defined areas may be preferred. Although this increases the expense of production and safety testing of the vaccine, it avoids the introduction of new strains into the area where immunization is implemented.

The search for *in vitro* characterization methods to differentiate between immunogenic types of *T. parva* has been intense but unsuccessful. An association between stocks with similar reactivity using

cross-protection characteristics and a panel of monoclonal antibodies has been reported (48), but has not been substantiated in other studies. Monoclonal anti-bodies highlight the antigenic diversity of *T. parva* (22,49), and to a lesser extent *T. annulata* (88). They also demonstrate the existence of mixed parasite populations in particular isolates or stocks (22), which probably contributes further to antigenic diversity during sexual reproduction within the tick. Similarly polymorphisms among stocks of *T. parva* have been demonstrated using DNA probes (3,4,22), pulsed field gel electrophoresis (63), and two-dimensional gels (93), and among stocks of *T. annulata* and *T. parva* using isoenzyme analysis (56).

The productivity of cattle vaccinated with cell cultures of *T. annulata* is not affected, although the vaccination of animals in late pregnancy or those under viral challenge is not recommended (45,74). In the case of *T. parva* it has been shown that cattle immunized by infection and treatment while on a falling plane of nutrition may have reduced fertility, but no effect on conception or calving rates was recorded in adequately fed animals (26). In another study, milk production and pregnancy were not disturbed (68). The benefits of the infection and treatment immunization are illustrated by the report of Berkvens et al. (10) who immunized many thousands of susceptible indegenous and cross-bred calves in Eastern Zambia and observed a very marked reduction in mortality under severe theileriosis challenge.

In general these vaccines are intended for use in improved cattle populations maintained in endemic and epidemic situations or in indigenous cattle in areas where the disease is epidemic. Infection and treatment immunization is expensive in adult cattle because of antibiotic costs and its application is recommended in calves over 2 months of age. It is appropriately deployed along with the strategic use of acaricides, and this integrated approach should be considered in the context of the control of tick-borne diseases in general. *Anaplasma* and *Babesia* species do not cause disease in young cattle, and exposure to these parasites during the period of natural protection allows them to develop immunity. However *Cowdria ruminantium*, which causes Heartwater, has a much shorter period of natural protection. With strategic acaricide use overwhelming tick burdens and disease challenge can be controlled during periods of tick abundance by frequent application. During dry periods when tick activity is low, acaricides may be applied at a reduced frequency. This approach is easier to apply in areas such as subtropical Africa, where a single rainy season confines the activity of adult ticks, and the occurrence of theileriosis, to a period between January and March. The treatment of specific disease outbreaks as they occur constitutes a third component of integrated control.

# 3. Immune Responses

## A. *Theileria parva*

The immune responses of cattle to *T. parva* have been more thoroughly characterised than those occurring in cattle infected with *T. annulata*. Antibodies directed against sporozoite, schizont, and piroplasm stages of *T. parva* can be detected in the serum of animals that have recovered from challenge with the parasite (16), but there is no evidence that these are relevant to protection under natural conditions. Following immunization by infection and treatment only low titers of antisporozoite antibodies are detectable in the serum, yet animals are resistant to homologous challenge (65). However, repeated exposure to infected ticks can result in the development of titers of antibodies that are capable of neutralising sporozoite infectivity (65) and this activity has been shown to be cross-protective between different stocks of the parasite (66). Sera from cattle that are exposed to heavy parasite challenge in the field are known to contain high titers of sporozoite-specific antibody (65), but it is difficult to predict the role of these antibodies in the immune status of these animals.

   High titers of antibody against the schizont stage of the parasite are detectable in animals immunized by infection and treatment, and first appear around the time of elimination of the parasite (16). The presence in serum of antischizont antibody is the most reliable measure of exposure to the parasite. However, animals immunized with heat-killed schizont-infected cells or semipurified schizont antigens produce similar titers of antischizont antibody but are fully susceptible to challenge, suggesting that these responses play no role in protection (32,100). The piroplasm stage of the parasite appears late in infection when pathology is already advanced. This suggests that although detectable in recovered animals, antibodies against the *T. parva* piroplasms are of little relevance to protection.

   These observations coupled with reports (64,94) that the transfer of serum from immune to naive animals fails to protect against challenge with the parasite suggest that serological responses play a limited role in recovery and protection from primary infection with *T. parva*. The observation of Emery (32) that immunity against the parasite can be transferred between chimaeric twins in the cellular fraction of thoracic duct lymph has strengthened the belief that protection is the result of cellular immune mechanisms. Immune animals usually develop a transient schizont parasitosis before eliminating the parasite, and after immunization by infection and treatment with live sporozoites cattle can resist challenge with up to $5 \times 10^8$ schizont-infected cells (36). Moreover, all successful methods of immunization have until now necessitated the establishment of the schizont stage of the parasite. Together with the

evidence that immunity is associated with cellular rather than humoral responses, these observations suggest that protection against the parasite is mediated predominatly by cellular mechanisms directed at the schizont-infected cell.

The development of techniques whereby bovine lymphocytes can be infected *in vitro* with *T. parva* sporozoites and maintained in culture (12) has greatly facilitated the study of cellular immune mechanisms directed at the parasite. Pearson et al. (71) demonstrated that irradiated infected lymphocytes induced proliferation in cultured autologous immune lymphocytes. They also observed that the proliferating cells were capable of killing autologous and to a lesser extent allogeneic infected lymphocytes. They concluded that the parasite induced an antigenic change on the surface of infected cells that stimulated cellular immune responses. However, since the responses were being measured *in vitro* the possibility existed that they were provoked by surface antigenic changes brought about by long-term culture. This question was resolved by the report of Emery and Morrison (31) that schizont-infected cells derived from various lymphoid tissues during lethal infection stimulated proliferation of autologous lymphocytes that had been cryopreserved prior to infection. Subsequent studies of bovine cytolytic responses to *T. parva* demonstrated that during the later stages of lethal infection peripheral blood mononuclear cells (PBM) contained cells that killed several allogeneic infected cell lines and a mouse tumor cell line, but did not kill autologous infected cells (33). In contrast, in animals undergoing immunization or challenge, PBM exhibited cytolytic activity restricted to autologous cell lines around the time of elimination of the parasite (33,36). This activity was later demonstrated to reside in the T cell fraction of PBM (34). Its restriction to autologous infected cells was reminiscent of that for cytotoxic responses of mice and humans to virus infections (95,106) and suggested that the induction of these responses might require the association of parasite antigens with class I MHC molecules on the cell surface.

The latter possibility has been examined in detail using serological reagents that identify polymorphic determinants on bovine class I MHC antigens. Three international workshops have grouped these reagents into over 30 specificities that are probably derived from two class I loci (9,35,97), BoLA-A and BoLA-B. Serological typing and a knowledge of parentage allows the identification of animals that are MHC haploidentical or that share individual class I MHC antigens. By examining cytolytic activity of immune PBM on infected target cells derived from animals of various MHC phenotypes, it has been possible to confirm that this activity is indeed restricted to target cells that share at least one class I MHC specificity with the donor (61). A direct role for class I MHC antigens in the generation of these responses was confirmed by the observation that a monoclonal antibody (mAb) specific for a mono-

morphic determinant on bovine class I MHC blocked the activity, while two class II MHC-specific mAbs had no effect. An additional observation of this study was that responses appeared to be biased toward one or other of the haplotypes in each animal, and that within the group of animals tested, certain BoLA specificities dominated as restricting elements.

A large body of evidence now exists to show that the major protective response to homologous challenge with *T. parva* is mediated by cytotoxic T lymphocytes (CTLs) that are restricted by class I MHC antigens. The phenotype of these cells has been defined as $CD2^+$ $CD4^-$ $CD8^+$ (41). However, the failure of some stocks of the parasite to immunize against challenge with others suggests that the parasite strain specificity of these responses may be important to protection. This question has been addressed by analyzing the parasite strain specificity of CTL responses in animals immunized with the Muguga and Marikebuni stocks of *T. parva*. Cross-immunity between these stocks is not reciprocal; cattle immunized with the Marikebuni parasite are resistant to challenge with both stocks, whereas a proportion of animals immunized with *T. parva* (Muguga) are susceptible to challenge with the Marikebuni stock (48). A study was carried out in five animals immunised with the Muguga stock and one animal immunised with *T. parva* (Marikebuni). The PBM from these animals were assayed for killing activity on autologous target cells infected with either parasite. Perhaps not surprisingly, it was observed that PBM from the Marikebuni-immunized animals were capable of killing targets infected with either stock. In two of the Muguga-immunized animals killing was restricted to autologous targets infected with the homologous parasite whereas PBM from the remaining animals of this group killed targets infected with either parasite stock. The results of this study suggested that the differences that have been observed in the capacity of Muguga-immunized animals to withstand challenge with Marikebuni might be related to the specificity of their CTL responses. This possibility is supported by a subsequent study, which involved the challenge of Muguga-immunized animals with the Marikebuni parasite after determining the specificities of their CTL responses (E. Taracha, B.M. Goddeeris, and W.I. Morrison, unpublished observations). However, these studies did not deal with the possibility that the capacity of the Marikebuni stock to protect consistently against challenge with *T. parva* (Muguga) was due to the presence within the stock of parasite strains with antigenic similarities to the Muguga parasite. This possibility has been addressed by the generation of CTL clones from animals immunized with *T. parva* (Muguga) (41). Analysis of the parasite specificities of clones derived from animals whose *in vivo* response was restricted to the Muguga stock revealed that this restriction was reflected at the clonal level (42,60). Similarly, animals whose PBM killed either stock yielded at least some clones that were cross-reactive (B.M. Goddeeris, un-

published observations.). In a study of the specificity of clones derived from a Marikebuni-immunized animal (62), it was observed that individual clones were capable of killing targets infected with either *T. parva* (Muguga) or *T. parva* (Marikebuni).

The results of these studies clearly suggest that at least two parasite determinants are recognized by CTLs derived from animals immunised with the stocks. One is present in both parasites, while the other is apparently restricted to the *T. parva* (Muguga) stock. The question remains, however, as to what factors influence whether an animal immunized with the Muguga stock will make a restricted or cross-reactive response. The observation that the specificity of CTL responses of mice to influenza virus is influenced by their MHC class I phenotype (95,99) suggested that a similar situation might exist in cattle. To investigate this possibility, the parasite strain specificity and MHC restriction of a panel of CTL clones derived from four Muguga-immunized animals were examined using MHC-matched or half-matched target cell lines infected with *T. parva* (Marikebuni) (43). It was found that clones that shared MHC restricting elements also shared parasite strain specificities. In contrast, clones that differed in their MHC specificity also differed in their parasite specificity, even when derived from the same animal. In agreement with earlier observations at the polyclonal level (61), it was also observed that the clones derived from each animal showed a distinct bias in their MHC restriction toward one haplotype.

These observations provide evidence that bovine MHC molecules can influence the specificity of parasite-specific CTL responses by selecting particular epitopes. It is known that MHC class I-restricted T cells recognize antigenic peptides associated with the restricting element on the cell surface (96). This association is the result of the processing of endogenously synthesised antigens (59,96), and X-ray crystallographic studies in the human have shown that the peptide binds to a cleft formed between two domains of the MHC molecule (11). It is possible that selection of parasite epitopes by bovine class I MHC may be a reflection of the relative affinities of different epitopes for the MHC binding site. Alternatively, the effect may be due to differences between animals in the frequencies of CTL precursors with specificities for certain MHC-peptide combinations.

## B. *Theileria annulata*

As might be expected for two related parasites with almost identical life cycles, bovine immune responses to *T. annulata* and *T. parva* show striking similarities. It has been shown that animals undergoing immunization with *T. annulata* develop neutralising antibodies against the sporozoite stage of the parasite (39). These responses are species-specific in that serum from animals immunised with *T. parva* do not neutralize

*T. annulata* sporozoites. The capacity to immunize successfully against *T. annulata* using attenuated schizont-infected cell lines suggests that responses against this stage of the parasite are important to protection. As in *T. parva* infection, although antibodies against the schizont stage of *T. annulata* can be detected in infected animals (72), these appear to be specific for the schizont rather than the infected cell surface, and there is little evidence to suggest that they play any role in recovery and protection.

The developments in the elucidation of cell-mediated responses against the *T. parva* schizont-infected cell gave rise to several investigations of the role of these responses in immunity to *T. annulata*. In one study (77) it was observed that animals undergoing primary exposure to *T. annulata* sporozoites manifested two waves of cytolytic activity against infected target cells. The first appeared about 2 weeks after infection and was restricted to autologous infected lymphocytes. The second wave of cytoxicity was not genetically restricted and was observed approximately 4 weeks after infection. These responses appeared to be associated with recovery from the disease in that they were rarely detected in calves that eventually died. Sporozoite challenge of immune animals was shown to give rise to similar responses, which appeared somewhat earlier after challenge.

In a more direct analysis of schizont-specific CTL responses in *T. annulata*, Innes et al. (47) examined the development and specificity of cytolytic activity in animals immunized with autologous or allogeneic *T. annulata*-infected cell lines. They observed that following immunization with allogeneic cell lines animals developed mild clinical reactions, and on day 9 exhibited strong CTL responses directed at the allo-MHC specificity. However, by day 23 the predominant CTL response was specific for autologous infected cells. In contrast, animals that received autologous infected cells exhibited severe clinical reactions and developed strong parasite-specific CTL responses, which were not genetically restricted until day 20 after inoculation. Both groups of animals were found to be immune to heterologous sporozoite challenge and developed CTL responses specific for autologous infected cells, which peaked at day 10 after infection. Blocking studies with class I MHC-specific sera later confirmed that class I MHC antigens were the restricting elements in genetically restricted CTL responses against *T. annulata* (E.A. Innes, P. Millar, C.G.D. Brown, and R.L. Spooner, in preparation).

An additional cellular immune mechanism directed at the schizont-infected cell has been reported for *T. annulata*. Preston (76) observed that adherent cells derived from PBM exert a cytostatic effect on *T. annulata*-infected cells. In a subsequent report (79), it was demonstrated that this activity occurred after immunization with sporozoites or schizont-infected cells, and peaked 3 to 4 weeks after primary or secondary exposure to the parasite. Cytostasis was effective against autologous or

allogeneic-infected cell lines, and did not appear to be restricted in specficity to the immunizing stock. Although the exact mechanism behind this effect was not determined, it appeared to be mediated by a soluble factor. Another activity has been observed in the serum of animals recovering from infection with *T. parva* (25) in which intracellular destruction of the schizont occurs.

Antibodies to the piroplasm and merozoite stages of *T. annulata* are detectable in animals that are recovering from infection (2,72). In view of the contribution that these stages make to the pathology of the disease, it is possible that these responses are of more relevance to recovery from infection with *T. annulata* than is the case with *T. parva*. However, piroplasm-specific responses are unlikely to contribute to the protection that is observed in immune animals.

# 4. Antigens

## A. *Theileria parva*

Considerable effort has been focused on the identification of antigens of *T. parva* that provoke protective immune responses. The observation of Musoke et al. (65) that sera from animals that have received repeated sporozoite challenges neutralize the infectivity of sporozoites *in vitro* and *in vivo* prompted a search for the target antigens of this activity. By generating monoclonal antibodies against the sporozoite and identifying those with neutralising activity (24,66), it was possible to identify a major neutralizing surface antigen of $M_r$ 67,000. This antigen has been shown to be restricted to the sporoblast and sporozoite stages of the parasite and there is evidence that it is invariant between strains. The gene that encodes the p67 antigen has been cloned and expressed in *Escherichia coli*. (69). In a recent study, a group of cattle immunized with an *E. coli*. fusion protein generated high titers of neutralizing antibody, and 6 out of 9 animals were protected against lethal challenge (67).

As already discussed, serum antibody specific for the schizont stage of *T. parva* is believed to play no part in recovery and protection from ECF. The use of mAb and recovery sera to identify parasite-specific target antigens for T cells antigens on the surface of schizont-infected cells has met with consistent failure. Since T cells recognize degraded antigens in association with surface MHC molecules, this is perhaps not surprising. The antigen binding cleft of class I MHC is believed to accommodate no more than 20 amino acids (11), and it is unlikely that a peptide of this size would be recognised by sera raised against native antigen.

It seems likely that in order to identify parasite antigens that drive T cell responses, it will be necessary to use T cells as screening reagents. Where T helper (TH) cells are concerned, this does not present a major

problem. This subpopulation recognizes processed exogenous antigens in association with class II MHC molecules (59). Thus screening can be achieved by the addition of parasite fractions or the products of recombinant expression libraries to cultures of immune T cells in the presence of autologous antigen-presenting cells. Progress has already been made in this area. In a study that involved the use of parasite-specific TH clones and parasite fractions prepared by hydroxylapatite chromatography and gel filtration it was possible to resolve three peaks of antigenic activity of approximate $M_r$ 43,000, 12,000, and 4200 Daltons (15). Further studies are underway to identify and characterize the antigen(s) responsible.

The identification of parasite antigens that provoke CTL responses is a considerably more complicated task. Since these cells recognize endogenously synthesized antigens that have been processed and associated with class I MHC (59,96), screening assays will require the delivery of parasite antigens to the cytosol. It is unlikely that this will be achieved by the use of soluble parasite fractions, but will require the expression of parasite antigens within appropriate target cells. This might be achieved by conventional transfection techniques or by the use of recombinant virus vectors, using specific genes or expression libraries of parasite DNA. Relevant constructs could then be selected on the basis of their capacity to render targets susceptible to lysis by *T. parva*-specific CTLs.

## B. *Theileria annulata*

Work in *T. annulata* has also focused on the identification of antigens on the sporozoite surface that are engaged by neutralising sera and mAbs (44). Williamson et al. (103) identified an antigenic complex comprising four protein doublets on the sporozoite surface using a neutralizing mAb. By screening a genomic expression library they isolated a 300 base pair (bp) fragment of parasite DNA that encodes the epitope. Immunization of a rabbit with a fusion protein prepared from the DNA clone gave rise to the generation of serum antibody capable of neutralizing sporozoites derived from three different stocks of the parasite. The potential of this antigen as a subunit vaccine is being explored.

Using mAbs raised against *T. annulata* schizont-infected cells, Sheils et al. (88) identified a surface antigen that appears to be associated with infection. The antigen varies between cell lines in the intensity of expression, and also seems to vary in size between $M_r$ 95,000 and 120,000 Daltons. Although the mAb that defines this antigen can mediate complement lysis of parasitized cells (78), it has not yet been possible to ascertain whether the molecule is encoded by parasite or host. Glascodine et al. (40) raised mAbs against *T. annulata* piroplasms, and the determinants recognized by these are restricted to merozoites and piroplasms. The possible significance of these antigens to recovery and protection from the disease will await their characterization.

## 5.  Conclusions

It seems likely that an effective subunit vaccine against either *T. annulata* or *T. parva* will require components that provoke antibody responses against the sporozoite stage and CTLs against the schizont-infected cell. Candidate sporozoite antigens are available in both cases, and are being evaluated for the generation of protective responses. Promising results in this area are already available in the case of *T. parva*. It is conceivable that these antigens alone, by inducing responses that limit the establishment of infection, might suffice as effective vaccines. It has been established that the severity of ECF is directly related to sporozoite dose (50,82,104), and at low challenge levels, naive animals are capable of mounting potent CTL responses against the schizont-infected cell. It is therefore possible that the use of sporozoite-based subunit vaccines could give rise to protective responses against both sporozoite and schizont stages of these parasites. Nonetheless, it is likely that the schizont-specific component of these responses would be, at least in the case of *T. parva*, restricted in specificity to the immunizing stock. In addition, titers of sporozoite-specific antibody would presumably need to be maintained at high levels in order to be effective at limiting sporozoite challenge.

It is probable that the induction of parasite-specific CTL responses in the absence of live challenge will require the use of recombinant antigen-delivery systems capable of giving rise to the expression of schizont-encoded antigens within cells of the vaccinated animal. Recombinant vaccinia viruses have been used to induce antigen-specific CTL responses (8), but there is some concern regarding the release of recombinant viruses into the environment. Although several vaccinia recombinants have been shown to be efficacious, none has yet been licensed for general use. Alternative delivery systems based on recombinant Salmonella are also available (84), and a recombinant *Salmonella typhimurium* has recently been reported to stimulate the generation of CTLs specific for the circumsporozoite antigen of the malaria parasite (1). As the genes for candidate schizont antigens become available they can be incorporated into these vectors for testing. The identification of these antigens remains a major obstacle to the development of subunit vaccines against both these parasites.

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