Principles of Animal Cell Culture

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Students Compendium

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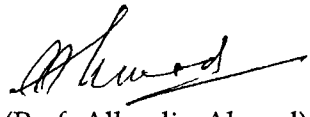
FOREWORD

Biotechnology has emerged as one of the most important branch of biological sciences. There is an upsurge of biotechnological research and its applications all over the world during the last two decades. Animal cell culture is one of the important tool of biotechnology as it has not only been used for the isolation and propagation of different viruses but recently it is being used for several advance studies in biotechnology, such as, the expression of different cloned genes, regulation of gene function, gene amplification, detailed analysis of toxic mechanism of action at cellular level, production of a variety of biomolecules at an industrial scale and screening of various useful materials like vaccines, antiviral agents and anticancer agents etc.

A course on animal cell culture has been included in various science and vocational courses of University Grant Commissions such as B.Sc. (Hons) Biotechnology, Bioinformatics, Industrial Microbiology and M.Sc. (Biochemistry). As there are a few books available on animal cell culture which can provide information to the students about all the possible topics of animal cell culture in a concise and systematic manner and thus students of the above disciplines are facing difficulty. Keeping in view the recent advances in animal cell culture and in order to meet the requirements of the courses Dr. B.K. Sinha and Dr. R. Kumar, have tried to present all possible aspects of animal cell culture in a concise and understandable manner. Each chapter has been provided with sufficient questions along with the key, which will help students to solve different problems of animal cell culture.



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PREFACE

Animal cell culture is playing a pivotal role in the various modern researches. Though the foundation stone of animal cell culture was laid for the isolation and characterization of different viruses but the recent developments in the field of Biotechnology and Molecular biology have given various new dimensions to this technology. Cell culture has provided a new tool for the better understanding of diagnosis and molecular biology in many areas of biological systems including bacterial, viral and parasitic diseases. The different cell cultures are being used in virology for the last five decades and one of its application has been utilized successfully in various types of vaccine production. To quote virologist Payton Rous "of late we have begun to study viruses not only for what they themselves mean, but for what they can reveal about the intrinsic cell structure, and functions through located injuries that they do". More recent discoveries such as production of various proteins and enzymes through recombinant DNA technology require the use of cell culture to study the exact gene expression, have fully utilized where the available evidences justify it and where their use is consistent with the effort involved and their routine application is recommended. With the advancement in tissue culture technology, scientists are now on the threshold of bringing sea changes in improving the life of human as well as livestock and thus it has become a source of unending fascination for different scientists.

The present work is directed primarily to the students of biotechnology, keeping in view the University Grants Commission syllabus on animal cell culture prescribed for vocational course both for undergraduate and postgraduate students and also for the scientists in the Medical, Veterinary and Biological sciences, whose curiosity and efforts are responding to the stimulus of current interest in biotechnology.

This book contains 20 chapters and each chapter has been written keeping in view of recent information on the subjects. Portion of the book have evolved through use of several lecture materials in courses which I have delivered in various colleges of Bihar and Jharkhand state having vocational courses in biotechnology. In different college, where I used to deliver lecture, I found that students are facing difficulty for want of a concise book on animal cell culture, which covers their full courses and thus I decided to write this book. This

notes give information to students they need to know in a handy and succinct format without overwhelming them with unnecessary jargon. It is hoped that it may have some value as a source of providing more readily obtained information on different topics. It is expected that some deficiencies might have persisted in this book, it is anticipated that these deficiencies may be eliminated by diligent supplementing readings and by classroom lecture materials. Though this is the first endeavour ,constructive criticism will be welcomed by the author to be included in the next edition.

Basant K. Sinha

ABBREVIATIONS

ACDP	Active Cell Death Process
ATP	Adinosine Tri Phosphate
BDNF	Brain Derived Neutrotropic Factor
CAD	Carbamoyl-Phosphate-Synthase Aspartatetranscarbamoylase Dihydroorotase
CaMv	Cauliflower Mosaic virus
CARD	Caspase Recruitment Domain
CFA	Complete Freund's Adjuvant
CHO	Chinese Hamster Ovary
CNTF	Ciliary Neurotrophic Factor
CSF	Colony Stimulating Factor
DHFR	Dihydrofolate Reductase
DMSO	Dimethyl Sulfoxide
DMEM	Dulbecco's modified Eagle's medium
ECF	Extracellular Fluid
EDTA	Ethylene Diamine Tetra Acetic acid
EGF	Epidermal Growth Factor
ELISA	Enzyme Linked Immunosorbent Assay
EPO	Erthropoietin
ETT	Embryo Transfer Technology
FACS	Fluorescene Activator Cell Sorter
FAK	Focal Adhesion Kinase
FGF	Fibroblast Growth Factor
FITC	Fluro Isothiocynate
FSH	Follicular Stimulating Hormone
GAGs	Glycosamino Glycans
GFAP	Glial Fibrillary Acidic Protein
GnRH	Gonatrophin Releasing Hormone
HAT	Hypoxanthine Aminopterin Thymidine
HB-EGF	Heparin Binding Epidermal Growth Factor
HbsAG	Hepatitis B Surface antigen
HBSS	Hank's Balanced Salt Solution
HEPES	[A-2(hydroxyethyl)-1 Piperaine Ethano Sulphonic acid]
HGF	Hepatotoxic Growth Factor
HMWK	High Molecular Weight Kininogen
hpt	Hydromycine phosphotransferase
ICSH	Interstitial Cell Stimulation Hormone

IGF	Insulin Growth Factor
IgSF	Immunoglobulin Superfamily
IL	Interleukin
IPTG	Isopropyl-β-D-thiogalacto Pyranoside
Is	Insertion Sequence
LH	Leutenizing Hormone
LMWK	Low Molecular Weight Kininogen
ME	Malic Enzyme
MEM	Minimum Essential Medium
MSC	Mesenchymal Stem Cell
MTX	Methotrexate
NF	Neurofilament
npt	Neomycin phosphotransferase
NT3	Neurotrophin3
PALA	N-Phosphonacetyl-L-Aspartate
PARD	[Poly (ADP-ribose) polymerase]
PDGF	Platelet Derived Growth Factor
PEC	Peritoneal Exudate Cell
PEG	Polythylene Glycol
PGD	6-Phosphogluconate Dehydrogenase
PGM	Phospho Glucomutase
PMSG	Pregnant Mare Serum Gonadotrophin
PPLO	Pleuro Pneumonia Like Organism
PTFE	Polttetrafluro Ethylene
PVC	Polyvinyl Chloride
RPMI	Rosewell Park Memorial Institute
RTF	Resistance Transfer Factor
SCID	Severe Combined Immuno Deficiency
SCNT	Somatic Cell Nuclear Transfer
SCP	Single Cell Protein
STAT	Signal Transducer and Activation of Transcriptase
TDC	Thymic Dendritic Cell
TCA	Tricarboxylic Acid Cycle
TGF	Transforming Growth Factor
Tdt	Terminal Deoxynucleotide Transferase
TK	Thymidine Kinase.
Tn	Transposon
TPA	Tissue Plasminogen Activator
TSH	Thyroid Stimulating Hormone

Contents

Detailed Contents

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Chapter 1

History of Development of Cell Culture

The culture of animal cells and tissues has now become a widely used technique in a spectrum of discipline ranging from environmental sciences to molecular biology. The term tissue culture is routinely used as a generic term to include organ culture and cell culture. The foundation for the development of cell culture technique was laid by Wilhelm Roux (1885) an embryologist, who showed that explants (fragments of tissue) of chicken embryonic cells can be maintained in warm saline *in vitro* for a few days. Arnold in 1887 demonstrated that explants isolated from frog can be maintained in warm solution for short time. Later on several attempts were made by various groups of scientists to improve the culture conditions and media to extend the life span of cells *in vitro*. After advent of antibiotics, cell culture became somewhat simpler to prepare. In recent years, the cell culture techniques have undergone meteoric sophistication heralding era of industrial biotechnology. The yearwise development of cell culture techniques are as follows:

1665 Hooke's observation in 1665 that vesicles and cavities observed in cork were cells, and this perhaps marked the beginning of cytology.

1674&1682 Malpighi (1674) and Greur (1682) also observed that plant cells contained some fluid like substances bounded by a cell wall.

1806&1830 Treviranus (1806) and latter Uugovon Mohe (1830) noted that meristem cells first undergo elongation and divide.

1897 Loeb demonstrated that blood and connective tissue cells survived in tubes containing serum and plasma.

1897 Ljunggren showed that an explant of human skin remained sufficiently viable in ascitic fluid. Later it became possible to successfully implant skin at a new site or location.

1902 German Botanist Gottieb Haberland first described cell culture in a nutrient medium containing glucose, peptone, and different salts.

1903 Jolly made detailed observations on *in vitro* cell survival and cell division using salamander leukocytes in hanging drop preparation.

1906 Beebe and Ewing observed cell division in explanted canine lymphosarcoma.

1907 Ross Granivelle Harrison cultivated amphibian spinal cord using frog lymph clot in hanging drop technique and demonstrated that axons are produced as extension of a single nerve cells and later he was in a position to cultivate nerve cells for several weeks. Harrison is regarded as the **"Father of tissue culture"** as he not only showed the method to keep tissue alive *in vitro* for several weeks but also demonstrated that the procedure was research oriented, which is a fundamental contribution in biological knowledge.

1910 Burrows cultivated chick embryo using chicken clot plasma. He opined that frog lymph does not form firm clot and is also difficult to procure it in larger amount.

1911 M. R. Lewis and W. H. Lewis made first attempt to replace natural media with synthetic media. The basic difference observed between natural and synthetic media was that the composition of natural media i.e., plasma and embryo extract was not fully defined whereas synthetic media was prepared from the known components.

1912 Alexis Carrel, a Nobel-laureate for his work in experimental surgery showed that composite culture media having blood serum, embryo extract and saline solution could further enhance the lifespan of the cells *in vitro*. In 1913 he introduced a flask to facilitate culture called **"Carrel flask"** under aseptic condition. (Fig. 1).

1916 Rous and Jones used "Trypsin" to free cells from tissue matrix.

1935 Moen described growth of a single animal cell such as guinea pig fibroblast into colonies.

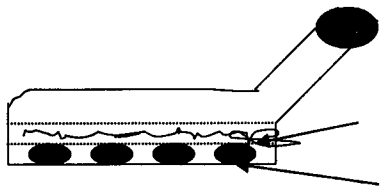

Fig. 1:- Structure of Carrel Flask.

1937 Simms and Stillman used trypsin to free cells from the tissue matrix in which these were embedded.

1947 Earls and colleagues showed that variety of cells can proliferate under in contact with a sheet of perforated cellophane. Earls and his colleagues showed that single cell of the L-cell line by treating primary mouse fibroblast with the cancer causing agent "Methyl cholanthrene" and showed that they formed clones of cells in tissue culture.

1948 Sanford Katherine, Wilton Earle and Gwendolyn isolated single cell from an established mouse cell line, designated strain "L". The parental L-strain was derived from normal subcutaneous areolar and adipose tissues of a 100 day-old C_3H/An mouse. The cloned strain L919 was established in March 1948 and the chromosome number in it varied between 62 to 68.

1949 Enders, Weller and Robbins singled the beginning of the new era and showed that poliovirus could be grown in non-nervous cells i.e., different kinds of human embryonic cells with production of easily recognizable cytopathic effect. Consequently polio vaccine was the first vaccine to be produced in cell culture and the first plaque assay for an animal virus in culture was done with polio virus.

1949 Joseph Morgan, HelenMorton and Raymond Parker produced a media called "Formula 199" which when supplemented with blood serum supported the growth of primary cell culture *in vitro*. In the same year Eagle and Karl Piez observed that for 100 cells per millilitre MEM was found to be nutritionally deficient but it is not so when 50,000 cells per/ml was cultured. This phenomena was known as "**population dependent growth**" and is explained by the experimental findings that cell cultured *in vitro* leak some compounds that they

synthesize when there are more than 50, 000 cell per ml. The leaked nutrient can reenter in another cell in close proximity and thus an intracellular level is maintained by cross-feeding between cell. However, when only a few cells are present in the culture, the leaked nutrient may be lost into the culture medium at a rate equal to its rate of synthesis. Because there is no cell in close proximity to supply the ingredients by cross-feeding, the cells that are present will be starved for the nutrient, despite the fact they can synthesize it.

An important breakthrough in cell culture technology came in 1950 when chicken embryo and tissue culture were adopted for production of vaccine.

1951 George Gey, Ward Coffman and Mary Kubicele derived a neutral epithelial cell line from the cervical carcinoma of a black woman named **Henreetta Lacks** which later became the well known "HeLa" cell line. In the same year Theodore Puck and Philip Marcus introduced a "FEEDER LAYER" method in which an almost confluent sheet of HeLa cells was first irradiated with X-ray to destroy their ability to divide but not their ability to metabolize. This irradiated cells served, as a feeder layer to provide essential ingredients needed for the growth of cell in cell culture.

1953 Frisch and Jentoft used trypsin for monkey testicular primary tissue culture.

1954 Zwilling used chelating agent (sodium versenate) in tissue culture. Levi Montalcini and associates showed that nerve growth factor (NGF) stimulated the growth of axons in tissue culture.

1954 Robertson used serum in tissue culture for the better growth of cells. In the same year Eagle demonstrated the specific aminoacid requirement of HeLa cell line and found that at least 12 aminoacids required for L-strain is essential for HeLa cell. These include arginine, cystine, histidine and tyrosine in addition to 8 aminoacids required for nitrogen balance in man such as isoleucine, leucine, lysine, methionine, phenylalanine, threonine, trptophane and valine. Only L-aminoacids were active

the D- enantimorphs had no demonstrable effect at physiological concentration.

1955 McAllister and Coriell described method of viable count used for cell culture. Oherry and Hull described the methods of suspension culture.

1956 Temin and Rubin developed a quantitative assay for the infection of chick cells in culture by purified Rous sarcoma virus. In 1956 only Bodian used overnight extraction of magnetically stirred monkey kidney cell in refrigerator.

1961 Hayflick and Moorhead derived a human diploid cell line W1-38. Initially they thought that this cell line has unlimited life span *in vitro* but later they observed that human fibroblast die after a finite number of division in culture. The limited replicate capacity of human cells in culture is sometimes called the "**Hayflick effect**"after the name of discoverer (Hayflik, 1965). The interesting thing about this phenomenon is that, it is very highly reproducible, it seems that cells had a built in time check which told them how long they have to survive?

1964 Littlefield introduced HAT medium for the selective growth of hybrid cells.

1965 HAM introduced a defined serum medium able to support the cloned growth of certain mammalian cells. In the same year Harris and Watkins produced the first heterokaryons of mammalian cells by the virus induced fusion of human and mouse cells.

1966 Abercrombie gave the interesting phenomenon of " **contact inhibition**" which is actually the inhibition of cell motility and also mitotic division that is observed when cells come in contact with one on solid support. Holley and Kiernan(1968) reported phenomenon of contact inhibition in 3T3 cells. Cancerous cells lack contact inhibition i.e., mitotic rate is not inhibited and thus culture cells tend to pile up forming irregular mass of several layer deep.

1967 Augusti-Tocco and Sato adopted a mouse nerve cell tumour (Neuroblastoma) to tissue culture and isolated clones that were electrically excitable and that extended

nerve fibre.

1970 Horibate and Harris described P_3k, a cell line of mouse plasmocytoma.

1975 Kohler and Milstein produced the first monoclonal antibody secreting hybridoma cell line.

1975 Sato and associates showed that different cell lines require different mixture of hormones and growth factors to grow in serum free medium.

1976 Wigler and Axel developed method for introducing single copy mammalian genes into cultured cells, adapting an earlier method developed by Graham and VanderEb.

2007 Mario Capecchi, Oliver Smithies of USA and Martin Evans of Britain received Nobel Prize in Medicine for their work on "Gene Targeting" commonly known as gene "knockout". They genetically manipulated mouse embryonic stem cell leading to laboratory rodent that replicate human disease.

Practice Assignment I

1. Hayflick effect refers to the :
   (a) Unlimited replication capacity of cells in culture,
   (b) Limited replication capacity of cells in culture,
   (c) Both of the above
   (d) None of the above.
2. HAT medium used for the selection of fused hybrid cells in hybridoma culture was introduced by:
   (a) Kohler and Milstein (b) Frish and Jentoft
   (c) Littlefield and Miller (d) Eagle and Karl.
3. All the statement for population dependent growth is true, Except:
   (a) The growth of cells in cell culture is better when number of cells are 50,000/ml.
   (b) The growth of cells in cell culture is much better when number of cells are about 100/ml.
   (c) The nutrient leaked by cells re-enter in another cell when there is close proximity.
   (d) The leaking nutrient is lost in the culture medium when the cells are not in close proximity.

4. The foundation for the development of cell culture technique was laid by:
   (a) Alexis Carrel (b) Sims and Salmon
   (c) Rous Harrison (d) Wilhelm Roux.
5. Use of trypsin to free cells from the tissue matrix for cell culture was described by:
   (a) Beebe and Ewing (b) Sims and Stillman
   (c) Gottieb Maberland (d) Jolly and Koch.
6. Contact inhibition refers to:
   (a) Inhibition of cell motility and mitotic division activity
   (b) Above events only occur when cell comes in contact with one on solid support,
   (c) It is a property of normal cells, not of transformed cells,
   (d) All of the above.
7. HeLa cell line was derived from a black woman named Henereeta Lack from :
   (a) Cervical carcinoma (b) Pharyngeal carcinoma
   (c) Oral carcinoma (d) None of the above.
8. For the first time formula 199 for tissue culture was prepared by:
   (a) Joseph and co-workers (b) Earls and co-workers
   (c) George and co-workers (d) Rous and Jones.
9. Cells used for feeder layer should:
   (a) Have ability to metabolise
   (b) Should not have ability to divide
   (c) These properties are obtained by exposing cells to irradiation
   (d) All of the above.
10. Cell culture technique became simpler only after advent of :
   (a) Antibiotics (b) Trypsin
   (c) Cell culture media (d) All of the above.

Answers:

1. -(b), 2. -(c), 3. -(b), 4. - (d), 5. - (b), 6. - (d), 7. - (a), 8. - (a), 9. - (d), 10- (d).

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Chapter 2

Natural Surroundings of Animal Cell

A living cell is the fundamental unit of living organisms, of both the plants and animals. It is a self - assembling, self-replicating and self-regulating tiny bags of lipid bilayer enclosing discrete organelles. The natural surrounding of the cell is the local biochemical, physiological and physiochemical *milieu* directly affecting the growth of the cells and their metabolism. All living cells are considered to have open chemical system (i.e., a system in which both matter and heat can exchange with the surroundings) of organic molecules operating on the principle of maximum economy of parts and processes. It promotes many consecutive linked organic reactions for the transfer of energy and /or the synthesis of its own components by means of organic catalyst that it produces itself. Being an open chemical system, each cell has a continuous exchange of chemicals and energy with its immediate outside medium called the external environment. The different behaviour of cells i.e., attachment, spreading, motility and biosynthetic capabilities are influenced by their natural surroundings. The cells remain busy in performing respiration (glycolysis), energy liberation (ATP) and in synthesis of energy rich compounds (ADP + pi + energy). The energy that cells absorb from their environment is transformed into chemical energy as living organism follow the rule of thermodynamics. The first law of thermodynamics states that if a system exchanges heat with or does work on, its surroundings, then a change in its internal energy occurs. This energy can neither be created nor destroyed. Living organisms thus cannot consume or use up energy, they can only transform one form of energy into another form. Wilson and Morrison (1966) suggested that the cell is an integrated and continuously changing system and when it ceases to be a cell in biological sense it becomes merely a mass of disintegrated matter.

In multicellular organism cells do not function in isolation but each cell serves the need of the whole organism. *In vivo*, cells in close contact interact with each other and the interactions are essential in the functioning of tissue organs. Cells are also in contact with a complex network of secreted proteins and carbohydrates called the extracellular matrix, which perform the following functions, i.e.,

1. fills the space between cells.
2. helps to bind cells together and,
3. provides a lattice through which cells can move and affects cell behaviour directly.

In addition, cells *in vivo* are in contact with hormone, and hormone like factors. This communication between different cell types is lost when cells are taken out and cultivated *in vitro* and thus cells remain in tremendous stress. Although the own system of cell system is working to ward off the condition of stress and would strive to maintain its internal conditions, but a cell with minute size and limited system could not work in fighting the stress condition in cell from its removal from the original source or host body. So, for the regulation and performance of various vital functions, cells need special surroundings because:-

(i) It needs nutrients and other essential things which are not synthesized by cells itself

(ii) As the cells are immobile, hence a mobile medium is needed for factor assessment to the cell.

In its natural surroundings, an animal cell is in close contact with other cells through cell junctions, extracellular matrix and extracellular fluids (ECF).

A. Extracellular Medium and Cellular Exchange:

Extracellular medium around the body cell consists mainly of (NaCl) dissolved in water. Its other constituents are carbonate (CO_3), bicarbonate (HCO_3), phosphate, sulphate of potassium, calcium, magnesium, chloride, iron, zinc, manganese, some organic acids and few proteins.

Extracellular mediums should have the following things for the survival of cells:

1. Nutrients :

Nutrients are the most important thing, a cell requires for its survival.

The various nutrients required are glucose, fats and fatty acids, lipids, phospholipids and sulpholipids, ATP and aminoacids.

Carbohydrate:- It is the major source of energy for cultured cells. It is degraded into carbon di-oxide and water in the cells. The most commonly available source is glucose. However, glucose is not the source of carbon. Lactic acid is the source of carbon. Glucose is degraded via-glucolysis, tricarboxylic acid (TCA) cycle and electron transport chain. It also produces many metabolic intermediates. Its molecular formula is $C_6H_{12}O_6$.

Lipids and its Derivatives :-These are heterogenous group of substances with the common property of poor solubility in water due to their nonpolar structure. These do not form long chains of monomers, and are the smallest macromolecules in the body. These can be divided into triglycerides, phospholipids and steroids (corticosteroids, testosterone, and progesterone).

Aminoacids:- The essential aminoacids are those aminoacids which are not synthesized in cells and thus forms the essential parts of the natural surroundings of the cell (Eagle 1955a). There are about 12 essential aminoacids which are provided to cell for their growth. These are arginine, cystein, isoleucine, leucin, histidine, lysine, methionine, phenylalanine, threonine, tryptophane, tyrosine and valine. In addition cysteine and tyrosine are also needed Deficiency of these aminoacids depletes the chromosome and the growth of cells are thus inhibited (Swim and Parker 1956). Besides the above mentioned aminoacids some cells have high requirement of glutamine.

Vitamins:- Several vitamins like folic acid, nicotinamide, pantothenic acid, pyridoxal, riboflavin and thiamine are necessary for cell growth and multiplication (Eagles 1955b). Most of them are known to form essential parts of co-enzyme. Cholin is necessary for the cell adhesion and for maintaining cytoskeleton. If cholin is not provided then rolling of the cells will occur. Retinol is also very important.

Minerals:- Macronutrients such as Na, P, O, Ca, Mg etc. are required. Micronutrients like ZN, Mn. Co. etc. are also required for maintaining the integrity of cells.

2. Hormones :

It is Greek word which means "to set in motion". Hormones are chemical messenger that are synthesized and secreted by ductless gland in minute quantities into the blood vascular system and are

transported to a remote target organ where they regulate the rates of specific biochemical processes e.g., Thyroid stimulating hormone (TSH), Follicular stimulating hormone (FSH), Growth hormones (GH), Prolactin, Progesterone, Testosterone, insulin, insulin like serum peptide, glucocorticoid, somatostatin and prostaglandin etc. Hormones either alone or in combination with other hormone or with low concentration of serum have growth promoting activity of certain cells (Fell 1954). Receptors for attachment of protein hormones such as Luteinizing hormone (LH) and FSH hormones are present on the plasma membrane of the cell. Binding of a hormone to a receptor in a target cell can be considered to be the primary event in the action of that hormone. Such a hormone receptor interaction will cause a measurable and distinct biological response for each hormone. The binding of the hormone to the cell surface receptor activates one or more second messengers, such as cyclic AMP (3', 5'-AMP). The second messenger is thought to activate another intracellular enzymes, protein kinase, which will influence the transport of cholesterol into the mitochondria. Receptor for steroid hormones are inside the cell. As the steroid hormones are fat soluble, so theses are able to enter in all cells of the body because the lipid cellular boundaries present no barrier to the steroid.

3. Plasma:

Plasma is a vital nutrient, which should be available in the environment of cells. Plasma is a constituent of blood, but it is devoid of the cellular parts of blood i.e., RBC, WBC and platelets. The various factors present in the plasma are protective system and plasma mediated kinin systems:-

The protective system which enables the cell to survive and it prevent from the attack of various micro-organisms, such as bacteria, viruses and fungi. The plasma contains all the nutrients listed above.

Plasma Enzyme Mediators:- This consists of four systems:

(a) Kinin System :- It is an enzymatic cascade that begins when a plasma clotting factor is activated following time injury. Two important kinins are Bradykinin and Kallidin. The precursor molecule is called kininogen and is normally available in globulin fraction of plasma protein. Two well known kininogens are (a) High molecular weight kininogen (HMWK) and Low molecular weight kininogen (LMWK). HMWK when acted upon by kallikrein gives rise to bradykinin. Bradykinin is a potent

vasoactive basic peptides and causes local vasodilation of inflammation. It is one of the main pain producing substance.

(b) **Clotting System** :-It is the most important haemostatic mechanism when there is an extensive injury to blood vessels. Coagulation occurs when the soluble plasma protein, fibrinogen is converted to a lattice of insoluble filamentous molecules by the action of thrombin called fibrin. Fibrin is laid down as a network of fine threads, which entangles the different blood cells. The freshly formed threads are extremely adhesive, sticky to each other to the blood cells, to the tissues and to certain extent foreign surface. This adhesiveness makes the clot and effective hemostatic agents. Stabilization of fibrin network occurs by an enzyme (Factor XIII), which is activated by thrombin.

(c) **Fibrinolytic System** :- Blood clotting as mentioned above is an essential mechanism for protection of the body in order to prevent excessive blood loss, but the clot formed in the tissues have ultimately to be disposed of as healing takes places. The dissolution of the clot i.e., fibrinolysis, which is a complex mechanism and is due to the action of the proteolytic enzyme called fibrinolysin or plasmin which degrades the fibrin threads. In the fibrinolytic system there is no free plasmin but blood plasma contains its inactive precursor plasminogen. Plasminogen is converted to plasmin by means of plasminogen activators which may be extrinsic or intrinsic. This is for removal of fibrin clot from injured tissue. In this system plasmin is the final end product.

Extrinsic activators of plasminogen are widely distributed throughout the cells and body fluids. The tissue activators probably occur in the microsomes. Urine also contains a plasminogen activator called urokinase.

Intrinsic plasminogen activator appears in the body as a result of surgical operation, violent exercise or injection of adrenalin etc. These promote fibrinolytic activity with dissolution of the clot within a few hours, through activation of the intrinsic plasminogen activator in plasma. Intrinsic plasminogen activator becomes adsorbed together with plasminogen on to fibrin clot so that when the system is activated the proteolytic activity of the plasma will be wholly exerted on the clot itself.

It has been suggested that in physiological conditions the clotting system of plasma is continuously forming small amount of fibrin which are deposited to form a thin layer on vascular endothelium, and that the fibrinolytic system is constant in action to prevent excessive fibrin formation. If the clotting system predominates there might be a tendency to bleeding. All this is speculative but if free plasmin circulates in large amounts in the blood it can cause bleeding by its capacity to digest several clotting factors, and it also interferes with fibrin polymerisation.

(d) **Complement System :-** Complement system consists of a series of serum proteins (about 20 in number) which circulates in the blood as an inactive precursors and are activated in turn. A complement components, when once activated, may then act as an enzyme, which cleaves several molecules of the next components of the complement in the sequence. This sequential reaction implies an amplification of the process, because one activated complement molecule activates many molecules in the next step, the complement reaction is thus a cascade reaction. The major purpose of the complement system is to provide a means of removing or destroying antigen, regardless of whether it has been coated with antibody or not. The other key function of the complement system is opsonization of microorganisms. The microorganism coated with antibody and/ or few fragments of complement (Immune adherence) are easily opsonized (opsonin means prepare food) by the different phagocytic cells (macrophages and polymorphonuclear leukocytes) as compared to without coated micro-organisms. But when all the components of the complements are attached with the homologous antigen-antibody complex, it results into lysis. It gives inflammatory response, helps in antigen-antibody reaction and thus helps in defence system.

4. **Creatinine:-** Most of the creatinine eventually excreted by cell originates from endogenous creatine. The aminoacid arginine and glycine combine to form guanidinoacetate in the liver, pancreas, kidney and small intestine. In the liver methionine provides a meth group for conversion of guanidinoacetate to creatine (Tyler 1972). Creatine circulates in the plasma and is taken up by muscle, where it stores energy in the form of phosphocreatine. Creatine is the sole precursor of creatinine. The conversion of creatinine from creatine is a nonenzymatic irreversible process that occurs at a rate of1. 6-2% daily (Hoberman *et al.* 1948).

5. Cytokines:- Some of the activities of immunocompetent cells are performed by the release of various soluble mediators, which are distinct from the antibodies. Such a class of substances is produced by stimulation of either lymphocytes or monocytes *in vitro* under different experimental conditions. All these cellular products had been collectively called "**lymphokines**" by Dumond *et al.* (1969). The cytokines produced by lymphocytes are also called "**lymphokines**"(Oppenheim and Rosenstretch 1976) whereas macrophage / monocyte are also capable to release mediators called "**monokines**" (Beller and Unanue 1977). Recently term "**Interleukin**" has been proposed for some of the mediators which acts between cells of immune system. Cytokines have a vital role in the initiation and regulation of various immune response (Kroemer *et al.* 1993) and is an important component for regulation of cell growth and differentiation. So, cytokines are potent multifunctional immuno-regulatory peptides produced by different cell types such as lymphocytes, monocytes, macrophages, polymorphonuclear leukocytes, fibroblast and endothelial cells. Paran *et al.* (1970) observed that some cytokine induces terminal differentiation of leukemic cells of human and mice into macrophage or granulocytes and thus this may serve as valuable tool for differential therapy in leukaemia.

Recently, great progress has been made in delineating the intracellular elements responsible for transmitting cytokine-dependent signals. A structurally unique family of protein tyrosine kinase termed as the Janus family of kinase and Signal transducer and activation of transcription (JAK/STAT), when linked with cytokine receptors, serves essential function in cytokine signalling. Ligation of cytokine receptors result in tyrosine phosphorylation and activation of receptor-associated JAK protein tyrosine kinase and cytoplasmic STAT transcription factors, which are translocated to the nucleus.

Interleukin-1(IL-1):- Interleukin-1 is a cytokine which was discovered by Gery *et al.* (1972). It is produced by the stimulated macrophage and monocytes. It has been found that it is also released by various other cell types, such as, lymphocytes, fibroblasts and endothelial cells. It is glycoprotein in nature and acts on wide range of cells including T-cells, B-cells, macrophage, fibroblasts, certain brain and liver cells. It binds to T-helper (Th_2) cells and activate them resulting into proliferation of T-cells leading to secretion of various proteins, like interleukin-2, interleukin-4, interleukin-5 and interleukin-10.

Interleukin-2 (IL-2):- Interleukin-2 is also a cytokine. When antigen is recognised by the T-cell receptors, IL-1 activates the T-cell and the activated T-cells secrets IL-2. Interaction of IL-2 with IL-2 receptors initiates the clonal proliferation of antigen specific-T-cells and their differentiation into memory and effector T-cells. IL-2 also stimulates the growth and activity of B-lymphocytes and natural killer cells.

6. Waste Material :- The different cells are also surrounded by the wastes and toxic materials which are produced by the cells itself. The surrounding fluids help to remove these waste and toxic materials and thus prevent the cell from its deleterious effects. The main waste materials are, nitrogenous waste, like urea, uric acid, toxins, nitrates, ammonia, sulphate and phosphates etc. Most of the sulphate and phosphates are produced metabolically from non-ionised sulphur of cystine and methionine and the less ionised phosphate of phospho-proteins.

7. Buffers:- The cells are vary sensitive to pH of their environment. Intracellular and extracellular fluids of living cells contain conjugate acid-base pairs which acts as buffers at the normal pH of these fluids. So, the buffers present in the plasma helps to regulate the pH. A buffer system is one which can accommodate the addition to it of moderate amounts of acids or base without marked change in its hydrogen ion concentration. . The ions of natural buffered fluids are of two types:-

(i) The fixed ions, whose concentration are unaffected by moderate variation in H^+ concentration.

(ii) The buffer ions, whose concentrations are considerably affected by attempted changes in H^+ concentration.

The major intracellular buffer is the conjugate acid-base pair $H_2PO_4^-$ – HPO_4^{2-} (pK^1=7. 2). Organic phosphates such as Glu-6-PO_4 and ATP also contribute buffering power. The major intracellular buffer in blood and interstitial fluid of vertebrate is the bicarbonate buffer system.

8. Growth Factors:- Growth factor is an organic compound which a cell must contain in order to grow but which it is unable to synthesize (Biggers *et al.* 1957). Different cells vary widely in their growth requirements, this differences in the requirement reflect differences in the synthetic ability of the cells. Growth factors are present in nanogram and picogram per millilitre of serum. These are proteins of highly specialized nature. These act as signals and facilitate cell

proliferation and differentiation. The mechanism in brief requires binding of a growth factor to a transmembrane receptor molecule present on the surface of the target cell. The intracellular portion of the receptor molecule then catalyses the production of molecules that act as intracellular signal molecules, relaying the stimulus to yet other molecules Growth factor receptors activate intracellular phosphorylation that lead to change in gene expression. Within 15 minutes of treatment with growth factor, *the early response genes* are induced, this induction does not require protein synthesis. In contrast, *the delayed response genes* require protein synthesis and the induction is delayed until at least one hour after the treatment with growth factor.

Various growth factors are essential to promote the growth of cells. These form the essential part of the natural surroundings of the cells. Growth factors first interact with specific receptors at the cell surface. Then some factors (e.g., RGF, insulin etc.) together with their receptors either are internalised into the cells where these are degraded by lysosomal enzymes or bind to internal receptors. Macromolecular synthesis may occur at the site of attachment through an internal messenger (as for certain peptide hormone for which surface synthesis cyclic AMP is a messenger) or through cytoskeleton, which connects all the cell constituents. Some of the growth factors are as follows:-

Epidermal Growth Factors (EGF):- These are isolated by Stanley Cohen in 1960 from sub-maxillary gland of mouse and are responsible for cell proliferation. It is also present in human urine. EGF isolated from human urine was initially named β-urogasterine. It differs from mouse EGF in that it has different aminoacid composition, a more neutral isoelectric point and a small molecular weight (5300-5500 kDa). It is a well characterised mitogen of 53 aminoacids (Carpent and Cohen 1990). The biological effector EGF both *in vivo* and in organ culture is well documented. It promotes the proliferation of numerous cell types including fibroblast cells, glial cells, mammary epithelial cells, vascular and corneal endothelial cells, bovine granulosa, rabbit chondrocytes *in vivo* and HeLa cells, SV_{40} cells and 3T3 cells *in vitro* (Rheinwald and Green 1977). Among the cells of ectodermal origin which responds to it are a variety of keratinocytes derived from skin, conjunctival or pharyngeal tissues. With the exception of haemopoietic cells the EGF receptors are detectable on a large variety of cell types or tissues. The gene for EGF receptor is located on the short arm of human chromosome -7 in the p14-p12 region (Knodo and Shimizu 1983).

Fibroblast Growth Factor (FGF):- During purification of various pituitary hormone it was observed that some pituitary preparation has mitogenic activity for various cultured cell types. This mitogenic peptide was called Fibroblast growth factor by Gospodarowicz in 1974. The biological effect of FGF includes proliferation of fibroblast cell, differentiation and senescence *in vitro*. It also induces the proliferation in embryonic development. Regeneration of most external body parts in lower vertebrates is one of the most dramatic example of cell proliferation by FGF. FGF increases the uptake of macromolecular precursors, the synthesis of protein, mRNA, tRNA and also induces polysome formation. FGF also stabilizes the phenotypic expression of cultured cells and extends its lifespan by delaying the ultimate senescence.

Platelet Derived Growth Factor (PDGF):- It is the principal mitogen found in mammalian serum and is released fromα-granule of platelet during blood clotting at the site of wound (Ross *et al.* 1986). PDGF elicits multifunctional actions with a variety of cells including mitogenesis of mesoderm derived cells, increased extracellular matrix synthesis and chemotaxis and activation of neutrophils, monocytes and fibroblasts. PDGF also acts as mitogen for dermal and tendon fibroblasts, vascular smooth musclecells, glial cells and chondrocytes. PDGF appears to interact with Transforming growth factor-1 in accelerating wound healing. However, oversecretion of PDGF may produce chances of artheriosclerosis and neoplasia. The mitogenic effect of all PDGF products have been tested in culture using Swiss mouse 3T3 cells.

Nerve Growth Factor (NGF):

Nerve growth factor was discovered by Rita Levi-Montalcini in the 1950. It is a dimer of 118 amino acid polypeptide and target-derived neurotropic factor, which supports the survival and growth of peripheral sympathetic and primary sensory neurons. NGF also induces a variety of effects in CNS cells including protein phosphorylation (Halegona 1980), the activation of gene expression (Leonard *et al.* 1987), the reduction neuronal loss after injury (Kromer 1987) and the chemotropic guidance of axons (Hagg *et al.* 1990). It also promotes survival and nerve process outgrowth of specific classes of neurons. e.g., Brain derived neurotrophic factor (BDNF), neurotrophin -3 (NT-3), neurotrophin-4 (NT-4) (Hohn *et al.* 1990).

Nerve growth factor (NGF), originally described as an essential supporting nerve growth especially in the peripheral autonomic and central nervous system has now been shown to have a large pleiotropic set of functions. It causes mast cell degranulation, in addition to nerve sprouting and growth, and achieves its effect through interaction at the cell surface with high affinity TrkA receptors. Injection of nerve growth factor *in vivo* causes mast cell growth, and NGF promotes basophils and eosinophils colony growth from human peripheral blood. This activity is synergistic with IL-5 and GM-CSF. In *in vitro* immunoglobulin synthesis NGF promotes IgG4 synthesis. It also acts to promote survival of mast cells by inhibition of apoptosis. NGF levels are elevated in serum and secretions of allergic patients, as well as in chronic inflammatory diseases. Mast cells, fibroblasts, glandular acinar cells and TH_2 lymphocytes all secrete NGF in addition to nerve cells. Nerve growth factor is partially responsible for bronchial hyper reactivity in a mouse model of asthma as evidenced by abrogation by anti-NGF of increased bronchial hyper reactivity upon repeated antigen challenge, as well as diminution in numbers of bronchial lavage eosinophils.

Recently, a number of studies have reported that NGF is more effective at promoting angiogenesis rather than nerve regeneration (Ohta *et al.*, 1991 and Santos *et al.* 1991). Angiogenesis is known to be essential for wound healing, female reproduction, embryonic development, organ formation, tissue regeneration and wound remodelling (Risau 1997).

Ciliary Neurotrophic Factor (CNTF):- Ciliary neurotrophic factor is a 22. 8 kDa protein first identified as a survival factor for neurons from the ciliary ganglion of chicken embryos. Its most of the actions are restricted to the nervous system including motor neurons, sympathetic ganglion neurons, sensory neurons, hippocampal neurons and medial septal neurons. CNTF also prevents degeneration of motor axons and outside the nervous system it maintains embryonic stem cells in an undifferentiated pluripotent state.

Insulin Growth Factor (IGF):- In 1921 Charles Herbert Best, (a medical student) with his brilliant teacher Frederic Bantic, Professor of Physiology discovered insulin because of its spectacular action on carbohydrate metabolism. Two years later Gey and Thalihimer 1924 found that insulin could help in the growth of cell *in vitro.* This was further strengthened by the observations that most of the mitogenic peptides isolated from blood and serum have an insulin like activity.

It was observed that insulin even in low doses i.e., 40 ng/ml initiates the DNA synthesis of human foreskin fibroblasts. Using BHK (Baby hamster kidney) cell it was observed by Imeniz de Ausa *et al.* (1973) that insulin (8 μg/ml) with serum could induce the cells to reach higher final cell densities than with serum alone. It was also observed that 10μgml insulin potentiates the effect of serum on DNA synthesis of BHK cells. Though for mammalian cells insulin is a poor mitogen but for chicken embryo fibroblast it is a good mitogen. Insulin also collaborates to other growth factor and activate the function of other growth factors.

Erythropoietin (EPO) :- It is a well known haemopoietic growth factor which stimulates erythropoiesis. It is a 34-kD glycoprotein and contains two disulphide bond and four site of glycosylation. These sites are important for biological activity. It is produced mainly by the tubular and juxta tubular capillaries of the kidney when anaemia occurs due to blood loss. Ectopic sources of erythropoietin production are polycystic kidneys, cerebral hemangioblastoma, uterine fibroma, renal carcinoma and hepatoma. In embryonic stage the main site of production is liver cell. The switch from liver to kidney is probably due to a gene switch. Erythropoietin promotes survival, proliferation and differentiation of erythroid progenitor cells (Krantz 1991). It stimulates committed stem cells including rubriblasts, prorubriblasts and young rubriblasts to increase their mitotic activity for more rapid production of erythrocytes and their release. It maintains the blood level by modulating the circulating erythrocytes.

Erythropoietin has been the fountain head in research on molecular mechanism of oxygen-sensing and hypoxia induced gene expression. The availability of recombinant human EPO for prevention and treatment of chronic anaemias is a turning point in clinical medicine. It is now commercially available and is administered in such patients like (i) chronic renal failure cases undergoing dialysis (ii) patient receiving anticancer chemotherapy which produces damage of RBM and anaemia and (iii) in patients of AIDS receiving chemotherapy.

TGEB:- It is made up of multisubunit, like actinin, it is a bone macrophage. It reduces the response of the most cells and inhibits the some growth factors.

Hepatotoxic Growth Factor (HGF):- It is one of the survival factors which are necessary for the normal growth and development of many tissues and organs. Its receptors encoded by c-met proto-oncogene, is a tyrosine kinase cell surface receptor. HGF/c-met system provides

primarily cross-talk between the epithelial and stromal components. This system also plays role in adhesion showing its effects primarily on VLA-A and VLA-5 integrins.

Serum:- Serum alone can provide various growth factors, hormones and other factors needed by the most mammalian cells for their long term growth and metabolism. The growth promoting effect of serum is stoichiometric, i.e., if other requirements are fulfilled, the number of cells produced in culture is proportional to the amount of serum added. For details Please refer chapter- 5.

B. Extracellular Matrix:

It is the immediate surroundings of the animal cells in tissues of multicellular organism. As the animal cells have no cell wall and so these are surrounded by a complex intracellular substance called extracellular matrix, whose constituents are secreted by the cells themselves. In various animal tissues the extracellular matrix is composed of several common components, such as fibrous structural protein, a large mucopolysaccharide and covalently linked polysaccharides, proteins in the form of proteoglycans, glycoproteins (mostly protein) and hyaluronan or hyaluronic acid. The fibrous protein remains embedded in the gel formed from polysaccharides called glycosaminoglycans (GAGs) which consists of repeating unit of disaccharides. One sugar of the disaccharides is either N-acetylglucosamine or N-acetylgalactosamine and the second is usually acidic (McDonald 1988). Extracellular matrix not only differs in different animal tissues but it also differs in different cell types, such as, neurofilaments are found only in nerve cells surroundings. The matrix thus, binds the cell together, influences their development, polarity and behaviours. The glucosamineglucans are a heterogenous group of negatively charged polysaccharide chains which are covalently bound to protein fibers to form asproteoglycan molecule. The fibres are interwoven in hydrated gel. These proteoglycan are also found on the surface of the cells, which they function as co-receptors to help the cells to attach to the matrix and respond to growth factors.

Ehrhart *et al.* (1966) studied the role of radiation on altering the extracellular matrix directly and through its action on resident growth factors such as transforming growth factor-beta. Extracellular matrix helps in binding the cells in tissues together and provide a lattice through which cell can move, particularly during the early stage of

differentiation. It has an important role in homeostasis, deterring malignant expression by controlling many phenotypic characteristics of the resident epithelial cells. Conversely, abnormal extracellular environment can disrupt the normal tissue controls and allow expression of malignant uncontrolled proliferation and invasion pass normal barriers. Future work can be taken to identify alteration in growth factor response proteinase expression and integrin expression as markers of malignancy.

Fibres which also constitutes the extracellular matrix are of two types, Structural fibres and functional fibres:

(a) Structural Fibres :- These are composed of **collagen** and elastin collagens. Collagens are fibrous protein. Collagens belong to large family of proteins containing at least 19 different aminoacids and is generally found at the cell surface. Each collagen is made up of α-helix triplex of three polypeptides (α-helix), which in turn are made up of multiple repetitions of the characteristic sequence, GLY-X-Y. The α-helixes unwind themselves and form a thick strands. Collagen is of two types:- Fibrilous collagen and Non-fibrilous collagen. Fibrilous collagen are of Type-I, Type-II and Type-III. These are made up of α-triplex helix e.g., cornea. The non-fibrilous collagen is not made up of α-triplex helix. It is linear and at the end it contains globular domain.

(b) Functional Fibres:- is composed of **fibronectin** and **laminin**. These multifunctional proteins are characterised by their ability to bind to several other matrix constituents at the same time.

Fibronectins are fibrous protein widely distributed in all fibrous tissue, in plasma and in other sites including the basement membrane (Rouslanti *et al.*, 1981). These consists of linear series of domain and give each polypeptide a molecular organization and stability. One molecules of fibronectin is formed by the two molecules of polypeptide each containing nearly 2500 aminoacids. The fibronectin molecules are divided into different domains. These domains bind to cell surface receptors, collages, fibrin and various proteoglycans. This is the basis of the "molecular glue" properties of the fibronectin. In the extracellular matrix, fibronectin is further cross-linked into fibrils by disulphide bonds.

Laminine is a distinct adhesion protein of the major structural components of the basal laminae synthesized in very early embryo, which do not contain collagen. It is also made up of two polypeptide chain and it helps to interact cell with environmental conditions.

Laminine has binding sites for cell surface receptors, type IV collagen and pertican. In addition laminine is tightly associated with another adhesion protein called entactin or nidogen which also binds to type IV collagen. As a result of these multiple interactions, laminine, entactin, type IV collagen and percolan form cross-linked networks in the basal lamina (Mecham 1991).

Function of extracellular matrix:

(i) Extracellular matrix is the framework of the cell by combining with the cells and tissues and forms structures with special mechanical properties, such as bones, cartilage, tendon and joints.

(ii) It influences the cytoskeleton and provides mechanical contact between cells.

(iii) It influences many cellular activities like, Cell proliferation, Cell metabolism, Cell-cell interaction and Cell growth.

(iv) It contains many proteins and that macromolecules contains many binding sites.

(v) It binds to the extracellular proteins and thus keeps the interaction with the environment.

(vi) It insulates cells and tissues from one another and thus minimizes friction at joints and facilitates the movements of cells.

(vii) It is responsible for the filtering properties of the kidney.

C. Cell-adhesion Molecules :

Cell adhesion molecule is the link between the cell-cell interaction and extracellular molecules (Wilson 1996). Signalling through cell adhesion molecules have long been of interest because of their importance in embryonic development, homeostasis, immune response, wound healing and malignant transformation, however, it is only recently realized that cell adhesion molecules are capable of transducing biochemical signals across the plasma membrane to regulate cellular functions. It is of following types:-

(i) Integrins:- Integrin is a cell adhesion molecule and is made up of two chains α and β. The α -chain is longer than the β- chain. Both the chains are linked with some extracellular matrix proteins, such as fibronectin, laminine and others (Buck and Horwitz 1987). When the integrin joins the extracellular protein thus it joins with the cytoskeleton of the cells. It is also connected with the cytoplasmic proteins like tulines. Integrin performs two activities i.e., both the cytoplasmic and environmental activities. It it combines with the cell substratum and

it helps in the transmission of signals between cell and environment. (Clark and Brugge 1995).

The extracellular proteins has three aminoacids- arginine, glycine and aspartic acid. RGD. These RGD molecules forms triplex codon which is same as the extracellular binding sites, so the extracellular protein binds to the integrin, when RGD binds the Mg^{++} and Ca^{++} ions. When the integrin joins the extracellular protein, it changes the conformation of the plasma membrane and stimulate Focal Adhesion Kinase (FAK). The FAK further stimulates the chain reaction inside the cytoplasm.

Cell Survival without Integrins:- When the cells are in suspension there is no signal transmission, so, in the suspension cells cannot survive.

(ii). Selectin:- Selectin is an another member of adhesion molecule. It is made up of a cytoplasmic domain and a large extracellular substrates which is divided into many domains and a end domain which is composed of lectin carbohydrate. It interacts with sugar ring of the oligosaccharide. It is of three types:-

Selectin E (endothelial) also known as $CD_{62}E$:-It is a 140-150 kD glycoprotein located with alfa-granules of platelets and also expressed on endothelial cells.

Selectin-L (leukocytes) also known as $CD_{62}L$:- It is expressed on leukocytes and binds to CD_{34} and helps in cell-cell adhesion. It mainly interacts with the blood cells and carbohydrate domains (lectin) which are responsible for its binding.

Selectin P (platelets) also known as $CD_{62}P$:- It is a member of LEC-CAM family of adhesion molecule that recognizes specific carbohydrate ligand and mediate an early response in the interaction of leukocytes with endothelium and platelets.

(iii) Cadherins:- These are the calcium dependent cell-cell interaction molecule. It is made up of five homologous domain, one transmembrane and one cytoplasmic domain. Cytoplasmic domain functions in the zipper like fashion. Cadherin is implicated in the development from the embryonic stage in the multi-cellular organisms. Thus these molecules are very important for the maintenance of tissue architecture in adult animals. The role of cadherins in linking the cytoskeleton of adjacent cells is analogous to that of integrins in forming stable junctions between cells and the extracellular matrix. Cadherins links to the bundles of actin filaments via the catenins (Cowin 1994). Like selectine adherins are also of three types, i.e.,

Cadherin-P (Placental), Cadgerin-E (Endothelial) and Cadherin-N (Neural).

(iv) Immunoglobulin Superfamily (IgSF) :- The immunoglobulin is composed of many domains (70-110 aminoacids). Many of the immunoglobulin molecules combine together to form a superfamily of the immunoglobulin. The IgSF interact only with a specific leukocytes and stimulated component of immune cells (e.g., macrophages). It can also interact with non-specific subunits which are non-immunogenic (e.g., neurons).

(D) Proteoglycans:

Proteoglycans are extremely large molecular complex and made up of carbohydrate (95%) and protein (5%) and act as packaging material. Numerous protein molecules are associated with an axis of hyaluronate and many of them carry additional polysaccharide chain. The polysaccharides found in proteoglycans typically contain acetylated aminosugars and are therefore referred to as glycosaminoglycans. The basic unit of the various glycosaminoglycans are repeating disaccharide units which contain a uronic acid and an aminosugar as building blocks. Because of their polar nature and their many negative charges, they bind large numbers of water molecules and cations, thus forming hydrated gels. The property of the amorphous ground substance of the extracellular matrix are mainly due to the proteoglycans.

(E). Cell Junction :

Specialized cell junctions occupy at many points of cell-cell and cell matrix contact in all times. It provides cell-to-cell communication and the exchange of small molecules between cells. Cell junctions can be classified in to three functional groups:-

1. Tight Junction (Occluding junction) :- As the name indicates, the junction formed between the plasma membrane proteins of most epithelial cells seal them together in such a way that virtually no space is left between the cells and that prevents passage of even small molecules. This junction forms a barrier and thus prevents diffusion of even dissolved ions and molecules. It also restricts the movement of dissolved material. Tight junction is made up of six units of coxines. Coxines are the tight junction protein and forms a passage between the adjacent cells (Citi 1993). Tight junctions are often found just below the free surface of epithelial cells. Best examples of tight junctions are

the tubular cells of kidney and Sertoli cells of testis, which form a barrier against extracellular diffusion between interstitial fluids and tubular fluids. There are two important functions of tight junction. First, tight junction forms seal that prevents the free passage of molecules between the cells of epithelial sheets and Second, tight junction separates the apical and basolateral domains of the plasma membrane by preventing the free diffusion of lipids and membrane proteins between them.

2. Anchoring Junction :- (a) Actin filament junction (b) Desmosome (c) Intermediate filament junction and (d) Communication junction.

(a)Actin Filament Junction:- Actin is the major cytoskeleton protein of most cell and was first isolated from muscle cells. Although actin was initially thought to be uniquely involved in muscle contraction. The polymerazation of actin forms actin filament, which is thin, flexible fiber, approximately 7 nm in diameter and upto several micrometers in length (Holmes *et al.* 1990). It is an actin cross-linked by another protein which can behave simultaneously as rigid and fluid channels (Sato *et al.* 1987). This may be of following types:

(i) Cell wall adherents junction,

(ii) Cell-matrix adherents junction and,

(iii) Separate junction (present in vertebrate only).

(b) Desmosomes:-

These connect cells, subjected to intense strength, such as epidermal cells of the skin, where the cell membrane of neighbouring cells are in close contact, a network of protein filaments form a plaque underlying the membrane of each cell. The protein plaque serves as anchors for other protein that penetrates the membrane and links the cell together. Intermediate filament also enters through cytoplasm between desmosome on opposite side of the cells, thus forming a continuous network of protein filaments through the tissue.

(c) Intermediate filament junction :- Every eukaryotic cells have network of protein collectively called cytoskeleton structure. This plays role in maintaining cellular morphology, structure and plays role in maintaining cellular transport, cell mobility and mitosis. As mentioned above actin filaments and microtubules are polymers of single type of protein, intermediate filaments are composed of a variety of proteins that are expressed on different types of cell. While intermediate filaments are present in both the embryonic and adult

forms of smooth, skeletal and cardiac muscles, these are most abundant in adult smooth muscles, which has led to its frequent adoption as a starting point for the purification and study. In adult smooth muscle these filaments form an interconnected network that links cytoplasmic dense bodies with membrane bound dense plaques. So the intermediate filaments are mostly needed to strengthen the cytoskeleton of cells in the tissues of multicellular organisms, when these are subjected to a variety of mechanical stress that do not affect cells in the isolated environment of a culture dish. Even though intermediate filaments may play a cytoskeletal role and remain insoluble in buffers containing high salt, these are not static structures. These may be induced to reversely disaggregate

Intermediate filaments protein are frequently used in the identification of various cell types, such as desmin of myogenic cells, vimentin of stromal cells, cytokeratins are indicative of epithelial cell, fibronectin of fibroblast and neurofilament of neuron or glial cells. This may be of following five types:

(i) Desmin :- It is an intermediate filament protein that is characteristically expressed in both the embryonic and adult forms of smooth, skeletal and cardiac muscles, but are most abundantly in smooth muscles of adult e.g., in mammalian uterine and stomach muscle cells, cardiac muscles and skeletal muscles. In adult smooth muscles these filaments form an interconnected network that links cytoplasmic dense bodies with membrane bound dense plaques muscle cells. Reconstituted desmin filaments have an average diameter of 100 Angstron which strongly suggests that this protein is indeed the major subunit of smooth muscle intermediate filaments (Geisler and Weber 1980).

(ii) Vimentin:- Brown *et al.* (1077) for the first time characterised vimentin as a major subunit of intermediate filament in chick embryo fibroblast having a size of 50 kD. The majority of cell of mesenchymal and non- mesenchymal origin and cell types grown in tissue culture, irrespective of the origin of the tissues, contain a class of intermediate filament, whose subunit is vimentin. Some smooth muscle cells contain vimentine instead of desmin as their major intermediate filament subunit e.g., mammalian uterine, stomach smooth muscles, cardiac muscles, purkinji conduction fibre of the heart and Sertoli cells of testis. The intermediate filament of cells of mammalian iris and lens epithelium is also composed of vimentin. The cell culture of mammalian and transformed cell lines which sole intermediate filament unit is

vimentin include the transfer cell line NIL8, Chinese ovary cell, all fibroblastic cells, macrophages, neuroblastoma, mouse 3T3 cells and human endothelial cells.

The insolubility properties of vimentin strongly suggests that these serve structural function in cytoplasm morphological. Various biochemical evidences suggest that vimentin filaments are associated with both nuclear and plasma membranes in culture fibroblasts and chicken erythrocytes. Possibly, vimentin filaments, by interacting with nucleus provide it with mechanical support and constrain it in place in a specific place in cell.

Nestin and vimentin are the main intermediate filaments in immature astroglial cells, whereas maturing astrocytes contain vimentin and glial fibrillary acidic protein (GFAP) (Eliasson *et al.* 1999).

(iii) Keratin:- The characteristic component of intermediate filament in epithelial cells and cells of epithelioid origin is keratin (Fuchs 1995). Keratin consists of a highly complex family of polypeptide with content of cysteine, a sulphur containing aminoacid. Keratin extracted in the presence of urea from living cells of epidermis or from cultured keratinocytes are composed of six to seven major polypeptide having molecular weight of 40 to 70 kD. Most keratin of living cells exists in reduced form that favours polymerisation and become increasingly cross-linked by intermolecular disulphide bonds as differentiation proceeds. The keratin in nails, claws and hooves is relatively soft and flexible and cracked easily when dehydrated.

(iv) Neurofilament (NF) :- It has been seen that isolated mammalian neurofilaments are composed of three neurofilament polypeptides designated as light (NF-L), medium (NF-M) and heavy (NF-H) with molecular weight of 60-70, 105-110 and 135-150 kD respectively. These neurofilament proteins form the major intermediate filaments of many types of neuronal axon, dendrites and perikarya. Neurofilament appear to function as a three dimensional structural lattice that provides tensile strength to axons.

(v) Glial Filaments:- Glial filaments are composed of only one major polypeptide of molecular weight 51, 000. It is found in glial cells and cells of glial origin.

(d)Communicating Junction:-It permits the molecule pass from one cell to another cell

(i)Gap Junction:- Gap junction is formed by membrane proteins that extend between adjacent cells to form a passage for exchange of small

molecules and ions. The exchange of ions permits one cell to affect the electrical activity of the adjacent cell. Tight junction may develop in gap junction (Griffith and Riley 1985). It is found in vertebrates and is exceptionally large protein channels forming water filled membrane pores extending from cells to cells called gap junction. It allows small molecule to pass directly from one cell to another cell forming a direct contact between cytosol of the cells. These special channels are much larger than the ordinary ion-channel in the membrane. It is found in most animal tissues including epithelial cells, endothelial cells and the cells of the cardiac and smooth muscle. Gap junctions are made up of transmembrane proteins called connexins (Beyer 1993). In the apical endothelial cells it performs its function by occules. Occules are the subunit of gap junction protein. In cardiac muscle and smooth muscles, gap junctions are crucial to the co-ordination of the electrical activities of the cells.

(ii)Chemical Communication :- It mainly occurs through endocrine system and nervous system. In the endocrine system, hormone is the main chemical messenger that conveys information from endocrine cells to the hormone sensitive target cells. Hormones are transported to target cells through the circulatory system and the tissue fluids.

In the nervous system the nerve cells (neurons) communicate with the other cells by means of chemical messenger. The nerve impulse transmitted in vertebrate is dominated by chemical synapses, which is crucial to the function of nervous system. When a nerve impulse arrives at a chemical synapse signal molecules called neurotransmitters i.e., acetylcholine are released from the nerve terminal and diffuses to the membrane of the target cells. Neurotransmitters have been divided into two main groups on the basis of molecular size, these are small molecule transmitter and neuropeptides. The small molecules transmitters are synthesized in the nerve terminals and neuropeptides, which consists of 340 aminoacids are synthesized in the cell body.

E. Cell-Cell Interaction:

Cell-cell interaction directly between cells as well as between cells and the extracellular matrix are crucial in the development and function of multicellular organisms. It depends on the various soluble and insoluble factors.

Soluble Factors:- This is due to the various secretions and their target sites. This secretions may be autocrine, paracrine or endocrine. In autocrine signalling cells respond to substances they release themselves

e.g., different growth factors by a responsive cell leading to continuous auto stimulation of cell division and thus the cancer cells are less dependent on growth factors from other cell. In paracrine signalling the secretions act locally upon adjacent cell e.g., catecholamine and other neurotransmitter which carry signals between nerve cells at a synapse. In endocrine signalling various secretions act on distant target cells at a distant body sites, such as various hormones. Other classical example is oestrogen, which is produced by the ovary and stimulates development and maintenance of the female reproductive system and secondary sex character.

Insoluble Factors:- Cell-cell interaction through insoluble ligands include various cell adhesion molecules (*vide supra*).

Practice Assignment II

1. Extracellular matrix :
   (a) Helps to bind cell together,
   (b) Fills the space between cells
   (c) Provides lattice for the movement of cell
   (d) All of the above.
2. In natural surroundings, cell remain in close contact with other cells through:
   (a) Cell junction (b) Extracellular matrix
   (c) Extracellular fluids (d) All of the above.
3. It is essential to know the natural surroundings of animal cell, because:
   (a) Cells removed from natural source remain in stress condition
   (b) Cells remain devoid of complex network of secreted proteins
   (c) Cells remain devoid of various hormone like factors
   (d) All of the above.
4. All the following statements about hormone are correct, except:
   (a) It is a chemical messenger
   (b) It is secreted in minute quantity
   (c) It is transported to target organ by various duct system of the body
   (d) All of the above.
5. Plasma enzyme mediators consists of:
   (a) Kinin system (b) Clotting system
   (c) Fibrinolytic system (d) All of the above.

6. All the statements about gap junction is correct, except:
   (a) Found both in vertebrates and invertebrates
   (b) It allows small molecules to pass directly from cell to cell
   (c) Also found in apical endothelial cells
   (d) It performs it function by occules.
7. Intermediate filament junction may be:
   (a) Desmin (b) Vimentin
   (c) Keratin (d) All of the above.
8. Chemical communication mainly occurs through:
   (a) Hormones (b) Neurotransmitters
   (c) Both of the above (d) None of the above.
9. Cell adhesion molecule is the link between :
   (a) Cell-cell interaction (b) Extracellular matrix
   (c) Both of the above (d) None of the above.
10. Functional fiber is composed of:
   (a) Fibronectin and laminin (b) Integrin
   (c) Collagen and elastin collagen (d) Selectin.

Answers:

1. -(d), 2. - (d), 3. - (d), 4. - (c), 5. -(d), 6. -(a), 7. -(d), 8. -(c), 9. -(c), 10. -(a).

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Chapter 3

Metabolic Capabilities of Animal Cell

Metabolic capabilities of the animal cell depends on the ease with which it can gain material from, or loose materials to, its surrounding environment. Transport of molecules across the membrane is regulated. The purpose of the hectic flow of the molecules and ions in and out of the cell is to facilitate defined molecular reactions, which beautifully orchestrate the rhythm of cellular chemistry, the key to the well being of cells and in turn the fitness of the tissues that are formed from those cells and further organs that are constituted from tissues. A cell can only grow and function if its requirement for oxygen, ions, aminoacids, vitamins, and a score of other chemical substances are met, and the waste or secretory products of its metabolism are removed. For these activities, cell possess a dynamic structure called cytoplasmic membrane, whose evidence is available from tracer studies concerning the rate of renewal of membrane components. The cytoplasmic membrane performs two important functions. First is the "Selective permeability" and the second is the "Exact defined mechanism" which provide surface for the location of membrane bound enzymes. The metabolic capabilitities of the cells depend upon the following factors:-

1. Chemical Exchange
2. Membrane Permeability
3. Transport Mechanisms
   (i) Active Transport
   (ii) Passive Transport
4. Anabolism
5. Catabolism
6. Bioenergetics.

1. Chemical Exchange

Every cell is surrounded by a plasma membrane. It constitutes a barrier between cell cytoplasm and the extra cellular factor (ECF). Transport across the plasma membrane may involve two aspects- membrane permeability and actual mechanism of transport.

2. Membrane Permeability

The permeability is selective. Only the nutrients like salts, hormones, O_2 and essential materials are permitted from outside to cross the plasma membrane to reach the cytoplasm. Harmful metabolic wastes, such as CO_2, nitrogenous wastes etc. are allowed to pass out across the membrane. The permeability of the membrane is due to the presence of lipid layers in its structure. Lipid soluble substances can pass through the membrane more readily than water soluble ones. Such as, a lipid-soluble fatty acid can pass through the membrane much faster than water soluble hydrochloric acid of the same concentration.

Besides serving as a semipermeable barrier plasma membrane also act as a Victoria one, in which the direction of flow is as important as nature of the compound itself. Such as, most cells of the body have higher concentration of potassium ion than their environment and if this concentration falls the cell draws potassium ion from its environment against the concentration gradient. Some substances pass through the membrane from a more concentrated solution to a lower concentration by simple diffusion. However, surprisingly all membranes are permeable to water, oxygen, and hydrogen, hence these are transported across the membrane freely, yet the flow of these substances are meticulously controlled. Cells quickly shrink in hypertonic medium and swell and burst in a hypotonic medium.

Exact Defined Mechanism :

Plasma membrane protein has exact defined mechanism. It has two types of protein, peripheral (extrinsic) and integral (intrinsic). The peripheral proteins are superficially located and many of them function as enzyme. The integral protein associated with the lipid bilayer penetrate into the interior of the membrane along with the fatty acids rich chains. These are tightly bound to the lipids and constitute the functional proteins not easily separable. All membrane bound enzymes and carriers are included in this category. All plasma and microsomal membrane have adenosine triphosphate enzyme,

whose activity depends on the presence of magnesium ion and it could further be stimulated by addition of Na^+ and K^+ ions.

3. Transport Mechanisms

Membrane of the cell forms physical boundaries between organelles and cytoplasm and between the cell and extracellular fluids. Transport of molecules across the membrane is regulated. Some molecules are passively and others are actively transported. Passive and active transportes in various membranes help cells to regulate their volume, internal pH and ionic composition. They concentrate metabolites which are essential for energy metabolism and biosynthesis concomitantly excluding toxic substances.

(i) Passive Transport :- This includes physical mechanism in which the forces that derive the substances across the membrane are supplied from the environment of the cell. This type of the transport requires neither activity nor energy expenditure by the cell. Passively transported molecules, such as ions, some solutes and water pass through the lipid bilayer by the process of diffusion, or pores bordered by channel proteins which have central core of water molecule or binding to transport protein that carry the substance across the membrane and release on the other side.

The diffusion through lipid bilayer follows the normal kinetic motion i.e., from region of higher concentration to the region of lower concentration. So here they ran 'downhill' along a concentration gradient. This does not involve covalent bond making and breaking reactions. Here free energy of the system will decrease.

Many water soluble solutes and ions such as K^+, Cl^- and HCO_3^- diffuses through water filled pores in the membrane. In the diffusion through water filled protein channel ions and molecules with polar covalent bond are bound to water molecules by electric forces. Here the channels are so narrow that only inorganic ions can pass through and for this reason, this is called ion-channels. The passive transport of ions through ion-channel is governed both by concentration diffusion and voltage difference between the inside and outside of cells.

The large hydrophilic molecules which can not pass through ion-channels are transported across the membrane by binding to transport proteins, carrier proteins which carry the molecules through the membrane. The binding alters the shape of the carrier protein in such a manner that the molecules become exposed to the fluid on the outer side of the membrane. The bond subsequently breaks and the molecule

diffuses away from the membrane.

(ii) Active Transport:- This mechanism is energy-dependent, forcibly transports a molecule against concentration gradients i.e., they ran 'uphill' along a concentration gradient and is effected through carrier proteins. It has the following characteristics:-

- Active transport depends on source of metabolic energy ATP. This can be exemplified by giving example of transport of K^+ and Na^+ between RBC and plasma. RBC has high concentration of K^+ and relatively low concentration of Na^+ whereas plasma around the RBC has low K^+ and high concentration of Na^+. Since the membrane of the RBC is permeable to both sodium and potassium ion, these tend to move down the gradient. But, Na^+ and K^+ diffusing down the gradient are pumped back. Since pumping in both the cases is against the concentration gradient, energy is required to do the work which comes from the glycolytic cycle.
- Active transport is solute specific transport system, such as RBC of some mammals transport D-glucose inward rapidly whereas transport of D-fructose is very slow.
- Activity of active transport depends on the concentration of substances being transported, such as rate of transport of glucose depends on the concentration of glucose in the surrounding medium. Rate of glucose influx increases with the rise of external concentration until a peak is reached when no further increase is possible.
- Active transport is direction specific, such as potassium ion in most cells is actively pumped in inward direction whereas sodium ion is always pumped in outward direction.
- This can be selectively poisoned, such as active transport of glucose in the kidney can be blocked by the inhibitor phlorizin, which is a glucoside obtained from the bark of a pear tree.
- In this system due to integrated action of active transport mechanism, internal solute and ion composition of cells is maintained at a remarkably constant level despite fluctuation in the surrounding medium.

Anabolism

Anabolism is the building or biosynthetic phase of metabolism. The enzymatic biosynthesis of various molecular components of cells as

nucleic acids, proteins, polysaccharides and lipids take place from their simpler building block precursors. Biosynthesis of organic molecules from simple precursors require input of chemical energy which is furnished by the ATP generated during catabolism. So, in anabolism the above mentioned complex cellular components synthesized by the cells are utilized for growth, repair, secretion and other functions. This biosynthesis is the constructive phase of the cell metabolism. Cells consume energy in this process. Anabolism is energy conserving or endergonic process.

Catabolism

Catabolism is the degradative phase of metabolism in which relatively large and complex nutrient molecules such as carbohydrates, proteins and lipids, coming either from the environment of the cell or from its own nutrient storage depot are degraded to yield smaller, simple molecule, such as lactic acid, acetic acid, carbon dioxide, ammonia and urea etc. Catabolism is accompanied by release of the chemical energy, inherent in the structure of organic nutrients, molecules and its conservation in the form of the energy transferring molecule adenosine triphosphate (ATP). So, in catabolism large molecules are broken down to small molecules releasing energy required for cellular function. This process is mainly performed in mitochondria. Catabolism is energy - releasing or exergonic process.

Thus metabolism is the sum total of energy gained and released in a living cell.

Bioenergetics

All living cells require energy for synthesis of cellular components and for carrying out various functions such as uptake of material, growth, development and movement. Cells are dependent on a supply of energy from the outside and continuously utilize energy to maintain their structure. The energy cycle of living beings is bioenergetics. Green plant can utilize energy from sunlight to form carbohydrate from carbon dioxide and water (Photosynthesis). This may be summed up like:

$$\text{Plant cells} \rightarrow \text{Solar energy} \rightarrow \text{Green plant}$$

Animal cells on the other hand are devoid of chlorophyll. These cannot use solar energy to synthesize carbohydrates and therefore they have to depend upon plants or animals. Approximately 60% of energy difference is immediately converted to heat energy. The most

important of these energy transport is adenosine triphosphate (ATP). ATP represents the direct energy source in most energy requiring processes in all cells of all organisms i.e., various microorganisms to human.

ATP is composed of adenine, ribose and three phosphate group and is thus a nucleotide with two extra phosphate group.ATP is relatively unstable molecule, and the outer most phosphate group can easily be split off by hydrolysis. The end product of this reaction. adenosine diphosphate (ADP) and inorganic phosphate (P_i, the anionic part of phosphoric acid) contain much less energy than the reactants ATP and water, consequently, energy is released and utilized to drive other reactions in the cell:

$$ATP + H_2O \rightarrow ADP + P_i + \text{energy}.$$

Practice Assignment III

1. In active transport:
   (a) The diffusion follows the normal kinetic motion
   (b) The diffusion follows against a concentration gradient
   (c) Both of the above (d) None of the above.
2. In passive transport:
   (a) The diffusion occurs through the lipid bilayer
   (b) Diffusion through water-filled protein channels
   (c) Both of the above (d) None of the above.
3. Passive transport across the membrane is determined by:
   (a) Concentration gradient (b) Electric forces
   (c) Potential energy (d) All of the above.
4. Chemical communication between cells occurs through:
   (a) Endocrine system (b) Nervous system
   (c) Both of the above (d) Non of the above.
5. In catabolism:
   (a) Cell synthesizes complex cellular components
   (b) Large molecules are broken down into small molecules
   (c) Both of the above (d) None of the above.
6. Anabolism is the:
   (a) Constructive phase of the cell metabolism
   (b) Endergonic process
   (c) Both of the above
   (d) None of the above.

7. Plasma membrane protein has exact defined mechanism, because of:
   (a) Peripheral (extrinsic) protein
   (b) Integral (intrinsic) protein
   (c) Both of the above
   (d) None of the above.
8. Plasma membrane :
   (a) Serves as semipermeable barrier
   (b) Decides the direction of flow
   (c) Both of the above
   (d) None of the above.
9. All the statements about animal cells are true, except:
   (a) These are devoid of chlorophyll
   (b) Can use solar energy
   (c) Can not synthesize carbohydrate
   (d) these depend upon plants.
10. Adenosine tripohosphate (ATP) is composed of:
   (a) Adenine (b) Ribose
   (c) Three phosphate (d) All of the above.

Answers:

1. -(b) , 2. -(c) , 3. -(d) , 4. -(c) , 5. -(b) , 6. -(c) , 7. -(c) , 8. -(c) , 9. -(b) , 10. -(d) .

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Chapter 4

Simulating Natural Conditions for Growing Animal Cells

These days different animal cells are being cultured to synthesize or produce a variety of biomolecules at an industrial scale. Other applications which may be included are enhanced synthesis of all specific proteins like enzymes, growth factors, hormones, interferon, gene amplification and regulation of gene function. For all the above mentioned works cells have to grow *in vitro*. As the different cells used for *in vitro* culture are taken out from the tissues or organs of living animals, so these cells remain under tremendous stress. Although cell's own system is working to ward off the condition of stress, but such a cell of minute size and limited system could not work in fighting the stress condition in cell from its removal from the source or host body. In order to survive the cells *in vitro* cells must be provided with the surroundings matching with the host system in which it has adapted to live and also the environment which supports its growth and proliferation quite effectively i.e., the atmosphere in culture must simulate the natural environment for proper growth (Paul 1959).

In practice it is very difficult to provide a cell with all the natural environment of host from which it has been derived. Thus careful study of the host system, from which the cells have been detached are essential in order to provide simulating natural conditions of the cells. For this a lot of aspects and phenomena are taken into account. Some of the common points which are taken into account are as follows:

1. Substratum :

The nature of the substrate or phase on or in which the cells may grow may be solid (glass, plastic etc.), semisolid (collagen, agar etc.) or liquid (suspension culture). In general two types of cells are present in culture. First, that grow in suspension culture and secondly,

anchorage dependent cells. Suspension culture does not require substratum to adhere with for its proliferation whereas anchorage dependent cells require substratum to adhere for their proliferation. The substrate used for the growth and proliferation of animal cells should simulate close enough to that provided to the cells *in vivo.*

Glass:- Glass is the most commonly used substrate used for the growth of cell culture. Glass may vary in its chemical composition. Such as, some glass may contain lead and arsenic, which are toxic to cells. Hence it is essential to find a brand of glass which has no toxic property. Rappaport and Bishop (1960) suggested that the extent to which glass permitted to adhere was related to the charge density on its surface. Glass preferably borosilicate of grade I, II or III facilitates the growing cells with a negative charge surface without being given any special treatment. The glass is cheap, easily washable without loosing growth supporting properties,can be sterilized in an ordinary laboratory with moist or dry heat. It should be optically clear, non-toxic and carry correct charge for cells to attach and grow. Glass Gas optical clarity and is not toxic.

Plastic:- The different plastic wares offer an alternative to glass and can also be used as substrate for growing cells in culture. It is cheap, optically clear and give a flat growth surface. The plastic may be grouped as Thermoplastic and Thermosetting plastic. The plastic used in thermoplastic are polystyrene, polyvinyl chloride (PVC), polyethylene etc. These are usually used to grow adherent cell culture which needs attachment to the surface. Being hydrophóbic plastic does not provide suitable surface for cell growth as cells will not adhere on them. Therefore, these are specially treated with gamma-radiation, chemicals or with electric arc in order to produce a charged surface to make it wettable. Sometimes the plastic is precoated with gelatin, collagen or polylysin to give a net positive charge for growth of neurons, muscle cells and endothelial cells.

The thermosetting plastics include PTFE (Polttetrafluro ethylene). These have got a hydrophobic surface and thus are used in growing suspension culture or transformed cells which do not require a cell surface attachment. The plastic substrate can be obtained in sterilized form but is expensive.

There are various factors on which the culture vessels are chosen for cell growth. The choice of culture vessel will depend upon cell yield, growth of cells as suspension or monolayer, whether the cultures are vented to atmosphere or sealed, cost, and the type of sampling and

analysis. The size may vary from Terasaki multiwell plates 1 mm^2 surface area, microplate 30 mm^2, 25 cm^2, 75 cm^2, 175 cm^2, roller bottle or microcarrier in the form of polystyrene beads.

Metals:- These are also used as substrate for holding cell culture, but these should be of very good quality and without any toxic effects. Metals used is generally molybdenum and chromium and treated stainless steel. These are supposed to be very resistant. For the first time palladium deposited on agarose was used as a substrate for the growth of fibroblast and glial cells.

2. Nutrients:

Nutrients are the substances that are used within the cells as metabolic substrate or co-factors for their proliferation and differentiation. It also provides functional activity of cells. Various nutrients are required by the cell and these should be supplied from outside as the cells or tissues have no capability to synthesize it like microorganisms. The various food which are provided to cell for their growth is also known as nutrients. All the living cells perform mainly two activities inside it. First, the synthetic activities(such as production of new cytoplasm and other organelles) and secondly the functional activity (such as contraction etc.). In order to perform this work the cells require carbon source, nitrogen source, minerals (sulphur and phosphorus), H_2 donor and acceptor and growth factors such as aminoacids, purine, pyramidine and vitamins etc. in optimal concentration. From the nutrients energy should be evolved and this is provided by the combustion of carbohydrate, especially glucose and fats. Nutrients should not be confused with the growth factor which is also required for the growth, Nutrients are required in large amount where as growth factor is required in trace amount i.e., nanogram. A given concentration of nutrients can only support a certain number of cells. Growth rate is always reduced when the nutrients get exhausted.

Nutrients have been classified in two groups : (i) Macronutrients or Macroelements and (ii) Micronutrients or Trace elements.

(i) Macronutrients or Macroelements :- It consists of the C, H, O, N, P, S, K, Ca, Mg, and Fe. Out of these six (C, H, O, N, P and S) elements are involved in the constituents of the carbohydrates, lipids, proteins and nucleic acid and remaining fours are involved in the cationic properties of enzymes and the cellular materials. K^+ ions is involved in the enzyme catalysis and also in protein synthesis. Ca^{2+} ions acts as a heat-shock element as observed bacterial endospore. Mg^{2+} ions act

as a enzyme catalysed reactions in ATP synthesis and also in the association of ribosome subunits. Fe^{2+} and Fe^{3+} ions are the component of cytochrome and also in the ring formation of Hb- molecule.

(ii) Micronutrients or Trace Elements :- These are present in trace amount and hence called trace elements. It includes elements like, Zn, Mn, Cu, Co, Ni etc. . Zn^{2+} ion remains present in the active site of enzyme and involves in the association of the carbomyl transference in *Escherichia coli*. Co^{2+} ion is present in vitamin D. So, essential balance of nutrients are necessary for the growth and development of the cultured cells.

3. Hydrogen ion Concentration (pH) :

The hydrogen ion concentration (pH) has direct effect on the cellular rate of reaction as pH influences cell survival, attachment, growth and function. Maintaining the correct pH is central to obtaining optimal cell growth and high yields, The more useful expression of hydrogen ion concentration is pH. The pH of a solution is equal to the negative logarithm of the hydrogen ion concentration in molarity (M)

$$pH = -\log [H^+ \text{ ions}]$$
$$= [\log (1/H^+)]$$

It is important that pH varies inversely with hydrogen ion concentration. When the hydrogen concentration in the blood increases, pH decreases and the cell moves towards acidosis and when the hydrogen ion concentration in the blood decreases, pH rises and the cell develops alkalosis. The acid base balance involves the following:-

- Extracellular and intracellular buffering,
- Regulation of the rate of alveolar ventilation to control carbon dioxide concentration,
- Regulation of renal hydrogen secretion.

Due to oxidative metabolism of the cell large amount of carbon dioxide are produced. Though carbon dioxide is not acid but when it combines with the water of cellular space resulting into formation of carbonic acid. This reaction is facilitated by the carbonic anhydrase enzyme of the red cell, which is as follows:

$$CO_2 + H_2O \rightarrow H_2CO_3 \Leftrightarrow H^+ + HCO_3$$

The optimum pH must be empirically determined for each types of cells, such as neutrophils grow best at a pH of 6. 0-8. 0, although

some acidophils have optimum as low as pH 3. 0 whereas alkalophils have optimum as high as pH 10.5, Optimum pH between7. 2 to 7. 4 is generally needed for mammalian cell. When the pH is changed from the optimum value growth rate of the cell in culture is diminished. This is due to the deleterious effect of the metabolic product of the cell which results into change of the pH and thus there is decrease in the rate of cell doubling which occurs independently of any prolongation of lag phase or decrease in the maximum population reached during stationary phase. If a cell type produces large amounts of Co_2 then media based on Hank's balanced Salt solution (0. 33g $NaHCO_3$ /litre) are more suitable than media based on Earle, s salt solution (2. 20g $NaHCO_3$/litre). *In vivo* intracellular and extracellular fluids of living cell contain conjugate acid-base pairs which act as buffers at the normal pH of these fluids. The major intracellular buffer is the conjugate acid-base pair H_2PO^{4-} - HPO_4^{2-} (PK'= 7. 2). Organic phosphate such as Glucose-6-phosphate and ATP also contribute buffering power in the cell. The major extracellular buffer inthe blood and interstitial fluid of vertebrate is the bicarbonate buffer system. This maintains the pH in the cell culture *in vitro* buffer system. Buffering capacity, head space and glucose concentration affect stability of pH.

The buffering capacity is normally maintained by sod. bicarbonate(5. 5%) in the CO_2-bicarbonate system (Geyer and Chang 1958) and it requires equilibrium with 5% CO_2., which is commonly used in the cell culture medium. It has a pKa level much below the physiological optimum. As the medium gets used, CO_2 is produced, lactic acid increases and pH tends to fall, which is restored by the bicarbonate buffer. Other examples of buffering capacity are : increased phosphates in the BSS; Wilbrionic buffer as in HEPES (N-2-hydroxyethylpiperazine-N-2 ethanesulfonic acid) either in addition to bicarbonate or as its substitute provides more effective buffering in the optimal pH range (7. 2-7. 4 at 37^0C) than non-HEPES supplemented media. Use of free amino acids, pyruvate and galactose in place of glucose are generally used in special media as from glucose pyruvic and lactic acids are produced which accumulate in media thus resulting into fall in pH.

Generally in the cell culture medium pH indicator, commonly phenol red is used to analyze the pH of environment in which cells are growing. Phenol red is yellow in acidic medium (pH 6. 8), tomato red at neutral pH (7. 0), red at an alkaline pH (7. 4) and blue at increased basicity (pH 7. 6) and finally purple at high pH.

3. Temperature :

Temperature indirectly holds the key to humidity distribution as the moisture holding capacity of air increases with increase in temperature by affecting the air densities. So, like optimum pH, optimum temperature is also required for the proper growth of the cell as the upper end of the temperature range tolerated by any given species of cell correlates well with the general thermal activity of the cell. Temperature should simulate the temperature of the body from which cells have been derived as optimal temperature varies from species to species. There is far difference between the culture of avian cells with that of mammalian cells as the optimum temperature of avian cell is 40^0C while that of mammal is 37^0C. Some anatomical variation may also need varying temperature, such as temperature of skin and testis require lower temperature than that rest of the body. The optimum temperature of any cell or organ is called ceiling temperature. Though it is a general rule that the rate of growth increases with increasing temperature until a maximum is reached and thereafter it begins to decline very sharply due to cell death. (Fig. -2).

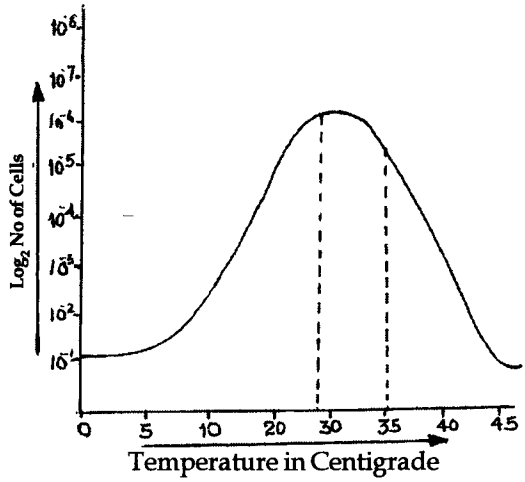


Fig. 2. Relationship between Growth and Temperature.

Arrhanius showed that the logarithm of the velocity of any chemical reaction (logK) is a linear function of the reciprocal of the temperature (1/t) since cell growth is the result of a set of chemical reactions; it might be expected to show this relationship (logK α 1/t). As the temperature also affect the rate of catalyzed reaction so cell growth is also related to temperature. At high temperature the enzymes of the cell denatures or becomes inactive whereas at low temperature

the Co_2 concentration increases and so pH decreases. Also osmolarity of cell changes which has detrimental effect on cell.

4. Humidity :

Proper humidity is also essential for cell growth as humidity distribution indirectly also has effect on temperature. As the moisture holding capacity of air increases with increase in temperature. For cell growth 100% humidity is essential.

5. Antibiotics :

Prevention of contamination by the different microorganisms (bacteria, mycoplasma and fungi) is the most important part of all animal cell culture. The risk of contamination during culture can be avoided by adding different antibiotics, such as penicillin (100 U/ml), streptomycin (100 mg/ml) or gentamycin (50mg/ml) for bacteria, and nystatin (50mg/ml) for fungi and yeast. The routine use of antibiotics is generally not recommended because:

- it may lead to a relaxation of aseptic technique,
- resistant microorganisms may develop,
- microbial growth may be controlled but biochemical alteration may be produced and
- it may have adverse effects on cell growth and function and can also reduce the longevity of cells (Litwin 1973) as many antibiotics have been found to be cytotoxic even at concentration approaching their effective levels. It has been seen that routine concentration of penicillin and streptomycin cause atleast 20% reduction of yield from cultures of human fibroblast compared with the same cells when grown in the absence of antibiotics for several passage (Goetz *et al.* 1979). Cytotoxicity is cell dependent and thus care should be exercised in selecting the appropriate concentration of antibiotics.

Cells should also be free from mycoplasma as mycoplasma is one of the important contaminating agents in cell culture growth and function and most importantly mycoplasma compete with cell nutrients in the culture medium. Contamination by mycoplasma can result in rapid depletion of the essential aminoacid arginine from the culture medium and an increased accumulation of ammonia (Stanbridge 1971). It is this depletion and imbalance in aminoacid composition of the medium which has serious consequences on the growth of cell culture. The contamination by mycoplasma can be avoided by adding various

antibiotics, such as, gentamycin, kanamycin and tetracycline.

6. Sterility:

For maintaining sterility complete sterilization of all glasswares, reagents and medium is an essential step. Sterilize is a latin word meaning "devoid off" or "barren". Complete aseptic environment is needed for the growth of cells *in vitro*. Efficient sterilization eliminates contamination. Thus, prevention of contamination by the different micro-organisms is an essential part of all animal cell culture. The risk of contamination can be eliminated by efficient sterilization methods, effective ascetic technique and antibiotics.

7. Osmolality :

It is the measure of depression in freezing point and elevation in the vapour pressure. Osmolality detect the quantity of media for any type of mistake in the amount of constituent taken. The growth and function of cells in culture depends on maintaining an appropriate osmolality in the culture medium (Kruse and Paterson 1973). Some cells (e.g., HeLa and other established cell line) can tolerate wide fluctuations in osmolality. In contrast primary cells and normal diploid cell strains are very sensitive to change in osmolality and high yields can only be obtained if the osmolality of the culture medium is kept within a narrow range. In the absence of evidence to the contrary the osmolality of the medium used for the culture of any particular type of cell should be kept constant ata value in the range 280-320 mOsm/ litre, normally 290-300mOsm/litre. By controlling osmolarity it is possible to achieve more reproducible cultures. The recommended osmolality of Iscov's modified Dulbecco medium is 300 ± 10 mOsm/ kg. Generally osmolality is maintained at the time of maintaining pH. If we are adding some amount of acid or base then some amount of salt must be added or omitted from the media e.g., HEPES (N-hydroxyethyl paparazine N-ethane sulphonic acid). To maintain osmolality some amount of Nacl is removed by the given formula :

$X = \frac{D\text{-}O}{32}$ [Where, X= amount of Nacl, D= desired and O= observed osmolality. (1mg/ml Nacl has osmolarity of32 mOsm/ml)].

8. Osmotic Pressure:

It is defined as the hydrostatic pressure which can be applied in order to balance the influence of solution in osmotically active solvent by

the semi permeable membrane.

Osmotic pressure also affects growth rate and for most cell the optimum osmotic pressure is about that obtaining within the animal. The maintenance of a constant ionic strength within the microbial cell is of great physiological significance since the stability and behaviour of enzymes are greatly influenced by this factor. Microbes have the ability to grow in media with varying osmotic pressure but to most of the microorganisms high sugar and salt concentration may be lethal, however, some moulds and yeasts have ability to survive in high concentration. The osmotic pressure in most biological fluids is mainly due to dissolved crystalloids and in mammalian and other tissues the greater part of it is accounted by sodium chloride.

9. Viscosity :

Mechanical damage of cells may take place during processing of cells during cell culture. Viscosity also affect the cell growth *in vitro.* Viscosity is enhanced by serum or carboxymethyl cellulose or polyvinyl pyrollodine which can prevent mechanical damage.

10. Surface Tension:

If oxygen is bubbled through the suspension culture the foaming results which can denature protein. So various antifoaming agents like silicone, Pluronic F-68 (Swim and Parker 1960) etc. are used to prevent foaming.

Practice Assignment IV

1. The various substratum used for the growing adherent cells are;
   (a) Glass (b) Plastic
   (c) Metals (d) All of the above.
2. The choice of culture vessels depends on:
   (a) Amount of cell yield (b) Type of sampling and analysis
   (c) Types of cell growth (d) All of the above.
3. Generally glass is preferred as substrate, because:
   (a) It is cheap (b) It is easily washable
   (c) It can be perfectly sterilized (d) All of the above.
4. The substrate used for growing suspension culture is:
   (a) Solid (glass and plastic) (b) Semisolid (Collagen and agar)
   (c) Liquid (d) All of the above.

5. The pH of the medium in which cells are growing, may fall because of:
   (a) Production of CO_2 (b) Production of lactic acid
   (c) Both of the above (d) None of the above.
6. Addition of antibiotics to check bacterial contamination may :
   (a) Affect cell growth and function
   (b) Reduce longevity of cell
   (b) Help in the emergence of resistant strain
   (d) All of the above.
7. Osmolarity is the:
   (a) Measure of depression in the freezing point
   (b) Elevation of the vapour pressure
   (c) Both of the above
   (d) None of the above.
8. To prevent viscosity the substance added in cell culture is:
   (a) Serum (b) Carboxymethyl cellulose
   (c) Polyvinyl pyrollodine (d) All of the above.
9. Simulating natural condition for growing cell means:
   (a) To provide the exactly the same environment from which cell has been derived
   (b) To provide the different environment from which cell has been derived
   (c) Both of the above
   (d) None of the above.
10. The ion which act as enzyme as catalytic reaction in ATP synthesis is:
   (a) Mg^{2+} (b) Ca^{2+}
   (c) Fe^{3+} (d) K^{+}.

Answers:

1. (d), 2. (d), 3. (d), 4. (c), 5. -(c), 6. - (d), 7. -(c), 8. -(d), 9. -(a), 10- (a).

Chapter 5

Serum and Serum Free Media

Role of Serum in Cell Culture

Classically the long term growth and metabolism of most mammalian cells is carried out in defined nutrient and salt mixture supplemented with biological fluids most commonly animal serum. Serum is an extremely complex mixture of many small and large molecules with different physiologically balanced growth promoting and growth inhibiting activities. Serum provides an enormous variety of substances necessary for cultivating wide variety of animal cells, such as hormone, growth factors, transport proteins and attachment factors etc. The growth stimulatory effect of serum on tissue culture was first shown by Gey and Thalhimer (1924). When initiating a culture it is usual practice to let the culture surface come into contact with medium containing serum before cells are added to the culture. In spite of containing various growth factor, foetal bovine serum is also known to contain substances which are toxic to some cell lines. Heat inactivation at 56 ^{0}C for 30 minutes in a water bath has been shown to inactivate these toxic substances without impairing the growth promoting properties of the sera (McIntosh *et al.* 1976). To sum up, the serum has the following advantages and disadvantages for the growth of animal cells in culture :

Advantages of Serum in Cell Culture:

1. Has basic nutrients in soluble or in protein-bound forms.
2. Contain certain hormones such as insulin and transferrin. Insulin is required for the growth of many cultured cells whereas transferrin acts as iron-binder for incorporation of iron by the cells in culture.
3. Serum contains many growth factors such as fibronectin, chondronectin, PDGF, TGF-b for stimulating cell growth and for specialized functions of cells.
4. Provides attachment of cell to the culture surface, probably by

supplying exogenous glycoproteins and spreading factors.

5. Binding proteins like albumin, hormones, vitamins, minerals, fatty acids etc. in the serum promote proliferation of cells.
6. Trace elements like Ca^{++}, Mg^{++}, Fe^{2++} found in serum promotes cell attachment.
7. Non-specific protection factors, e.g., protection against mechanical damage during agitation and aeration of cell suspension due to sheer forces provided by viscosity.
8. Protein inhibitors
9. By maintaing the buffering capacity cell culture it maintains the -pH and,
10. Provides appropriate Osmotic pressure.

Disadvantages of Using Serum in Cell Culture

1. Difficult to get in large quantity, as supply is always less than demand.
2. Foetal calf serum (FCS) is expensive, contains high level of arginase which depletes the medium arginine (Mellman Cristofalo 1972), an essential amino acid in the medium. Serum from new born or adults contain high levels of antibodies and thus pose similar problems.
3. Batch to batch variation in serum supplied occurs with respect to a number of components and this variation results in widely different plating efficiencies, affects the growth and yields, gives inconsistent result, even if other culture conditions are optimal for growth (Esbee *et al.* 1973). The batch of serum can also determine which components from the medium will become limiting e.g., the batch of serum is critical in division-limiting concentration of cystein (Corfield and Hay 1978). Standardization is difficult, time consuming and expensive.
4. The serum itself may be cytotoxic e.g., the enzyme polyamine oxidase in serum may react with polyamine such as spermine/ spermidine secreted by highly proliferative cells and form cytotoxic polyaminoaldehyde. It is for this reason horse serum is preferred over FCS and human pregnant serum which contain more of polyamines.
5. Serum may be containing inadequate levels of cell specific growth factors which have to be supplemented, and overabundance of

others which may be cytostatic.

6. Serum is not the most natural medium for culturing cells. Therefore *in vitro* results may not be extrapolatable, or may not be as dependable as an *in vivo* experimental result.
7. It is highly risky to use serum as it is very likely to carry contaminating microorganisms into laboratory.
8. Sterilization problems associated with filtration of colloids and particulate substances are required to be faced with.
9. It may inhibit the growth of insect cell line (Goodwin 1975) and some cell types e.g., epidermal keratinocytes.
10. Specific cell line requires specific foetal calf serum, so selection and use of foetal calf serum is determined by the requirement of the cell line.

Serum Free Media:

The development of a defined cell culture environment often termed serum free or chemically defined media. Seing the various disadvantages of serum in culture medium, for the first time White in 1946 tried to develop chemically defined or serum free medium. Later Eagle *et al.* (1958) investigated the essential nutritional requirement of various mammalian cell and developed Eagle's medium. Ham and and Mckeetian (1979) extensively worked out the nutritional requirement of cell and developed Ham's medium (1965) to eliminate the requirement of serum in cell culture. The undefined nature of serum supplements and their variations in quality makes the serum free media one of the goals of cell culture. Recent developments in formulating media show that a wide variety of cells can be cultured in the absence of a serum supplement, provided various combination of hormones, nutrients and purified proteins are added to the medium to replace serum with little perceivable lag time (Barnes and Sato 1980) as cells in serum free media are generally more sensitive to osmotic pressure and hydrogen ion concentration. The serum free media must be supplemented with the various ingredients, attachment and spreading factors. The main constituent of serum free medium are: adhesion factors (treatment of plastic growth surface with fibronectin, laminin and pretreatment with polylysine), hormones (growth hormones), growth factors (EGF, PDGF, FGF, TGF, TNF-α etc.), protease inhibitors (soyabean trypin inhibitor) trace minerals, minerals, glucose, bovine serum albumin, transferin and aminoacids etc. Serum free media often require addition of fibronectin (1-50 mg/

ml) before many cells can attach to culture surface. A minimum of 15 ng of adsorbed fibronectin/cm^2 is required for spreading of an established type of cell such as BHK (Hughes *et al.* 1979). Commercially available serum free media are MCDB 105 (optimized for human diploid fibroblast), MCDB 202 (optimized for chicken embryo fibroblast), HIES and Bigger's medium (BGJ) etc. The serum free media must contain the following factors:

Attachment and Spreading Factor:

Serum contains many of the attachment and spreading factors. Most non-transformed primary cells are anchorage - dependent and need to attach to a solid substrate in order to grow. Animal cells and extracellular matrix used in labs, like glass and plastic are both negatively charged. Cross-linking with glycoproteins/fibronectin or divalent cations Ca^{++} and Mg^{++} provide necessary electrostatic attraction for cells to bind to the matrix. The transformed and haemopoetic cells are anchorage- independent and do not need this. Chondronectin is required for adhesions of chondrocytes and laminin for adhesion of epithelial cells. Fibronectin is also involved in binding/attachment. Culture medium supplemented with 10% (v/v) foetal calf serum contains approximately 2-3 mg fibronectin/ml (Hayman and Rouslathi 1979) and a large proportion of fibronectin adsorbs to culture surface within a few minutes (Grinell *et al.* 1977). Fibronectin in serum forms immobilized fibrillar net and thus cells anchorage is facilitated. They attach to the fibril and also to each other. Fibronectin molecules have many discrete domain having tetrapeptide sequence Arg-Gly-Asp-Ser (recognition sites/peptides). The binding sites of fibronectin have high affinity for the cell surface, collagen, fibrin, sulfated proteoglycans, other extracellular proteins, polysaccharides. Adsorption of purified fibronectin to culture dish frequently results in tight adhesion of cells to dishes and development of stress fibers. Its growth promoting action to mammalian cells was reported by Orly and Sato (1979).

Collagen enhances growth or differentiation of numerous mammalian cell types and many cells grow rapidly on a collagen matrix than on glass plate. It is commonly used for the culture of hepatocytes as freshly isolated hepatocytes attach to collagen with maximum efficiency and spreading is more rapid than on any other cell culture system.

Growth Factors of the Serum:

Serum alone provides various growth factors which are essential for the growth of cells. Serum derived growth factors may act on cells at a specific distinct stage of differentiation, e.g., Colony Stimulating factor (CSF), act synergistically on several cell types, Epidermal Growth factor (EGF) promote proliferation of many cell types, such as, fibroblasts, epidermal and glial cells and acts as inductive signals in embryonic development. A single cell type may respond to a number of serum derived growth factors e.g., fibroblasts may respond to fibroblast growth factor (FGF), platelet derived growth factor (PDGF), insulin like growth factor or somatomedins.

FGF was first demonstrated by Gospodarowicz in 1974 in the bovine pituitary gland, which stimulates growth of fibroblast, stimulates proliferation of many cell types, inhibit differentiation of various types of stem cells, and act as inductive signals in embryonic development. There are 7 subtypes.

Transforming growth factor(TGF) are of two types : TGF-α and TGF-b. TGF-α (Lin-3 protein) is found in *C. elegans* whereas TGF-b potentiates or inhibits responses of many cells to other growth factors, depending upon cell types. It regulates differentiation of some cell types, and act as inductive signals in embryonic development. There are multiple subtypes like bone morphogenetic proteins (BMGPs), Decapentaplegic protein (in drosophila), Vg1 protein in Xenopus.

Insulin-like growth factor (IGF-I, IGF-II) or Somatomedin can often replace the action of insulin in lower concentration.

Certain cell types require specific growth factors which are not present in serum such as the hormones Erythropoietin. T-lymphocyte of the immune system require Interleukin-2 (IL-2) and Interleukin-3 (IL-3) required for proliferation of activated T-lymphocytes and haemopoietic colony stimulating factor (CSFs), required for proliferation and survival of various types of blood cell precursors. For details of growth factors please refer Chapter- 2.

Hormones:

Since serum can only meet the requirement of various hormones, serum free media must be supplemented with various hormones, however, actual requirement of hormone is difficult to predict. Insulin is essential for growth nearly for all cells in culture. Its growth promoting action was discovered by Gey and Thelhimer (1924). It is

sensitive to inactivation by cysteine and has very short half-life and so large amount is required in the medium i.e., 100 μg/ml and in some cases 5 to 10μg/ml. Actual hormone requirement is difficult to predict.

Glucocorticoid hormones such as hydrocortisone and dexamethasone can either stimulate or inhibit cell growth depending upon cell types. Its growth promoting action was reported by Macieira-Coelho (1966). Density of 3T3 cell line (derived from mouse embryonic fibroblast), inhibited by cortisol was increased by glucocorticosteroids. Glucocorticoids also initiates DNA synthesis and proliferation of cell by fibroblast growth factor, growth on human lung fibroblast, HeLa cell and synovial cells. Other steroid hormones are oestradiol, testosterone, and progesterone which are also commonly used in serum free media. Thyroid hormones, thyroxine (T_3) and triiodothyronine (T_4) are also essential to support growth of some cell types.

Binding fFactors

Essential and low molecular weight substances are carried into cells by binding factors like albumin, transferrin. Albumin protects cells from mechanical damage, provides optimal colloidal osmotic pressure and viscosity, buffer, carries into cells vitamins, fatty acids and cholesterol. Transferrin is involved in transport of iron. Iron, Fe+++ binds to transferin to form ferrotransferrin and gets transported into cells in vesicles. Low pH causes dissociation of Fe+++ from ferrotransferrin; the apotrasferrin so formed is returned back to outside cell.

Fatty Acids

Serum provides lipids in various forms to cultured cells. These forms include cholesterol, phospholipids, triglycerides, fatty acids, and various esterified forms of these lipids. As serum free media will be devoid of these factors, so synthetic media require essential fatty acids i.e. phospholipids, cholesterol in the absence of serum or in the presence of dilapidated albumin. Cholesterol is the essential constituents of the cell membrane and thus is required by many cell line for its proper growth, such as HeLa cells, procine kidney cell line and human kidney cells etc. requires cholesterol. Prostaglandin E and F2 are are also involved in cell growth, possibly acting in conjunction with EGF and other growth factors. Various cells like vascular

endothelium, smooth muscles and corneal endothelium require phospholipid when serum is not provided in the medium (Fujii *et al.* 1983).

Trace Elements

The importance of trace elements for the growth of cell in cell culture was demonstrated by Shooter and Gey (1952). Since Cu, Zn, Mo, Mn, Cobalt and selenium are found in serum and thought to be involved in activating enzymes and protecting against free radicals which cause damage to DNA thus these trace elements must be added in serum free media. Mckeenhan *et al.* (1976) observed that selenium is essential for the growth of the WI-38 diploid human fibroblast.

Antiprotease

Tissue cells, particularly endothelium, myeloid cells release protease. These have to be neutralized. Serum antiprotease arrests proteolysis, particularly after trypsinization.

Miscellaneous Factors:

Besides the above ingradients some more substances may be needed in serum free media which may be specific to a particular cell culture system. Such as, for the clonal growth of a strain of Chinese hamster cells spermidine, spermine and putrescine are required. Protamine is an important component of serum free media which is required to promote attachment of cell to glass substrate. Addition of 2-mercaptoethanol was found to stimulate the proliferation of mouse leukemia cells. Synthetic polymers like Polyvinyl pyrolidones can be a good substitute for serum.

Nagata (1978) observed that the polyethylene glycol (PEG) which is generally used as fussogen can stimulate the growth of mouse-human-human heterohybridomas at a concentration of less than one hundredth of that used for fusion.

Advantages of Serum Free Media :

(i) Improved reproducibility between cultures and avoidance of batch to batch variation of sera.

(ii) Standardization of media formulations among different laboratories.

(iii) Improved economy.

(iv) Easier downstream purification of produce from cultured cell.

(v) Less protein interference in bioassay.

(vi) Avoidance of serum toxicity.

(vii) No serum proteases to degrade sensitive proteins.

(viii)Prevention of fibroblasts overgrowth in primary cultures.

(ix) Selective cultures of differentiated and functional cell types from heterogeneous population of primary cultures.

(x) In absence of serum, the phenotype of cell can be well controlled.

Disadvantages of Serum Free Medium

(i) Supplementing medium with hormones and growth factors would be more expensive than using serum.

(ii) Serum free media are highly specific to one cell type as cell type from each specie has its own characteristics requirements and that each grows best in a medium that has specially optimized to its own needs. Thus, additional and different selective media will have to be used for each cell type. This will be expensive.

(iii) Reliable serum free media, with few exceptions, are not readily available commercially.

(iv) Ultra high purity of reagents, water and critical control of pH and temperature are required as compared to that with serum containing media.

(v) Cell growth is often slower, saturation density may be lower, maximum generation may be less.

From the above facts it is clear that both, i.e., the media having serum and serum free media having various serum supplements have its own advantages and disadvantages. But in the present time for the production of different pharmaceuticals for therapeutic and diagnostic use at commercial scales requires chemically defined serum free media, but some cells do not survive in chemically defined medium as their nutritional requirement is not fully understood.

Practice Assignment V

1. Use of serum in culture media has the following advantages, except:
   (a) Provides nutrients in soluble or in protein-bound forms
   (b) Provides hormones such as insulin and transferrin. Transferrin acts as ion-binder.
   (c) It contains many growth factors such as fibronectin,

chondronectin, PDGF, TGF-b for stimulating cell growth and for specialized functions of cells.

(d) It may be contain inadequate levels of cell specific growth factors which have to be supplemented, and overabundance of others which may be cytostatic.

2. Use of serum free media has the following advantages except:
   (a) Cell growth is often slower, saturation density may be lower, maximum generation may be less.
   (b) Easier downstream purification of culture produced
   (c) Less protein interference in bioassay
   (d) Avoidance of serum toxicity.
3. The serum used in culture medium is generally obtained from :
   (a) Foetal calf (b) Adult cattle
   (c) Adult human (d) All of the above.
4. Foetal calf serum contains:
   (a) Approximately 2-3 mg fibronectin/ml.
   (b) Contains high level of arginase
   (c) Both of the above
   (d) None of the above.
5. Cytotoxicity of serum may occur due to
   (a) Reaction of polyamine oxidases with polyamines
   (b) Formation of polyaminealdehyde
   (c) Both of the above
   (d) None of the above.
6. Disadvantage of serum free media is:
   (a) It is highly specific to one cell type
   (b) Require addition of different substances according to cell type
   (c) Both of the above
   (d) None of the above.
7. Addition of serum in media is advantageous, because:
   (a) It provides peptides and other growth regulating hormone
   (b) It is used to increase cell mass
   (c) Provides essential low molecules of nutrients
   (d) All of the above.
8. Addition of serum in media has following advantages, except:
   (a) May protect against proteolytic action of trypsin
   (b) May contain inadequate level of cell specific growth factor

(c) Acts as carrier of many water soluble nutrients
(d) Cell is protected in agitated cell culture.

9. Cell culture medium without serum has the following advantages, except:
   (a) There is no batch variation of the serum
   (b) Variety of cells can be cultured in serum free medium
   (c) Ultra high purity of reagents are required
   (d) Less protein interference in bioassay.

10. Serum free media has the following advantages:
   (a) It is highly specific to one cell type, so additional and different selective media will have to be used for each cell type.
   (b) Reliable serum free media, with few exceptions, are not readily available commercially.
   (c) Ultra high purity of reagents, water and critical control of pH and temperature are required
   (d) All of the above.

Answers:

1. -(d), 2-(a), 3-(a), 4-(c), 5. -(c), 6. -(c), 7. -(d), 8. -(b), 9. -(c), 10. -(d).

Chapter 6

Cell Culture System

Cell culture system involves the culture of cell of a particular organ of animals that has grown in artificial media. Though the culture of cell is more difficult as compared to the culture of various bacteria and fungi, yet by providing appropriate nutrition and environment cell culture is being done successfully.

Cell Culture System has Following Uses :-

- It is used for culture and identification of such microorganisms which can't grow on artificial media and requires live cell to grow such as viruses.
- Used for the development of different vaccines and diagnostics.
- It is used to study the expression of different cloned gene.
- Used for the study of toxicity as an alternative to live animal test.
- Here the experimental conditions can be strictly controlled. It provides the opportunity for detailed analysis of toxic mechanism of action at the cellular level. Results from initial *in vitro* study can lead to more efficient experimental design for animal experiment which could result in economy in terms of money and time.
- By employing appropriate metabolic competent cells, organ and species specific effects can be evaluated.
- The use of human tissue or cell *in vitro* systems may lead to more accurate comparison between animal and human data using cell system data as an intracellular interaction with critical macromolecules and of response.
- Cell culture system may offer the setting of or observing the effects of compounds on cells in an artificial environment without interference by various inimical factors present in the intact organisms.

- Cell culture can also be utilized for toxicological testing, assay of by products and as models for screening of potential useful materials like antiviral agents, antifungal and anticancer agents etc. (Balls and Horner 1982).
- Cell culture allows a significant reduction in number of animals needed and are more economical.

Disadvantage of Cell Culture System:

- It requires trained personnels, strictly sterile conditions and reliability which has not been fully evaluated yet.
- Because of the restrictive source of the cells used in culture, such systems are not capable of predicting or identifying the more susceptible organs in the intact animals or how the intact animals will respond to chemical insults to various organs.
- Cell culture can never replace the more complex live animals in demonstrating the overall impact of virus, vaccine or toxin.
- When it is needed to observe the possible side effects of a new treatment one has to use live animals as normal body metabolism, various hormones might disrupt the action of treatment which cannot be observed in cell culture.

There are mainly three types of cell culture system:-

1. Primary cell culture
2. Secondary cell culture
3. Continuous cell culture.

Primary Cell Culture:

Primary cell culture refers to culture of cells obtained directly from the tissues or organ aseptically collected from different apparently healthy animals. From these tissues or organs the cells can be disintegrated by mechanical, chemical or enzymatic digestion. When these cells are induced to grow *in vitro*, on suitable tissue culture media, cell cultures are formed and is called Primary cell culture. These cells grown *in vitro* as adherent monolayer on a solid substrate or as a suspension in the medium. From one animal several bottles and flasks culture can be prepared. The primary cultures are usually heterogeneous and have low growth rate, but are still preferred over the continuous cell lines because these are more representative of the cell types in the tissues from which theses are derived and in expression of tissue.

Actually the cells which form tissues *in vivo* may be of epithelial, connective, muscles and nervous types. When explant from any part of the body is taken it will contain cells belonging to one or other of the above mentioned types. But when the cells are cultured *in vitro* these are placed in a totally different environment and the factors which previously determined their characteristic structure and activities *in vivo* no longer operates. Thus, the loss of histological organization which occurs in cell cultures is often accompanied by loss of the specific structural and functional attributes (which typified the individual cells in the intact animal). The cells which were recognizably different from each other *in vivo* may appear very similar in culture. The morphological structure of cells in culture may be of following types:-

- *Epithelium type*:- Epithelial cells are polygonal, forming sheets, cells multiply mitotically forming a continuous thin layer, often one cell thick, i.e., monolayer, when a suitable surface is available to them, such as glass surface, plastic etc. (Fig. -3).
- *Epitheloid type* :-Certain cells have round outline and resemble epithelial sheets but do not form sheet.
- *Fibroblast type*:- Fibroblast generally have a more angular shape than epithelial cells. It is thin and elongated, regularly oriented parallel to each other with several pointed processes. These form open network of cells rather than a tightly packed cells. After infection, become randomly oriented and circular (fig. -5).
- *Connective tissues type*:- These include the true connective tissue i.e., fibrous tissue, cartilage and bone. *In vivo* these are characterized by having large amount of fibrous and amorphous extracellular materials. The haemopoietic tissues i.e., bone marrow and lymphoid tissue, which are densely populated by a variety of cells of the myeloid and lymphoid series are supported by a mesh of fine collagen and reticular fiber.

Disadvantage of Primary Cell Culture:

- Mixture of cell population are present (i.e., heterogeneous cells).
- There may be presence of contaminating viruses originating from animal source from where it has been taken.
- Requires recurrent animal sacrifice for preparation of fresh primary monolayer cell culture.

2. Secondary Cell Culture :

The cells of primary cell culture may not last long due to various factors such as, depletion of nutrients and accumulation of metabolites etc. Thus, for its survival it is essential that it should be subcultured in a separate tissue culture vessel having fresh medium. When subculture is done from primary monolayer culture it is called Secondary Cell Culture. Subculture means transferring of cell from primary cell culture by taking adherent cells by enzymatic digestion. For this the adherent confluent monolayer of primary cell culture are rinsed twice with calcium-magnesium free physiological buffer saline (PBS) and then treated with Trypsin- Versene Glucose Saline (TVGS) solution. The excess fluid is drained off and the flasks and bottles are incubated at 37^0C for 10-15 minutes with the residual TVGS. The cells are dispersed by pipetting several times and 10^6 cells per ml. and then resuspended in appropriate volume of growth medium (Dulbecco's MEM) containing 10% foetal calf serum. The cells are further seeded as required into Screw -capped tubes, Leighton tubes and Roux flasks with 1, 2 and 100 ml quantities respectively. The seeded glasswares are incubated at 37^0C and observed every hour for 24 hours interval under an inverted microscope until confluent monolayers are formed.

These days secondary cell cultures are very popular because of their easy availability, maintenance and growth. These are quite useful in virological, immunological and toxicological research.

4. Continuous Cell Culture:

Continuous cell cultures are available in the form of immortal cells which can be grown many times in artificial media. It has several advantages:-

- It has increased growth rate
- Reduced serum dependence
- Reduced anchorage dependence and
- Has increased cloning efficiency.

Disadvantages of Continuous Cell Culture:-

- It has greater chromosomal instability
- It has loss of tissue specific markers and
- Has altered cyto-morphology.

Tissue Culture

Many animal cells with special care can be induced to grow outside their organ or tissue of origin. The *in vitro* cultivation of organs, tissues and cells under controlled conditions is collectively known as **tissue culture**. In early experiments fragments of tissues were studied and this gave the name tissue culture. The term **cell culture** refers to cultures derived from dispersed cells that have been taken from the original tissue. The development of cell culture technique has reduced the cumbersome and expensive use of animals and embryonated eggs as means of conducting various researches, diagnostic works as well as propagation of many animal and plant viruses.

Requirement of Tissue Culture Laboratory :

Basic requirement for a cell culture laboratory can be classified into the following major areas:-

(i) Equipment for tissue culture laboratory

(ii) Cleaning and sterilization of glassware

(iii) Requirement of media and its preparation

(iv) Working area for aseptic manipulation of cells and

(v) Equipment for routine maintenance of cells.

(i) Equipments for Tissue Culture Laboratory:- The equipments may be classified as essential equipment and Desirable equipment. The details of equipments along with their use has been depicted in Table-1. Besides the equipment mentioned in the table different specialized glassware and pipettes for the precision and convenience of cell culture is required (Sanford *et al.* 1950).

(ii) Cleaning of Glassware and other Materials:-

Good laboratory technique demands clean glassware, because even the most carefully executed piece of work may give an erroneous result if dirty glassware is used. Dirt may contain various bacteria and fungi, which are the most common contaminants observed in the cell culture. There are thousands of species of bacteria which can live on or in almost any material and environment from soil to water, air and even on us. Bacteria can live in temperature above the boiling point of water (Thermophilic bacteria) and in cold that would freeze blood (psychrophilic bacteria). Some of the bacteria may have a hard protective covering around them called spore. These spores are generally very much resistant to different adverse circumstances and

may survive for weeks and even years. Fungal spores are lightweight and ubiquitous in the environment. If a fungal spore comes in contact with cell culture media, contamination of the cell culture is inevitable. Besides that mycoplasma is also one of problematic contamination of cell culture as atleast 20 distinct *Mycoplasma* or *Acholeplasma* species have been isolated from contaminated cell culture. Importantly, this kind of contaminant represents a significant factor in compromised or invalidated research findings and enormous economic losses in research time, material and industrial production. Though primary cell cultures are rarely contaminated (0-4%), but continuous cell lines are frequently contaminated (50-90%). *Mycoplasma* sp. once in cell culture, which often multiply in the medium, form a very close association with individual cell. Indeed, cells have specific receptors for some mycoplasma on their surface (Stanbridge 1971).

The different glassware for use in tissue culture must be resistant to acids, alkalies, free from traces of heavy metals, ions and alkalinity. Borosilicate glass-U. S. P. Type-1 or Pyrex type glasses are the only types that should be used. These glassware should be perfectly cleaned with non-ionic detergents alkalies, oxidizing acids or by ultrasound cleaning equipment. Non-ionic detergent is efficient and economical for routine cleaning of glasswares and are oftenly used in tissue culture. A wide variety of non-toxic detergent solutions are available commercially, such as microsolve, 7X Haemosol, Stergene, RBS25 or Decon 75 and Laxbro solution etc. Most of these detergents can be easily washed off from the glassware thereby minimizing the toxicity to the cells during tissue culture. Although alkalies and acids are not necessary for cleaning purpose, the use of chromic acid is still followed in many laboratories. The new glassware should always be soaked in dilute acid solution (1% HCl or HNO_3 solution) for several hours as these are slightly alkaline in reaction which could affect the growth of cells in tissue culture. It should be kept in mind that while using cleaning aids such as brushes etc., this should be in good condition. The use of worn out brushes will lead to serious abrading of the glassware.

(iii) Sterilization of Materials used in Tissue Culture:- Animal and plant cell cultures are expected to be free from all microbes and asepsis is a primary requirements for tissue culture work (Leifert and Cassells 2001). Thus, proper sterilization of all the materials which are going to be used must be done properly. Sterilization is the process of freeing an articles from any types of microbes (both pathogens and non-

pathogens). Cleaned glassware can be achieved by the use of dry heat, moist heat, chemicals and by irradiation. Before sterilization all the glasswares should be fully dried and necessary plugging should be done with cotton, then with aluminium foil and then finally wrapped in craft papers. Generally all the glasswares for use in tissue culture is sterilized by hot air sterilization (dry heat) which can be done in a suitable hot air oven. The holding period of hot air sterilization is one hour at 180^0 for bacteriological work and at 160^0 C for three hours for virological works.

For sterilization of material which can be destroyed by dry heat, such as, rubber cork, rubber gaskets, media and different solutions, rubber gloves etc. should be sterilized by autoclaving. The high latent heat of the steam which rapidly transfers its energy to materials being sterilized is the method of choice for sterilization.

Membrane filters have been widely used for decades to sterilize liquids used in cell culture such as serum and other proteinous material. Filters with 0. 45mm pore size have been recognized as the standard for retaining of microorganisms and are used to recover bacteria and other micro-organisms from many samples and environment.

Table-1: List of Equipments along with their Use.

Equipments	Uses
A. Essential Equipments	
1. Laminar Air flow cabinet (Horizontal or vertical)	Sterile working area.
2. Inverted binocular microscope	Examination of tissue culture growth.
3. Triple glass distillation set	Distilled water.
4. Deionizer	Deionized water.
5. Autoclave	Sterilization of media.
6. Hot air oven	Sterilization of glassware.
7. BOD incubator	Incubation of tissue culture
8. Refrigerator	Storage.
9. Deep freeze (-80°C)	Storage.
10. Bench top centrifuge	Centrifugation.
11. Magnetic stirrer	For trypsinization.
12. Different filtration set (Seitz filter, Millipore membrane filter)	Sterilization by filtration.
13. Liquid nitrogen vessel	Cryopreservation.
14. Refrigerated microcentrifuge	For centrifugation of cells.

Equipments	Uses
15. Micropippettes of various volume (0.05µl - 1000 µl).	For pippetting.
16. Pipette washer	For pipette washing.
B. Desirable Equipments:	
1. Air conditioner	For maintaining temperature.
2. Digital pH meter	pH adjustment.
3. Electronic balance	For weighing.
4. CO_2 incubator	Incubation.
5. Haemocytometer	Counting of cells.
6. Ultracentrifuge	High speed centrifugation.
7. Cell counter	Counting of cells.
8. Cell harvester	Harvesting of cells.
9. Vaccum pump	Filtration.
10. Fluorescein Activated Cell Sorter	Cell separation.
11. Vortex mixture	For mixing.
12. Orbital shaker	For shaking cells during trypsinization.
C. Special Glassware:	
1. McCartney bottle	For culture.
2. Roux flask	For culture.
3. Leighton tubes (5"x8")	For culture.
4. Serum bottles (100ml &500 ml.)	For processing and storing serum.
5. Assorted Enamel & Aluminium trays	For keeping different article

Chemical sterilization is achieved by washing with 70% Ethanol, Chloroform or Ethylene oxide vapour and is not routinely used in tissue culture laboratory.

Gamma irradiation is particularly useful for packed plasticware and certain heat-labile materials. Gamma-irradiation of 2. 5 Mrad (Co^{60} source) are employed for plasticware sterilization. Microwave irradiation is suitable for recycling of disposable plasticware, which can be recycled 5-10 times without altering the surface properties.

Often, liquid and the environment are carefully checked for sterility and the user is well protected by providing appropriatework stations, seat, clothing and masks, but the pipettors are generally forgotten. However, with the air displacement pipetting is one of the most common laboratory task and include a number of potential contaminating risks. This contamination can occur through handling of infectious and toxic agents, such as bacteria and in situations where

the sample or specimen is at risk through contamination from other samples or environmental factors. Pasteur pipette or serological pipettes with a piece of cotton as a filter in the upper end have traditionally been used together with pipetting aids featuring a filter of 0. 45 or 0. 2 mm. However, modern displacement pipettes, especially electronic pipettes with disposable tips enable fast, more accurate and precise pipetting and dispensing. Various tip manufactures have developed aerosol-barrier tips which are useful in contamination sensitive work. These tips have a porous filter positioned inside the tip (Reibold 2005). During pipetting air flows through the filter, aimed to reduce the flow of aerosol or liquid in to the pipett barrel and subsequently to the next sample (carryover contamination).

Good laboratory practices, pipetting slowly and carefully minimize aerosol formation and foaming which is important to avoid contamination of the pipettor and the subsequent sample. The risk of microbial contaminationcan also be reduced by using laminar airflow cabinet.

(iii) Requirement of Media and its Preparation :- When living cells are excised from the original tissue or animals they are deprived at a stroke of all these physiological supporting and protecting mechanism. If they are kept to alive *in vitro* for their survival, proliferation and differentiation they must be provided with appropriate nutritional, hormonal and stromal factors which resembles their milieu *in vivo* as closely as possible, in much the same way as an astronauts must be enclosed within a protective capsule and equipped with life support mechanism if he has to survive in outer space. The nutritional requirement of cells are not necessarily the same throughout the culture cycle and for optimum results it may be desirable to alter the formulation of culture medium at some stage in culture cycle such as addition of retinoids can promote adhesion of cells which adhere weakly to substrate and retinal or retinoic acid can be added at up to 1 mg/ml for the improvement of cell adhesion and their effect may be due to influence attributed to the synthesis of specific glycoconjugates of the cell surface (Adamo *et al.* 1979).

Media may be classified into two distinct categories, i.e., natural media and synthetic media.

Natural Media :

Natural media are those media which are prepared from the natural sources. From the history of organ and tissue culture it seems that

for the growth of tissue and organ *in vitro* natural media were used. The natural media used were of generally three types:-

(ii) Coagulum or Plasma Clot:- This is obtained from the heparinised blood from chicken and other animals and then plasma is separated from this by centrifugation at 2000 rpm for 15 minutes. It is commercially available in liquid and lyophilized state.

(iii) Biological Fluids:- This includes the serum derived from the human chord blood, amniotic fluid, pleural fluid, insect haemolymph and foetal calf serum. Bovine amniotic fluid has been used as tissue culture medium for the cultivation of poliomyelitis and other viruses (Enders 1953). Before use toxicity of biological fluids should be checked.

(iv) Tissue extract:- Generally chicken embryo extract is used but sometimes extract of leukocytes (Fischer 1951) and other tissues like liver and spleen are also used (Fischer 1939). The growth promoting effect in cell fraction of chicken embryo was demonstrated by Kutsky and Harris (1953).

The classical natural tissue culture media contain different nutrient, salt mixture supplemented with biological fluids, most commonly animal serum.

Basal Media:

Basal media consists of carbohydrates, aminoacids, vitamins, inorganic salts and many other constituents. Many basal media are now commercially available and have been established for serum supplemented cultures.

Complete Media:- Basal media when supplemented with 5 to 20% foetal calf serum it becomes complete media. Basal media may also be supplemented with glutamine which acts as a major carbon source for most cells in culture.

Synthetic Media (Chemically Defined Media):

As early as in 1943 White tried to develop chemically defined synthetic medium. In 1950 Joseph Morgan, Helen Morten and Raymond Parker prepared a medium called Formula 199 which when supplemented with blood serum, supported the growth of most primary cells cultured *in vitro*. Glucose is added as a source of energy and as precursor to provide metabolic intermediates. Serum proteins may function as hormone, and are only required in small animals. Later,

Ham (1965) obtained clonal growth of mammalian cell in a chemically defined synthetic medium.

The synthetic media are generally prepared artificially by mixing various ingredients.

This has virtually replaced the use of earlier natural media as they are simple to constitute from substances essential for survival of cells and each component is well defined chemically and is easier to formulate the medium with greater reproducibility. The shelf life of chemically defined media is also more than natural media and requires less stringent facilities. As different types of cells have different growth requirement so a universal medium for all cell type is not possible. The requirement differs with species, among animals of same species and even among different tissues of an individual and hence the choice of any medium strictly depends on the types of cells to be cultured. Synthetic media must contain source of carbon, nitrogen, minerals, H_2 donor and acceptor, essential amino acids, nucleotide precursors (purine and pyramidine bases), vitamins, O_2 and CO_2 gas phase and various growth factors. Synthetic media may be used with serum or without serum.

Irrespective of specific modification, the common constituents of all the chemically defined media should must have basic requirement and these can be grouped as follows:-

(i) **Amino Acid :-** Essential amino acids which are lysine, histidine, leucine, isoleucine, valine, metionine, threonin, phenylalanine and trytophane are not synthesized by adult vertebrate animals and thus are obtained from their diet. So, all these nine essential aminoacids must be added in the culture medium used for the growth of animal cells. In addition, cysteine, glutamine and tyrosine aminoacids are also needed by the cells, which are synthesized by the specialized cells in the intact animals are also to be added in the culture medium for cell growth. The glutamine act as carbon and energy source and virtually required by all mammalian cells in culture regardless of type. As glutamine is labile in solution so this has been a major concern to many tissue culture scientists. Some scientist have found that glutamine is most stable when stored at 4^0C.

(ii) **Vitamins:-** Vitamins which forms the essential parts of co-enzyme involved in cell metabolism are generally not synthesized by the cells and hence should be included in the medium. Fat soluble

vitamin like Vitamin-A is essential for epitheloid cell types to maintain them in undifferentiated state. Vitamin limitation is usually expressed in terms of cell survival and growth rate rather than cell density.

(iii) Ions:- Ions are required for maintenance of osmotic balance on internal pH, transport of essential nutrients and as cofactors for various enzyme reaction. Bulk ion includes Na^+, K^+, Mg^{+2}, Ca^{+2}, Cl^-, SO_4^{-2} and PO_4^{-3}. Bicarbonate ions are added in isotonic concentration to prevent osmotic imbalance. Trace elements like copper, selenium, zinc, vanadium etc., are also required for normal and optimal growth of cells.

(iv) Organic Nutrients:- This include carbohydrate, (like glucose, is needed for energy requirement of growing cells), protein (for normal development and growth of growing cells) and supplementary metabolites like fatty acids, nucleotides etc. which are essential for proper growth of cells must remain in the media.

(v) Antibiotics and Antifungal Drugs :- Antibiotics like Penicillin G sodium (50 U/ml), Chloromycetin (10 μg/ml), Tetracycline (10 μg/ml), Kanamycin (for PPLO control) (100 μg/ml) etc., and antifungal drugs like Mycostatin (25 μg/ml) and amphotericin B (Fungizone) (2. 5 μg /ml) must be added in the media in order to prevent it from different bacterial and viral contamination as prevention of contamination by microorganisms including PPLO is an essential part of all animal cell culture (Litwin, 1973). PPLO or Mycoplasma are also the important contaminantof cell cultue. These not only compete with the cells for nutrients but also cause rapid depletion of essential aminoacids, i.e., arginine from the culture medium and an increased accumulation of ammonia (Stanbridge 1971).

Antibiotics can control the growth rate and reduce longevity of animal cells in culture. Routine concentration of penicillin and streptomycin cause at least a 20% reduction in yield from cultures of human fibroblast compared with the same cells grown in the absence of antibiotics for several passage (Goetz *et al.* 1979).

(vi) Other Factors :- In addition to satisfying nutrient requirements, the culture requirement, must include congenial physiochemical properties that fall within acceptable limit for cell survival and multiplication. Many protective agents are often added to the culture medium to protect the cells from damage caused by osmotic gradients, shear and interaction. Temperature, pH and

osmolality of the culture medium are the main physical factors that influence the cell survival.

The chemically defined culture media were based on the analysis of plasma which led to the formation of Medium 199 that contained more than 60 ingradients (Morgan *et al.* 1950). Other most commonly commercially used media available are Minimum Essential Media (MEM) of Eagle's (1955), RPMI (Rosewell Park Memorial Institute)-1640 (Moore 1967) HAM's F-10 medium (Ham 1963). Eagle's MEM is a more enriched media and can be used for primary monolayer call culture. The composition of these media has been tabulated in Table-2.

Table-2: Composition of the Different Tissue Culture Media.

Ingredients (per 1000 ml.)	Medium 199 (Hayflick *et al.* 1964.).	Eagle's medium (Dulbecco & Freeman 1959)	RPMI 1640 (Moore, 1967)	HAM medium (Ham 1963).
Sodium chloride	8.0 gm	6.4 gm	6.0 gm	7.4 gm
Potassium chloride	0.4 gm	0.4 gm	0.4 gm	0.285 gm
Magnesium sulphate	0.2 gm	0.2 gm	0.1 gm	0.153 gm
Disodium phosphate	0.06 gm	-	1.512 gm	0.29 gm
Monosodium phosphate	-	0.124 gm	-	-
Monopotassium phosphate	0.06 gm	-	-	0.083 gm
Calcium chloride	0.14 gm	-	-	0.044 gm
Ferric nitrate	0.1 mg	0.1 mg	-	-
Glucose	1.0 gm	4.5 gm	2.0 gm	1.1 gm
Adenine sulphate	0.01 gm	-	-	-
l-Arginine	0.07 gm	0.084 gm	0.2 gm	0.211 gm
l-Histidine	0.02 gm	0.042 gm	0.015 gm	0.021 gm
l-Lysine	0.07 gm	0.146 gm	0.084 gm	0.029 gm
di-Tryptophane	0.02 gm	0.016 gm	5.0 mg	0.6 mg
l-Tyrosine	0.04 gm	-	-	-
l-cystine	0.02 gm	-	0.05 gm	-
l-leucine	0.12 gm	0.105 gm	0.05 gm	13.0 mg
l-isoleucine	0.14 gm	0.105 gm	0.05 gm	3.0 mg
Ferrous sulphate	-	-	-	2.0 mg
Copper sulphate	-	-	-	0.004 mg
Zinc sulphate	-	-	-	0.051 mg
dl-Phenylalanine	0.05 gm	0.066 gm	0.015 gm	5.0 mg
dl-Methionine	0.03 gm	0.030 gm	0.015 gm	4.0 mg
dl -Serine	0.05 gm	0.042 gm	0.03 gm	11.0 mg
dl-Threonine	0.06 gm	0.095 gm	0.02 gm	4.0 mg
dl-Valine	0.05 gm	0.094 gm	-	4.0 mg
dl-Glutamic acid	0.15 gm	-	0.02 gm	15.0 mg
dl-Aspartic acid	0.06 gm	-	-	13.0 mg

Ingredients (per 1000 ml.)	Medium 199 (Hayflick *et al.* 1964.).	Eagle's medium (Dulbecco & Freeman 1959)	RPMI 1640 (Moore, 1967)	HAM medium (Ham 1963).
dl-alpha Alanine	0.05 gm	-	-	9.0 mg
l-Proline	0.04 gm	-	0.03 gm	12.0 mg
l-Hydroxyproline	0.01 gm	-	-	-
Glycine	0.05 gm	0.030 gm	0.01 gm	8.0 mg
Glutamine	0.10 gm	0.030 gm	-	0.146 gm
Sodium acetate	0.05 gm	0.58 gm	-	-
Adenosine Triphosphate	10.0 mg	-	-	-
Tween 80	0.005 ml	-	-	-
Guanine	0.3 mg	-	-	-
Xanthine	0.3 mg	-	-	-
Hypoxanthine	0.3 mg	-	-	-
Thymine	0.3 mg	4.0 mg	-	4.0 mg
Uracil	0.3 mg	-	-	1.0 mg
Pyridoxal	0.025 mg	4.0 mg	-	-
Pyridoxine	0.025 mg	-	1.0 mg	-
Niacin	0.025 mg	-	-	0.206 mg
Niacinamide	0.025 mg	4.0 mg	-	-
p-aminobenzoic acid	0.05 mg	-	1.0 mg	0.615 mg
Inositol	0.05 mg	7.2 mg	0.035 gm	-
Riboflavin	0.01 mg	0.4 mg	0.2 mg	0.541 mg
Thiamin	0.01 mg	4.0 mg	1.0 mg	0.376 mg
Calcium pantothenate	0.01 mg	4.0 mg	0.25 mg	1.0 mg
Choline chloride	0.5 mg	4.0 mg	1.0 mg	0.715 mg
Biotin	0.01 mg	-	0.2 gm	0.698 mg
Folic acid	0.01 mg	4.0 mg	1.0 mg	0.024 mg
Calciferol	0.1 mg	-	-	1.0 mg
Cholesterol	0.2 mg	-	-	-
Alpha tocopherol	0.01 mg	-	-	-
Menadione	0.01 mg	-	-	-
Ribose	0.5 mg	-	-	-
Deoxyribose	0.5 mg	-	-	-
Adenylic acid	0.3 mg	-	-	-
Polymyxin B sulphate	20000 U	20000 unit	20000 unit	-
Neomycin sulphate	10000 U	10000 unit	10000 unit	20000 unit
Phenol red	0.02 gm	15.0 mg	5.0 mg	10000 unit
l-lysin	0.1 mg	-	0.04 gm	1.2 mg
Glutathione	0.05 mg	-	-	0.029 gm
Ascorbic acid	0.05 mg	-	-	-
l-Tyrosin	-	0.072 gm	0.02 gm	-
l-Cystine	-	0.048 gm	0.05 gm	0.28 mg
			0.84 gm	0.3 mg
Sodium bicarbonate	-	-	-	1.0 mg
Lipoic acid	-	-	0.005 mg	
Vitamin B12	-	-		

Maintenance Media:

Media for maintenance of cellular metabolism without stimulation of multiplication is required for cell culture. The ideal medium for this purpose should, without renewal, maintain cellular metabolism and preserve cellular morphology unaltered for at least 10 to 14 days of incubation at 37^0C. At the same time it should be economical, reproducible and stable on storage.

Hank's Balanced Salt Solution (HBSS):

This is very common synthetic medium used by the different tissue culture laboratory to maintain cells in a viable state for short period of time. Apart from furnishing the inorganic ions essential to life of all cells, HBSS provides an aqueous environment in which the correct osmotic pressure is established, chiefly by the sodium chloride, the pH is maintained at 7. 2 to 7. 4 by the buffering action of the sodium bicarbonate and a convenient source of energy is also available in the form of glucose. The indicator, phenol red is included at a concentration which is not toxic to cells but is sufficient to give visual warning of a significant change in the pH. The formulation of HBSS is as follows:-

Table 3. Composition of Hank's Balanced Salt Solution.

Ingredients	Amount (mg/L)
Sodium chloride (Nacl)	8000
Potassium chloride (KCl)	400
Calcium chloride ($CaCl_2$, $_2H_2O$)	185
Magnesium sulphate ($MgSO_{4,7}H_2O$)	200
Di Sodium hydrogen phosphate(Na_2HPO_4)	47.5
Potassium dihydrogen phosphate (KH_2PO_4)	60
Sodium hydrogen carbonate ($NaHCO_3$)	350
Glucose	1000
Phenol red	17
De-ionized water	1000 ml.

The prepared HBSS is sterilized by positive pressure filtration and is stored at 4^0C.

Primary Monolayer Tissue culture technique:

The procedure usually involve growing cells as monolayer sheet upon glass surface which are subsequently maintained in appropriate nutrient media so that these remain viable for a prolonged period. The steps involved in the tissue culture are as follows:

1. Source of Cell :- The ultimate source of cell for tissue culture is intact animal. The cells may be derived from a number of organs and tissues which may be foetal, infant and adult origin. Monkey kidney, Chicken fibroblast, Hamster kidney is oftenly used for tissue culture. Neonatal or young animals should be selected as cells from them are easier to establish and grow better *in vitro* than those from mature and aging animals. Fat and necrotic tissues should be removed completely during dissection.

If primary monkey kidney monolayer tissue culture is prepared then kidneys are removed from a monkey freshly killed by exsanguination. These are kept into a flask containing 200 ml. HBSS. Kidneys are decapsulated, cleansed of fat and connective tissues and are cut in half longitudinally. The medulla is removed (to minimize heterogeneity of cells) and discarded with adherent connective tissue The whole tissues are removed in a other container containing HBSS and then tissues should be chopped in order to have smaller pieces (about $1mm^3$) with sterilized scissor and forceps. Care should be taken that with minimum damage should occur to the cells.

2. Cell Dispersion :-Dispersion of the pre-existing cell mass is an essential step in the production of tissue culture. This can be done either enzymatically or mechanically. Before enzymatic treatment tissues are minced to 1-3 mm size and washed two to three times with neutral HBSS in a refrigerated centrifuge. The ordinary centrifuge will kill the cells because of the generation of heat and friction. The following methods are commonly used for cell dispersion:-

A. Enzymatic Digestion:

Different tissues will require differant treatment in order to obtain disaggregation. This statement is more true for adult tissues probably due to the development of large amounts of intercellular materials of a specialized nature. However, the following methods are used for the disaggregation of tissues for tissue culture:

(i) Digestion with the Proteolytic Enzymes:- For this trypsin is generally used. The use of trypsin for cell dispersion was introduced by Frish and Jentoft (1953) and its action depends on the ability of the enzyme to digest intercellular proteinaceous material, thereby allowing adjacent cells to be detached from each other while maintaining their individual structural integrity. Generally 0. 25% solution of crude trypsin or 0. 01-0. 05% pure trypsin having pH 7. 2-7. 4 at 4 to 37^0C is commonly used. Although trypsin is highly effective

in achieving cell dispersion, it is at the same time somewhat inimical to the living cells, thus it is important to expose cells to trypsin for as short a period as possible. For sensitive types of cells trypsinization may be preferred at 4^0C (Ham 1980). Thus, overtrypsinization or undertrypsinizationshould be avoided. Frothing during trypsinization should also be avoided. The principal of operation is therefore to expose cells to its action for the shortest possible time consistent with adequate cell dispersion, at the same time employing sub-optimum condition of pH and temperature. Simplified procedure of trypsinization of monkey kidney tissues was proposed by Bodian (1956). Here the minced tissues are kept in a conical flask having HBSS and sterile stainless steel or silicone-covered magnet. And the flask is kept over a magnetic stirrer so that the magnet rotates at a fairly low rate. The whole set is kept at 4^0C for overnight stirring. Aliquots of suspension can then be removed by pipetting or centrifugation under refrigeration.

(ii) Digestion with Collagenase :- Collagenase is a protein with a high degree of specificity for collagen (Harper, 1980) and used for the disaggregation of chiefly adult tissues, for the preparation of primary culture. It is some times also used for the separation of cell chiefly of embryonic and malignant origin. Generally it is not used for epithelial tissue as it is damaged by the action of collagenase, whereas it has no effect on fibrous tissues. Collagenase acts by disintegrating collagen which forms the intracellular matrix. The crude collagenase which is used in the concentration of 200-2000 U/ml also contain non-specific proteases.

B. Dispersion by Chelating Agents:-

(i) Sodium Versenate (E. D. T. A. -Ethylene Diamine Tetra acetic acid):- This is used as tetra sodium salt. The separation of cells by chelating action of sodium versenate was introduced by Zwilling (1964) and its chelating action depends on its ability to combine with divalent cat ions such as Ca^{++} and Mg^{++}. As Ca^{++} and Mg^{++} ion plays an important role in the attachment of living cells so removal of these ions by substances which bind them (chelating agent) may disrupt tissues very easily. Versene is used as 0. 02% solution in buffer saline lacking Ca and Mg salt at pH 7. 2 to 7. 4. Generally it is used for disruption of cells from continuous cell line and diploid cell strain monolayer because it has mild action. Though, Melnick *et al.* (1955) used EDTA to disaggregate fresh kidney tissue but now a days it is rarely used for fresh tissue.

(ii) Trypsin-Versene Mixture:- Generally 0. 25% trypsin and 0. 02% versene is used at pH 7. 2-7. 4. This solution can be prepared in Ca^{2+} and Mg^{2+} free PBS but the following solution is preferred for retention of maximum viability:

Sodium chloride (NaCl)	122 mM
Potassium chloride (KCl)	3.0 mM
Di-sodium hydrogen phosphate(Na_2HPO_4)	1.0 mM
Glucose	4.0 mM
EDTA	0.02 % (w/v)
Tris (hydroxymethyl) aminomethane	2.0 % (w/v)
Phenol red	3.3 mM

To this solution trypsin is added to make the final concentration 0.25%. Trypsin solution is sterilized by filtration through a 0.2mm sterile filter. Since trypsin solution is subject to self-digestion, it is essential to divide freshly prepared solution into small aliquots and store frozen until required.

C. Mechanical Disintegration:- Where the tissue is susceptible to various enzymes, mechanical disintegration may be adopted for cell separation. Sieving process for preparation of dispersed cell suspension by mechanical method was described by Shannon *et al.* (1952). Here the piece of the tissue is taken on a stainless steel sieve and is then pressed with all glass syringe barrel with rotating movement. While rotating it is essential to add HBSS or MEM to prevent it from drying. The processed material may be collected in a sterilized beaker. This method is still in use for separation of thymocytes from thymus.

3. Assessment of Viable Cells:- To ascertain the viability of cells, exclusion of dyes provides a convenient measure of cell viability (Patterson 1979). For this, a number of vital staining technique are available to differentiate between viable and non-viable cells. Various dyes such as methylene blue or various tetrazolium salts, neutral red are taken up by living cells and indicate metabolic activity of the cells Other group of dyes, such as trypan blue, erythrosine B, eosin Y can also be used to test the ability of viable cells to exclude dye. Commonly used method in tissue culture laboratory to test the viability of cells are trypan blue dye exclusion test. Trypan blue has added advantage that it can be used with both living materials and also materials fixed with glutaraldehyde. This is the only dye which give reproducible results both before and after fixation. Here one drop (0.4%) is mixed

with the one drop of cell suspension. Living cells do not take dye and thus remain colourless whereas dead cell take the dye and thus becomes blue in colour.

4. Counting of Cells and Adjustment of Cell Concentration:- Various methods have been used for counting of the cells before actual cell culture is done. These are:

(i) By Packed cell volume (PCV):-. For this 10% cell suspension is centrifuged at 100 r. p. m. for 10 minutes. The basis of cell count is 1.0 ml PCV obtained after centrifugation at 1000 r. p. m. for 10 minutes which contain approximately $2x10^8$ cells (Waymouth 1956). The disadvantage of this method is that it takes no account of the proportion of non-viable cells and gives total count of the cell. . So, the suspension contains a high proportion of dead cells giving a false estimation of the useful material. The advantage of this technique is that it is quicker than direct counting and is at least as meaningful when there is excessive cell clumping

(ii) By Haemocytometer :- Counting of cell with haemocytometer is done by the standard method. Cell suspension in balanced salt solution containing approximately 2. $5x10^5$ cell/ml is prepared. Counting is done using the all four large square of the corner as done in case of white blood corpuscles. If greater than 10% cells appear clustered, cells are dispersed by vigorous pipetting in the original cell suspension and entire procedure of counting is repeated

(iii) By Plating:- Assessing the ability of individual cell to multiply and give rise to colony in isolation can be enumerated. This is done by employing various serial dilution of the cell suspension, plating on suitable medium and determination of survival by counting the colonies they form on the medium.

(iv) By Electronic Cell Counter:- This has been developed to speed up blood cell counting procedure. This instrument is suitable for counting various animal cells grown in culture. A single count including 1000 or more cell is completed in approximately 20 seconds. The counting error is less than 1 to 2 per cent.

(v) Colorimetric Method :- Recently, a colorimetric method using MTT [3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide] (Sigma, USA) assay (in the concentration of 2-5 mg/ml in PBS) can be used both to measure viability after release of cytoplasmic content into the medium from artificially lysed cells and for microscopic visualization within the attached cells.

5. Suspension of Cell in Tissue Culture Medium:- A wide variety of culture media can be used for the culture of any given type of cells. The most suitable starting point is to use the medium which has previously been reported to support the growth of the particular type of cells. The number and volume of cells, to be inoculated, depend on the volume of the type of culture vessels, such as culture tube, culture flasks and diameter of Petri dishes. Thus, the cell concentration used is therefore a compromise. However, for setting most monolayer culture 5. 0×10^4 to 1. 0×10^5 cell/ml. is usually satisfactory. The required concentration of cells are added in the culture flask and is incubated at 37 ^{0}C. Cells are examined next day under inverted microscope. Depending upon the type of cells used in the primary culture various attachment factors, hormones and growth factors are also included. Careful replenishment of medium during the cell culture is an important aspect of maintaining culture cycle, because of the following reasons:-

(a) Replacing essential nutrients which are depleted by cell growth.

(b) Removing any dead cells and erythrocytes.

(c) Removing product of metabolism which inhibit growth or survival

(d) Assisting in control of pH.

It should be kept in mind that whenever fresh medium is added it should have the same temperature as the culture. The fresh medium should have a pH and osmolality optimum for cell growth. Another approach to change of medium, especially during the later stage of exponential growth, is to feed culture with a modified medium as during this stage of culture, nutrient and growth requirements are not the same as at the beginning of the culture.

6. Attachment of Cells:- The attachment of cell to culture surface is fundamental to the traditional monolayer cell culture. The proliferation of anchorage dependent cells occur only after attachment to suitable culture surface. (Grinell 1978) and thus the cells start to settle immediately after these are suspended in growth medium. Irreversible attachment to the culture vessel wall is accompanied by a significant number within the first hour. Attachment of cells in culture is a multistep process and involves the followings:-

(a) adsorption of attachment factors to the culture surface,

(b) contact between the cells and surface,

(c) attachment of the cells and the surface and

(d) spreading of the attached cells.

To ensure uniform setting of cells, culture should be placed upon a levelled surface. If the culture is not levelled in the beginning, the resulting cell sheet will be uneven.

7. Growth of the Cell Monolayer:- The cells spread out as a layer and the cell density is high. Here sheets of contiguous cells about one cell thick is maintained. The growth of such cell is limited only by the availability of space. A uniform distributed pattern of cells is observed on the floor of culture vessels 24 hours after incubation (Fig. - 3 and 4). Growth depends upon the doubling and generation time of the

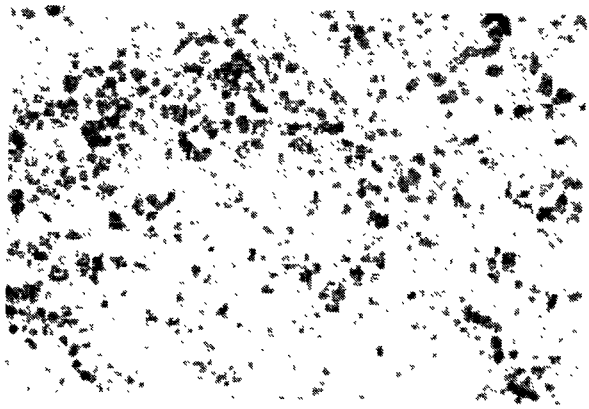


Fig. 3. Healthy Monolayer Vero Cell on 5th Day Post Seeding

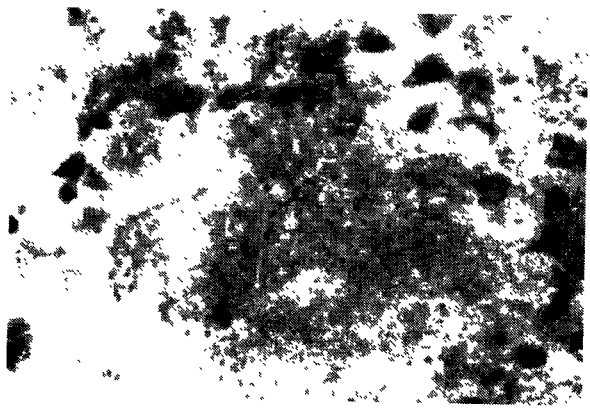


Fig. 4. Vero Cell Infected with Vero Cell Adapted Rinderpest Virus Showing CPE (syncytium formation) on 5th Day Post Infection (May-Grunewald & Giemsa staining).

cells **Doubling time** is the time interval in which a given population of cells doubles in its number, whereas **generation time** is the time interval between the two consecutive division of a cell. Scaling up of the cultured cell can be done by different scaling methods, such as roller bottle system, microcarrier beads, artificial capillary perfusion system etc., in which the total surface area becomes available for higher cell attachment.

8. Preservation of Animal Cell Culture:- For maintaining the cell culture for longer duration of time, it requires suitable preservation. By suitable preservation loss of cell culture can be reduced to great extent and this can be done by cryopreservation. Freezing by cryopreservation protects the cells from changes due to the genetic drift and minimize the risk of contamination. In cryopreservation cells are stored at -180^0C using liquid nitrogen in a state of suspended animation until they are needed. The modern cryogenic storage system make storage, tracking and retrieval of sample efficient and easy. Cost of cell culture is also be reduced by suitable preservation of cell culture.

Healthy cells are suspended in a solution of either glycerol or dimethylsulfoxide (DMSO), with a high concentration of serum and then cooled at definite rate in liquid nitrogen vapour and then placed in liquid nitrogen cylinder. Without the presence of DMSO, freezing is lethal to most mammalian cells. Damage is caused by mechanical injury by ice crystals, concentration of electrolytes, dehydration, pH change and denaturation of protein. DMSO minimizes the above lethal effects by lowering the freezing point and allows for a lower cooling rate. The function of DMSO is to reduce the water content of the cell. DMSO is a small molecule which is soluble in lipids and thus enters in cell quickly by diffusion across the lipid bilayer of the plasma membrane. In the presence of DMSO ice crystal inside the cell cytoplasm is not formed. In absence of DMSO ice crystal will be formed inside cytoplasm resulting into rupture of cell membrane and lyses of cell (Brown and Nagle 1965). The high serum concentration probably contribute to cell integrity by maintaining the intracellular protein concentration of cells rendered permeable by the DMSO. Glycerin has much the same effect as DMSO and is used by the some tissue culture laboratory.

Applications of Tissue Culture

- To study processes taking place in animal cells, e.g., metabolic regulations, cell physiology.

- To enable cultivation of viruses for their study and production of vaccines
- Easy growing of cells *in vitro* has led to a spurt in the activity to apply cell culture technology to synthesize or produce a variety of biomolecules at an industrial scale, which otherwise would have been difficult.
- Cultured cells are used for reconstruction of damaged tissues or replacement of non-functional cells or tissues.
- Cultivation of patient's own cells *in vitro* in order to generate enough cells and/or to genetically manipulate the cells to replace a missing function, to overcome problem of immune rejection and of scares tissue source. Parkinson's disease may one day be cured using cultured cells.
- Antigen-specific killer T cells grown *in vitro* can be used in immune suppressed patients. May be useful in as immune-suppression therapy, possibly in prevention of HIV cases turning into blown up cases by using specific killer T cells.
- Scanning of anti-cancerous drugs in tissue culture, as a clear correlation between *in vitro* and *in vivo* activities of chemotherapeutic agents was demonstrated in 1950.
- Use of tissue culture avoids many ethical objections raised against animal experiments and also allows experiments on human tissues which otherwise could not be done *in vivo*

Disadvantages :

- Tissue culture requires strict aseptic conditions and skilled persons and techniques.
- Cost of animal tissue culture and labour, often may be high and prohibitive.
- The growth and metabolic regulatory mechanism that exist under *in vivo* conditions are absent in culture condition.
- Cell lines may not reflect the actual conditions *in vivo* and results may not be reproducible in the living animals
- Tissue culture may not always be possible.

Types of Cell Culture :

Anchorage Dependent Cell Culture:

Adherent cells are said to be anchorage-dependent and attachment

to a substratum is a prerequisite for their proliferation. They are generally subject to contact inhibition, i.e., they grow as an adherent monolayer and stop dividing when they reach a density that they touch each other. Most cells with exception of mature haemopoetic cells and transformed cells grow in this way.

Cells bind to surface through specific cell surface receptors to attachment proteins absorbed to the surfaces. Two factors in culture medium is essential for adhesion of cell to culture surface - divalent cat ions and protein (Grinnell 1978). The protein molecule essential for full adhesion of cells to a culture surface is now known to be a glycoprotein (Yamada and Olden 1978). In the absence of divalent cations and proteins, cells attach to a culture surface only by a non-specific adsorption (Grinnell *et al.* 1977). Serum provides some of these adhesion factors that are essential components of extracellular matrix. Glycoprotein is found in the serum in culture medium as cold insoluble globulin or is secreted from certain cells as fibronectin (Hughes *et al.* 1979). The prominent attachment and spreading factors for monolayer cells are extracellular matrix proteins, such as fibronectin, laminin, vitronectin and different collagen types. Laminin and fibronectin are intrinsic mitogens and are differentiation factors for the maintenance of normal cellular functions. The receptor-binding domain of fibronectin is a tripeptide, L-Arg-Gly-Asp (RGD). Fibronectin mainly promotes attachment and differentiation of mesenchyme derived cells including fibroblasts, sarcoma, granulocytes, kidney epithelium, adrenal cortex epithelium, CHO cells, myoblasts etc.

Laminin enhances attachment of cells of ectodermal and endodermal origin, such as bronchial and secretory epithelial cells, neuronal cells, hepatocytes etc. Infact laminin suppresses overgrowth of fibroblasts in primary cultures of epitheloid cells.

Contact Inhibition :

It is the inhibition of cell motility and also of mitotic activity that is observed when cultured cells come in contact. This is observed in cultures growing on solid support such as glass surface. As long as cells float freely in the nutrient medium they generally divide every 24 hours. However, when they come in close contact in a monolayer, the rate of mitosis slows down, there is inhibition of cell division. The inhibition depends upon some unknown signal between cells coming in contact. Movement of a cell in the direction of a contact

tends to be prevented - phenomenon called **Contact Inhibition** (Abercrombie 1970).

Non-anchorage Dependent Cell Culture :

Non- anchorage dependent cells do not attach to the substratum and therefore, remain suspended in the medium and are called suspension culture. Cells cultured from blood, spleen and bone marrow adhere poorly, do not adhere and grow in suspension. Suspension cultures are easier to propagate as subculture in liquid medium are poor in divalent ions and stirred constantly. This does not require dilution with medium and is useful in experimental virology. Suspension cultures are done in spinner flasks or fomenters depending on the scale on which one intends to obtain cells.

Adult or Embryonic Tissue

Tissues from almost all parts of the embryo are easy to culture whereas tissues from adult are often difficult to culture. Widely used embryonic cells include Mouse embryonic fibroblasts, 3T3 cell and Human foetal lung fibroblasts cell line such as MRC-5 etc. Despite practical advantages of embryonic cell lines, it is important to remember that these cells may not behave in the same way as the adult cells.

Primary Monolayer Chicken Embryo Fibroblast Cell Culture :

For primary monolayer chicken embryo fibroblast 10 to 12 chicken embryos are taken from about 10 days old embryonated eggs. Eggs shells are fully cleaned with 70% ethyl alcohol. The whole content of the eggs are taken in a sterilized Petri dish containing magnesium free Hank's balanced salt solution. Embryos are removed with the help of forcep. Head and leg of the embryo are pinched off and trunk is transferred in other Petri dish having HBSS. Bodies are washed off and freed from red blood cells and other by transferring it in several petridishes having HBSS in a row. The internal organs are removed and discarded and the remaining portions are washed three times with HBSS. The embryos are chopped into very small pieces with sterile scissors. These pieces are now transferred in a sterile conical flask containing 0.5 gm of glass beads (710-1180 μm diameter). The glass beads may be resuspended in 1 ml. PBS and autoclaved in a trypsinization flask before use. Now 20 to 30 ml. Ice cold 0.2% (v/v) trypsin (1:250) in HBSS is added and the flask is swirled at room temperature for 30 seconds. Supernatants having flakes and RBC are discarded. Further 15-20 ml. trypsin is added and swirled at room

temperature for 30 seconds and the liquid phase is removed in a sterile container. In this way, trypsin, glass beads and gentle agitation combine the elements of both enzymatic digestion and mechanical dispersion. This material is centrifuged at 300g for 5 minutes at 4^0C. Then supernatants are discarded and cells resuspended in 10 ml HBSS + 5% foetal calf serum. It is resuspended in 25 ml. HBSS + 5% foetal calf serum and the cells should be placed in a fresh sterile conical flask (with beads treated as above) and incubated on ice for one hour with swirling every 10 minutes. This incubation allows any residual trypsin to continue the digestion, in conjunction with further mechanical dispersion. After incubation cell suspensions are filtered through a cell sterilized strainer. Cells pelleted and resuspended in 20-30 ml HBSS +10 % foetal calf serum. Cells are counted and viability checked as described earlier and finally cells are plated in tissue culture flask and incubated. Tissue culture flask is examined under inverted microscope for any growth, if needed medium is changed to remove any dead cells, red blood cells etc. When full growth takes place sheet of fibroblast can be observed (Fig. - 5).

Plating or Cloning Efficiency :

Plating efficiency is the percentage of those cells plated out on to culture medium that give rise to viable colonies i.e., ratio between colonies formed and cells inoculated. It is used to denote the percent of cells set down which form colonies visible to unaided eye within 8 days or less. The culture plate is observed under the inverted microscope and single cell is marked on the outside of the plate with a fine marker to keep track of their regeneration potential. This ensures the isolation of pure single cell clone. In preparing culture, if a known volume of suspension is transferred to each plating dish, it may be possible to assess the plating efficiency (PE) quantitatively using formula :

$$PE = \frac{\text{Final number of colonies/plate}}{\text{Initial number of cell unit/plate}} \times 100$$

Plating efficiency varies between cell types from as low as 0.1% to as high as 80-90%When a particular cells have a low plating efficiency it can be enhanced by using micro carriers which enables attachment of the minimum number of cells or starting culture and sets optimal number of cells. The culture volume can be maintained at 50% of the final dilution for the first 3 days of culture and then fresh medium is

added to reach the final volume. In this way the population of cells can be cultured at greater densities during the period the culture is most susceptible to dilution. Lasly, at the most basic level, a plating efficiency is the percentage of those protoplasts plated out onto culture medium that gives rise to viable cell colonies. Other parameters of obtaining PE are:

- Using a conditioned medium or synthetic medium designed to permit growth from a low initial density,
- Avoiding plating cells too long in stationary phase,
- Harvesting cells during exponential growth phase,
- Exposure of cells to temperature not exceeding 37^0C and
- Incubating the plate in diffused light or darkness.

Growth Curve of Cloned Population :

Single cells of HeLa cells are deposited in Petrie dishes in nutrient medium and incubated in the standard fashion. At various time intervals microscopic count of cell number is made. Growth curve shows all initial lag phase of approximately 20 hrs followed by linear log. reproduction. The dynamics of Feeder system are of special interest. Feeder system is a powerful adjunct as it may permit the initial clone cultivation of cells after which it may be much easier to isolate cloned stock and discover means for their adaptation to grow without feeder.

Feeder Layer :

Sometimes on the substrate surface a monolayer of special kind of cell is used partly to supplement the culture medium for growth and partly for conditioning of the substrate by cell product. These monolayers are called feeder layers since they also fed growing culture An approach to culture protoplast at low density is the Feeder layer technique. The cell of feeder layer is generally irradiated to destroy its multiplication capacity, other factors remain the same. Raveh *et al.* (1973) prepared a feeder cell layer by exposing tobacco cell suspension protoplast to an X-ray dose of $3x10^3$ R which inhibited division of cells but allowed them to remain metabolically active. MacPhersion and Bryden (1971) used mitomycin C treated cells as feeder layer. Thus we see that the feeder layers are the culture of cells that do not have the ability to grow continuously in culture but are able to provide the initial stimulus to the inherent cells by way of providing cell growth factors, cell contact and they may also digest potentially toxic

cell debris but at the same time prevents differentiation of the pleuripotent cultured cells (Anderson 1992 and Dang *et al.*, 1995). The culture of murine and stem cells usually require feeder cells (Evans and Kaufmann 1981 and Thompson *et al.* 1998). The Feeder layer may consist primarily of fibroblasts obtained either from adult or foetal tissues, but the later is preferred. For mouse embryo fibroblasts, normal fetal intestine, glial cells etc.

Feeder layers consisting of newly formed hybridomas were used for optimal growth and hybridoma production. The most common Feeder layer consist of :

- Murine peritoneal cells
- Cells derived from mouse, rat or guinea pigs
- Irradiated spleen cells
- Human fibroblasts, human peripheral monocytes or thymus cells.

The high metabolic activity of the Feeder cells which enables them to condition the medium effectively despite their total loss of reproductive ability is understandable in view of the nature of the X-ray inactivating process. The total amount of energy absorbed by a cell irradiated with 4000 r is 4000x2. $03x10^{-6}$ calories/g an amount of energy equivalent to a temperature rise of 0.008 C. Hence the effect of the irradiation in multiplication must be confined to the production of a block one or a very few of the metabolic chains necessary for growth. Coman *et al.* (1950) have demonstrated that cancerous epitheloid cells differ from their normal counterparts in being deficient in the ability to bind Ca++. This property prevent the invasive cells from adhering together so that their natural amoeboid motion results in extensive migration. Supernants from a number of different cell type tissue culture have been shown to have angiogenic activity (Klagsburn *et al.*·1976). Feeder layer or media conditioned by primary cells have been used to increase hybridoma survival and clonal development e.g., conditioned media produced by growing a mouse macrophage like cell line in Iscove's modified Dulbecco's medium and 2% foetal bovine serum is found to be ideal for conditioning media in hybridoma protocol.

Preparation of Feeder Cells:

From the eviscerated bovine foetus (60-70 days gestation), 1 cu mm pieces of subdermal tissues are placed in 25 cm flask containing small drops of re-calcified bovine plasma and then incubated for one hour

Fig. 5. Characteristic Fibroblast Cells Prepared from Skin and Stained with Crystal Violet (x 1000)

for fixation of the tissues prior to adding media. Dulbecco's MEM + 10% serum + antibiotics is added for the growth of the fibroblasts. Confluent cultures are trypsinized and reseeded for several passage for cell multiplication. The harvested cells are frozen in aliquots in the freezing medium contained in 10% DMSO. Frozen aliquots are thawed and further grown to confluency. Confluent cultures are treated with 10μg/ml mitomycin in D10 media for 3 hours. This treatment inactivates cell DNA so that the fibroblasts do not divide when seeded as feeder layer. The mitomycin treated cells are trypsinized and frozen in small aliquots, thawed and seeded atleast one day before seeding embryo.

WI-38 of normal human diploid strains in phase-III is used as feeder layer. This can be prepared by trypsinizing confluent WI-38 monolayer cultures and suspending the cells in Eagle's minimum essential medium at a concentration of $5x10^4$ viable cell/ml. Two inches petriplate is set with 10ml volume of this suspension and incubated overnight at 37^0C in an atmosphere of 5% CO_2 in air to form the feeder layer.

Scaling up of Animal Cell Culture:

The primary objective of scaling up of mammalian cell culture is to obtain high yield of cells from small culture volume. Laboratory routine cell culture can be used for virus isolation, studying cell morphology and functions, study of different agent on cell on a fairly small scale. Thus, scaling of animal cell culture is needed for getting

high yield production of cells, viruses or cell products, thus, deriving benefit of cell cultures for meeting the demands of society.

Scaling up of Anchorage-dependent Cell Culture :

Rous flask or plastic flask is the commonly used substrate for routine cell culture. As both the above mentioned flasks have only one surface for cell attachment, so there is a low surface area to volume ratio. The largest Rous flasks or plastic flasks provide maximum surface area of 175-200 cm^2 and needs 100-150 ml. of medium to grow $2x10^7$ diploid cells or $1x10^8$ heteroploid cells. Scaling up of anchorage dependent cells requires suitable substrate (glass or plastic), to attach themselves while growing. The scaling up of anchorage dependent cells can be done by Roller bottles, Hollow fiber system, Glass bead reactors and by Micro carrier system.

Roller Bottle System:- In this system the bottles are placed horizontally on rollers and are rotated at the rate of 10-20 rph, while cells are attaching and then at 6-8 rph. The cells adhere evenly round the glass surface and eventually spread to form a monolayer covering. This system provides a much larger area than the culture flask and also ensures a much higher surface area to volume ratio. Further, the surface area of roller bottle can further be increased by the spiral film method and the glass tube method. Cells growing on culture surface often use medium more efficiently than the same calls growing in free suspension (Ham and Mckeehan 1979).

Hollow Fiber System:- In this bundle of synthetic hollow fibers is used to fill up the vessels similar to roller bottle and thus offer a matrix similar to vascular system of the body and cells in tissue like density can be grown. Fibers of acrylic polymer of about 350 mm in diameter with 70-75 mm thick wall are enclosed in a cartridge and encapsulated at both ends. Once the fibers are packed, medium is pumped through the lumen and it then perfuses through the fibers wall which are porous enough to allow the passage of macro-molecules of molecular weight between 10, 000 and 1, 000, 000 daltons. A very high surface area to culture volume ratio of 30 cm^2/cc can be achieved and cells 10^8/ml can be maintained.

Glass Bead Reactors:- In this method the cells are seeded and allowed to attach on glass beads of 3-5 mm diameter, which are tightly packed in a glass column. The medium is kept in another glass column. The medium is either pumped in the glass column of the beads either through a peristaltic pump or using airlift driven system is used. When

the culture is confluent, the medium can be drained off, the beads washed with buffer and trypsinized. The cells grow on the surface of glass beads. Glass beads are tightly packed in such a way so that glass bead may not slide around and damage cells but with enough space to allow medium to flow freely through at a rate which is not likely to damage cells by setting up sheer forces. Such reactors have been scaled up to 1001. This method also allows aeration by perfusion method as the medium is kept separate from the cells.

Microcarrier System :- The concept of culturing anchorage dependent cell on a small sphere called microcarrier was first given by vanWezel (1967). In the microcarrier cell culture system, cells are seeded and allowed to grow on microcarrier beads and then these beads are kept in suspension by stirring as the suspension cultures are set up. Various substances, such as, polystyrenes, polyacrymide, sephadex and gelatine are being used for microcarrier system. Carriers can be held in either static or packed beads through which nutrient fluids are pumped as they can be fluidized as particular suspension. This provides a homogeneous culture environmentwith high cell densities as well as simple medium cell suspension (Natiapetian 1986). Recently, microcarrier having a surface layer of denatured collagen has been used. Collagen is soluble at the extremes of pH and when made into a particles is isopycnic with water. This provides the same type of surface area as found *in vivo*. Such a surface is important for maximum plating efficiency, growth and function of certain cell types and lends itself to unique possibilities for harvesting cell from microcarrier culture. Now a wide range of micro carriers are commercially available. The advantage of microcarrier system can be used to obtain high yields of cells from small culture volume. Cultures can often be initiated with 10^5 cells/ml or less and at the plateau stage the yield is usually more than 10^6 cells/ml. This high yield of cells per unit culture volume and the large increase in cell numbers during the culture cycle (10-fold or more) make microcarrier system an attractive technique for production of cells from a wide range of culture volume.

Scaling up of Suspension Cell Culture :

Some of the cells, like cells of haemopoietic tissues and hybridoma cell normally grow in suspension either as single cell or in small clumps of cells derived from single cells. Scaling up these types of cells which remain as suspension culture is easy. This can be done by stirred tank reactors or air lift fomenters. The cells grow in suspension and these

are kept in suspension either by externally driven or internally driven stirrers.

It is essential to maintain oxygen supply and pH in both the systems.

Oxygen is only sparingly soluble in culture media. A typical culture of $2x10^6$ cells would deplete oxygen of the culture media in just one hour. For the supply of oxygen surface aeration, sparging, membrane perfusion and membrane diffusion are done. In surface aeration, the oxygen present in the head space is available to the cells and if a 10:1 head space to medium volume ratio is used, e.g. in a culture flask, enough oxygen is available to support 10^8 cells for 450 hours. The oxygen transfer rate is greatly enhanced by stirring and a head space to medium ratio 2:1 can be used. In sparging method, gas is supplied by bubbling using a fish tank air pump but it is important to keep the rate flow (5cc /1 /min) so that bigger size bubbles are formed which are less damaging to cells than smaller sized bubbles because of surface energy release when burst. In membrane perfusion system, medium is separated from the cell and then vigorously gassed. In membrane diffusion system, long length of silicone tubing is used to diffuse the gas to the medium. This method is expensive and inconvenient to use.

The pH is maintained by using the CO_2 - bicarbonate system and this requires equilibration with 5% CO_2. In a closed culture vessel, build up CO_2 pushes the pH to acidic side and therefore 10 fold greater head space is needed which is not affordable for large scale culture vessel. Therefore, continuous flow of air supplied through one filter and extracted through another filter, is required.

Stirred Tank Reactor:

In this type of reactors either small flask or a long vessel is used. In both the types a stirrer is used. It provides a relatively homogeneous culture environment. Generally, the large culture vessels are made up of stainless steel rather than of glass. The stirrer is moved by a motor fitted at the top of the vessel. The main advantage of the stirred type reactor is that it allows better and efficient control of mixing and provides a relatively homogenous culture environment (Bliem and Katinger, 1988). Here cells are only periodically exposed to the impeller discharge flow and for only a short time interval.

Airlift Fermentor System:

For stirring large volume of the culture medium without damaging

the cells in contact to the stirrer by sheer force or heat, airlift system is used. This is mainly used for culture of freely suspended animal and insect cells (Handa *et al.* 1987). This system is also like the stirred tank system where large stainless steel tank is used but differ in that they are designed to induce a cyclic flow of culture around the vessel. Here, bubble column principle is used to agitate and aerate the culture. Air bubbles are introduced into the bottom of the culture vessel via a sparger. An inner tube is placed inside the vessel and bubbles lift the medium as aerated medium has a lower density than non-aerated medium. The medium and cells which spill out from the top of the inner tube then circulate down the outside of the vessel. This way non-mechanical stirring of the cells is achieved.

Spinning System in Fermenters:

Spinning system moves the content with a unique oscillatory movement that guarantees low sheer forces while making optimum mixing. This system has the following advantages

(i) No heat is imparted to cell suspension,

(ii) Enables high expression for even the most adherent or resuspended cell and

(iii) Provides high surface to volume ratio ensuing improved oxygenation.

Practice Assignment VI

1. Cells of cell culture differ from the native organization in a tissue because:
   (a) Only those cells survive *in vitro* which can adopt to the environment
   (b) Cells undergo transformation and dedifferentiation
   (c) Both of the above
   (d) None of the above.
2. In primary monolayer cell culture all the statements are true, EXCEPT ;
   (a) Cells are arranged in monolayer
   (b) There are overlapping of cells
   (c) Phenomenon of contact inhibition is observed
   (e) Anchorage activity is present.
3. In Trypan blue dye exclusion test:
   (a) Viable cells take blue colour, whereas dead cells remain unstained

(b) Dead cells take blue colour, whereas viable cells remain unstained

(c) Both viable and dead cells take blue colour

(d) Both viable and dead cells remain unstained.

4. Feeder layer cells are:
   (a) Live with no mitotic activity
   (b) Live with mitotic activity
   (c) Dead and no mitotic activity
   (d) All of the above.
5. Cells used for feeder layer techniques :
   (a) Are generally irradiated cells
   (b) Takes part in supplying nutrients to underlying cells
   (c) Both of the above
   (d) None of the above.
6. Separation of cells from organ/tissue for cell culture may be done:
   (a) By using different proteolytic enzymes
   (b) By different mechanical means
   (c) By using different chelating agents
   (d) All of the above.
7. Cells for primary monolayer cell culture is derived
   (a) Directly from organ/tissue of live animal
   (b) Directly from other cell culture tube
   (c) Directly from transformed cells
   (d) All of the above.
8. Separation of cells are done by using ;
   (a) Trypsin
   (b) Versene (EDTA)
   (c) Trypsin + Versene mixture
   (d) All of the above.
9. Factors responsible for adhesion of cells to culture surface are:
   (a) Glycoprotein found in serum
   (b) Divalent cations
   (c) Non-specific adsorption
   (d) All of the above.
10. Cryopreservation of cell culture is done at:
    (a) -180^0C in liquid nitrogen
    (b) -120^0C in deep freeze
    (c) + 4^0C in an ordinary refrigerator
    (d) None of the above.

11. Scaling of cell culture is done to :
    (a) Enhance the quantity of cell culture
    (b) Enhance the quality of cell culture
    (c) Both of the above
    (d) None of the above.
12. Scaling up of suspension culture is done by :
    (a) Airlift method
    (b) By stirring method
    (c) Both of the above
    (d) None of the above.
13. Scaling up of anchorage dependent culture is done by:
    (a) Roller tube method
    (b) Microcarrier system
    (c) Hollow fiber system
    (d) All of the above.
14. Complete medium consists of :
    (a) Basal medium with calf serum
    (b) Basal medium without calf serum
    (c) Both of the above
    (d) None of the above.
15. Sodium versenate acts by :
    (a) Chelating action (b) Proteolytic action
    (c) Mechanical action (d) All of the above.
16. Trypsinization of cells by overnight stirring of cells at 4^0 C was introduced by:
    (a) Shannon (b) Bodian
    (c) Zwilling (d) Waymouth.
17. Anchorage dependent cells are:
    (a) Adherent cells on the substrate
    (b) Cells in suspension
    (c) Both of the above
    (d) None of the above.
18. In microcarrier system cells:
    (a) Grows on monolayer on the surface of small spheres
    (b) Grows as monolayer on the surface of Roux flask
    (c) Grows as monolayer in the glass culture tube
    (d) All of the above.

19. The advantage of microcarrier system includes :
    (a) High yield of cells from smaller culture volume
    (b) Culture can be initiated from 10^5cellc/ml or less
    (c) Reduced cost, increased productivity and reduced contamination
    (d) All of the above.
20. The separation of cells by chelating action of sodium versenate was described by :
    (a) Van Wezel (b) Bodian
    (c) Zwilling (d) Pizarro.

Answers:

1. -(c), 2. - (b), 3. - (b), 4. - (a), 5. - (c), 6. - (d), 7. -(a), 8. - (d), 9. -(d), 10.-(a), 11. -(a), 12. - (c), 13. -(d), 14. - (a),. 15. -(a), 16. -(b), 17. -(a), 18. -(a), 19. -(d), 20.-(c).

Chapter 7

Secondary Culture: Transformed Animal cell

Secondary Cell Culture :

The cells in a primary culture multiply and form a confluent layer. When subculturing is done from the primary cell culture it is called secondary cell culture. This is accomplished by removing the bathing medium first from the primary culture bottle and the cell layer is washed twice with phosphate buffer saline (PBS) without calcium and magnesium. The PBS fluid is removed from bottle. Then buffered salt solution having 0.25% trypsin is added to cover the surface of the cell layer. Trypsin will break both the bonds holding the cells together and those involved in attachment to the surface. The resuspended cells are centrifuged, trypsin solution decanted and cells are inoculated in another tissue culture bottles containing growth medium. Cells first attach to the surface of the bottle and starts growing until confluent sheet of cells is formed.

When the cells are more fragile, instead of trypsin, solution of versene (1:5000) is sometimes used to cover the surface of cell layer. The cultures are placed in the incubator so that the versene washes over the cell layers and the bottle is shaken at about 5 minutes intervals until the cells are completely dispersed in the fluid. The cell suspension along with the versene is collected and centrifuged at 600 rpm for 5 minutes in the ordinary centrifuge as refrigeration of the centrifuge is unnecessary at this stage. After centrifugation supernatant is removed and the cells are resuspended in a growth medium. Screw capped bottles may be seeded by adding 10 ml. growth medium containing 1, 00, 000 cells per ml to each bottle and are incubated at 37^0C. The confluent monolayer of cells grow within 3 to 5 days time and then ready for use.

Cell Line :

When primary cell culture is subcultured successfully at the first time a cell line arise. The cell line cultures consist of lineages of cells originally present in the primary culture. Since the primary culture has heterogeneous population, the cell line arising from this may also have heterogeneous population.

Cell Strain:

A cell strain can be derived by selection or cloning of cells having specific markers or properties from a primary cell line or an established cell line. Cell strain is defined as a population of cells derived from animal tissues subcultured more than once *in vitro* but lacking the property of indefinite serial passage while preserving the chromosomal karyotype characterizing the tissue of origin. Human cells cultured *in vitro* can retain the diploid karyotype through 50 transfers over a period of eleven month, when the cells degenerate and die.

Transformed or Immortalized Cell Lines (Continuous Cell Line) :

After several sub culturing or passaging, cells that undergo phenotypic and genotypic change and develop the capability of an unlimited number of cell division *in vitro* as long as they are supplied with nutrients are called transformed cell. Such cells are subcultured indefinitely and are known as continuous immortalized cell lines. Senescence is a dominant trait and immortalization is due to genetic mutation. Examples of continuous cell lines are HeLa cells obtained from a human carcinoma and CHO (Chinese Hamster Ovary) etc.

Characteristics of transformed and immortalized cell line (Continuous cell line) are :

- Altered Cytomorphology - smaller cell size, more round, higher ratio of nucleus to cytoplasm, less adherent.
- Increase growth rate - Population doubling time reduced from 36-48 hrs to 12-36 hrs.
- Reduction in anchorage-dependence
- Reduced serum and growth factor dependence.
- Increase heteroploidy (chromosome number varies from cell to cell) and aneuploidy (divergence from euploid donor).

- Cells grow in multi-layers, grow more tightly, i.e., no contact-inhibition.
- Increase in cloning efficiency.
- Increase in tumorogenecity.

Disadvantages of Continuous Cell Line

- The process of generating cell line involves selection.
- These cells have reduced cell size, higher cloning efficiency, increased chromogenecity and variable chromosomal numbers.
- Cells of continuous cell line usually have more chromosomes than the cells *in vivo* from which they arose and their chromosome compliment may undergo contraction in culture. The culture is said to be heteroploid, i.e., having inappropriate number of chromosomes and cells of such cultures are obviously mutants.

Established Cell Line:

Established cell line is defined as a population of cells that may be grown *in vitro* for an indefinite period, the chromosome number is different from the number characterizing the source of cells. An established cell line is characterized by its heteroploid karyotype. Established cell lines can arise in diploid cell culture by a process called transformation. In the culture, a single altered cell divides and multiplies forming a cluster/focus of cells. These heteroploid cells grow rapidly and eventually replace the diploid cells. Transformation may occur in three ways:

- Spontaneous transformation from diploid to heteroploid.
- Transformation of diploid cells in presence of a carcinogen, ionizing radiation, alkylating agents.
- Transformation due to infection with certain viruses e.g., EBV.

There are rare instances where established cells are diploid. For example, cells of BHK 91 (Baby Hamster Kidney fibroblast cell line 91 described by Macpherson & Stocker 1962) are diploid and capable of growing apparently for indefinite period. The difference between cell strain and cell line has been depicted in Table-4.

Table:-4. Difference between Human Cell Strain and Cell Line

Character	Cell Strain	Cell Line
Chromosome number	Diploid	Heteroploid
Sex chromatin	Retained	Not retained or variable
Histotypical differentiation	Partially retained	Not retained
Growth in suspended culture	Unsuccessful	Generally successful
Pathological criteria for malignancy as determined by biopsy of cells inoculated into hamster or human terminal cancer patient	Negative	Positive
Limitation of cell multiplication (life of strain or line)	Limited	Unlimited
Spectrum compared to corresponding primary tissue	Same	Often different
Cell morphology compared to corresponding primary tissue	Same	Characteristically different
Acid production	More than that produced by equal number of cell line cells	Less than that produced by equal number of cell strain cells
Ease of establishment	Usually successful	Difficult

The difference between normal and transformed cells have been present in Table-5.

Table. 5. Difference between Normal and Transformed (cancerous cells)

S. N.	Properties	Normal Cells	Transformed Cells
1.	Anchorage	Have anchorage property and thus stick with glass surface	No anchorage property and thus floats in the medium.
2.	Contact inhibition	Present and thus forms monolayer sheet.	Absent thus piling formation takes place.
3.	Ability to bind Ca^{++}	Present, thus cells bind together.	Absent, thus have invasive property and migrates extensively (Coman *et al.* 1955).
4.	Requirement of serum	Requires more serum i.e., 10-20% (6-12 mg protein/ml)	Low serum requirement as it produces a factor similar to migration inhibition factor present in the serum.
5.	Cell densities	Lower cell density	Higher cell density.
6.	Agglutination by Plant lectin	Less agglutinable, but mild treatment with proteases makes them agglutinable.	Agglutinated by plant lectin.

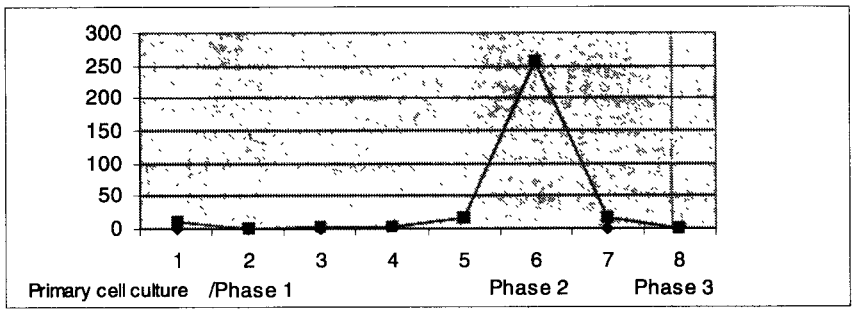
Formation of cell strain and phenomena of cell alteration after several subcultivation has been shown in Fig. -6.

Cumulative Number of Cells:- It is calculated as if all cells derived from the original culture had been kept at transfer.

Cell transformation can occur due to :

- Oncogenic viruses
- Radiation
- Certain chemicals.

Occur in serial growth of cell without evident exposure to any agent. Radiation and chemicals cause transformation by inducing mutation.



Phase 1 or the Primary culture :- terminates with the first confluent sheet

Phase 2 :- It is characterized by luxuriant growth necessitating many subcultivations. Cells in this phase are termed cell strains. An alteration may occur at any time giving rise to cell line where potential life is infinite. Cell strains enter Phase 3

Phase 3:- Here cells are lost after a finite period of time.

Fig. 6:- Diagrammatic Representation of the History of Cell Strain and the Phenomenon of Cell Alteration.

Properties of Transformed Cells :

A. Cultural

- Increased culture thickness.
- Random cell orientation.
- Increased saturation density.
- Decreased serum requirement.
- Increased efficiency of clone formation.
- Increased edge indentation and refractility.
- Decreased anchorage dependence.

B. Cell Surface

- Decreased large external transformation - sensitive (LETS) protein causing disorganization of cytoskeleton.
- Shorter gangliosides.
- Reduced glycosyl transferase activity.
- Reduced adhesion to plastic.
- Increased receptor mobility (Lectins).

- Increased agglutination by lectins.
- Loss of receptors for hormones and toxin.
- Increased and unregulated transport activity (e.g., glucose, phosphate, nucleosides etc.).
- Increased glucose binding protein.
- Presence of new antigen.

C. Biochemical Characteristics

- Increased protein production.
- Disaggregated microfilaments and myosin.
- Increased aerobic glycolysis.
- Decreased intracellular concentration of cyclic AMP.
- Increased abnormal collagen synthesis.
- Resurgent fetal function.

D. Other Characteristics

- Production of tumor by10^6 or fewer cells inoculated subcutaneously in immunologically accepting animals (syngeneic or nude mice).
- Formation of chromosomal abnormalities.
- Absence of aging.

Not all transformed cells have all the characteristics listed above. Some kind of transformed cells are malignant. Common properties of malignant cells are

- Loss of anchorage dependence and protease production.
- Reduction of LETS.

State of Differentiation of Cells in Culture

Animal cells in culture retain at least in part the state of differentiation they had in animal but it may not be easily recognized. Cells are so usually identified by descriptive names such as fibroblasts or epitheloid that bears little relation with cells of origin. However specific products can sometimes be formed, e.g., collagen from fibroblasts, casein from mammary gland cells, specific hormones from pituitary cells. A differentiation potential may also be expressed *in vitro*. Skin keratinocyte differentiate into squamous cell and 3T3 cells (skin fibroblasts) into fat cells.

Cloning of Animal Cells

For specific studies it is desirable to obtain a pure line of cells and the best way to ensure this is to grow the line from a single cell. The first technique of cloning was given by Sanford *et al.* (1961). Clones can be obtained from most cell strains or cell line by transferring cells to a new culture at a very high dilution. For cell line, the proportion of cells which survive and give rise to colonies (efficiency of plating) approach 100% but for primary cultures and cell strains it is low. However, this can be increased by a feeder layer of similar cells made incapable of multiplication by x-ray irradiation or mitomycin as these cells are still metabolically active and supply factors that enable the unirradiated cells to survive and multiply. The efficiency of plating can also be increased by introducing individual cells into very small volume of medium (as in a sealed capillary tube or in small drop of medium surrounded by paraffin oil).

Aging

Culture of cells obtained from animals undergoes some kind of cellular *senescence* and finally dies. Culture senescence is absent in transformed cells. Aging *in vitro* is related to aging *in vivo*. Fibroblasts obtained from human donors of increasing age show decreasing life span *in vitro*. Fibroblasts from patients with Werner's syndrome (premature aging) have a shortened life span and undergo fewer generation *in vitro*.

Culture senescence is due to:

- Accumulation of unrepaired damage in cellular constituents.
- Accumulation of mutations in DNA lowers the growth ability of cells and finally death. Accidental error in translation causes changes in proteins that can influence information transfer such as aminoacyl-t RNA synthetase, and DNA & RNA - polymerases may also cause progressive deterioration in cellular proteins even independent of mutation.
- The key to senescence may be absence of *efficient selection* against cumulative damage, because the cultures of untransformed cells required seeding at a relatively high cell density and the growth of the cells soon become limited by density-dependent growth inhibition.
- Another factor, which also determines the life span in vitro, is *terminal differentiation*. The human keratinocytes on 3T3 feeder

layer normally survives for about 50-cell generation and at the end they differentiate into squamous cells, die. If the culture is grown in the presence of epidermal growth factor (EGF) their lifespan increases to about 150 generation, apparently because differentiation is delayed. Transformation may prevent aging because it prevents terminal differentiation.

Practice Assignment VII

1. In secondary cell culture:
   (a) Cell is cultured directly from intact animals
   (b) Cell is cultured from primary cell culture
   (c) Both of the above.
   (d) None of the above.
2. Established cell line:
   (a) Grow for an indefinite period
   (b) Can grow only for a definite period
   (c) Both of the above
   (d) None of the above.
3. Established cell line has:
   (a) Altered cytomorphology
   (b) Reduction in anchorage dependence
   (c) Reduced serum dependence
   (d) All of the above.
4. Cell strain:
   (a) Cannot multiply indefinitely
   (b) Preserve the chromosomal karyotype of parent cell
   (c) Both of the above
   (d) None of the above.
5. All the statements for suspension culture is true, Except :
   (a) It adheres to the substrate
   (b) It is easy to propagate
   (c) Does not require dilution with media
   (d) Cell culture from blood, bone marrow remain in suspension.
6. Adherent cell culture :
   (a) Is anchorage dependent
   (b) Subjected to contact inhibition
   (c) Both of the above
   (d) None of the above.

7. In contact inhibition:
   (a) There is inhibition of cell motility and mitotic activity of cell
   (b) It occurs only when cells come in contact
   (c) Both of the above
   (d) None of the above.
8. Normal cells:
   (a) Have anchorage property (b) Show contact inhibition
   (c) Both of the above (d) None of the above.
9. In established cell line:
   (a) Cells grow in multiplayer
   (b) There is increased cloning efficiency
   (c) There is increase in tumourogenicity
   (d) All of the above.
10. Transformed cells:
   (a) Have increased saturation density
   (b) Have increased receptor mobolity
   (c) Have chromosomal abnormality
   (d) All of the above.

Answers:

1. - (b), 2. - (a), 3. - (d), 4. -(c), 5. - (a), 6. -(c) 7. - (c), 8. - (c), 9. - (d), 10.- (d).

Chapter 8

Commonly used Cell Lines and their Uses

For the first time the need of cell culture was felt for the growth of different viruses for their studies but now-a-days various mammalian cell cultures are used for the production of biologically active substances in large scale. Until the mid 1840, the technique for vaccine production was in infancy. Negri developed small pox vaccine by collecting skin of calves or sheep affected with small pox as source of virus. An important breakthrough in cell culture technology came in 1950 when chicken embryo and tissue culture paved the way for vaccine production. With the advancement in tissue culture technology, use of continuous cell line have taken a long strides in evolving different cell lines for various uses like bulk vaccine production, growth of different viruses, to study the gene expression, anti cancerous agents, production of different biologicals and pharmaceutical agents and other drugs which may have cytotoxic effect. Though the preferential use of cell culture of bovine, canine and swine origin is undoubtedly more accepted for homologous vaccine production, yet the use of heteroploid line from normal healthy and free from oncogenicity, have been used because these grow faster, give more cells and can also be grown in suspension. The cell line MA104 (derived from rhesus monkey kidneys) has been proved to be an efficient cell line for cultivation of rotavirus. Various cell lines help in the study of pattern of release of different microorganisms, e.g., secondary chicken embryo fibroblasts infected with *Rickettsia prowazkii* are packed with rickettsia 72-96 hours after infection, at which times some of the cells suddenly break up. Release continue for many hours where as in *Rickettsia rickettsia* as early as one hour of infection intact cells begin to release infectious rickettsia. Rickettsia fail to accumulate in host cell cytoplasm, there is a substantial build up of rickettsia in the growth medium and there is rapid spread of initially uninfected cells (Wisseman *et al.* 1976). Some of the cell types along with their important properties for the expression of heterologous proteins have been depicted in Table-5.

Table:-5. Different Cell Types with Their Properties for Heterologous Protein Expression.

Cell types	Properties
Chinese Hamster Ovary cell (CHO)	Express tissue plasminogen activator (tPA),Factor VIII, β and γ interferon.
Chicken embryo fibroblast	Vaccine for FMD, influenza, measles and mumps.
Calf kidney MDBK (Madin Durby Bovine Kidney cell)	Vaccine for IBR (Infectious bovine rhinotracheitis).
Dog kidney cell line	Canine distemper vaccine.
Duck embryo fluid	Vaccine for rabies and rubella.
Erythrocytes cell line	Expresses haemoglobin.
Human mammary epithelial	Expresses milk proteins.
Human kidney	Express urokinase.
Human leukocyte	Production of Interferon.
Mouse embryo fibroblast	Expresses fibronectin.
Myoblast	Expresses muscle cells.
Primary pig kidney (PK-15)	Hog cholera vaccine.

Different cell lines developed from normal and neoplastic cells of human origin, cell line from normal and neoplastic animals and mammalian cell lines excluding human and marsupial have been listed in Table-6.

Table:-6. Different Cell Lines of Normal and Neoplastic Origin of Man and Animals.

Cell Lines of Normal Human Origin

Tissues	Cell strain	References
Amnion	FL-E	Fogh & Lund (1957)
Amnion	ND-M	Fernandes (1958)
Amnion	A1 to A5-E	Zitcher & Dunneback (1957)
Appendix	Chang E	Chang (1954)
Conjunctiva	Chang E	Chang (1954)
Oesophagus	ND-E	Syverton & McLaren (1957)
Foreskin	Fs4-F	Swin & Parker (1957)
Heart	ND-E	Giradi *et al.* (1958)
Intestine	407 E	Henle & Deinhardt (1957)
Kidney	Chang E	Chang (1954)
Liver	Chang E	Chang (1954)
Liver 407 E, R, E		
Tonsil	T16-E	Frans (1957)
Uterus	U12-F	Swim & Parker (1956)
Nasal mucosa	DMB-E	Jordan (1956)

[HeLa cell line has 70 to 80 chromosomes as compared to normal chromosome number which is 46]

Neoplastic Cells of Human origin :

Carcinoma (Pharynx)	HEP-2E	Moore *et al.* (1955)
Carcinoma (larynx)	HEP-2I	Moore *et al* .(1955)
Adeno carcinoma (lungs) LAC-E	Maben	Berman *et al.*(1957).
Lymphosarcoma (lung)	Detroit-116P	Seegmiller *et al.*(1967).
Carcinoma (lymph node)	HEP 3-E	Moore *et al.* (1955)
Carcinoma (mouth)	KB	Syverton And McLaren (1957)
Reticulo endothelial	H.Em	Moore *et al* .(1955)

Normal Animal Cell Lines:

Bovine kidney	RB1-E	deBrion & Gruest (1957)
Bovine kidney	MDBK-F	Madin & Darby (1958)
Bovine Embryo - Diaphragm	ND-E	Warren & Cochins (1957)
Pig Kidney	PK-15	Stice (1955).

Neoplastic Animal Cell Lines:

Carcinosarcoma of rat	Walker 256	McCoy & Neuman (1956)
Sarcoma of mouse	S- 180	Foley & Droleh (1956)
Myeloma of mouse	MPS-11	Laskov and Scharff (1970)
Lymphosarcoma of mouse	MB-111	deBruyn and Gey (1952).

Mammalian Cell Lines (excluding human and marsupial):

Chinese Hamster ovary	CHO	Kao and Puck (1968)
Baby Hamster Kidney	BHK 21	Macpherson and Stoker (1962).
Hamster lung	C, P and V	Ford and Yerganian (1958).
African Green Monkey Kidney	BSC1 (VERO)	Hopps *et al.*(1963).
Guinea Pig Heart	CSL 212	
Guinea Pig Lung	CSL 213	
Chinese Hamster lung	DON	
Rabbit Skin	CSL 218	
Rabbit Kidney	RK 13	
Rabbit Cornea	SIRC	
Mouse lymphosarcoma	MB-111	deBruyn and Gey (1952)
Mouse (Swiss) embryo	3T3	Todaro and Green (1963).

Genetically Homogenous Cell Strain:

1. Pure mammalian cell strain designated 'L' was clonally derived by Sanford *et al.* (1948) from an established fibroblast strain originally explanted from subcutaneous tissue of a C3H mouse.
2. Strain HeLa cells - a stable strain explanted from an epidermoid cervical carcinoma by Gey (1952) and established continuous cell culture used cultivation for Polio virus.
3. KB cell line from human epidermoid carcinoma tissue by Eagle (1955).
4. Maben cells :- Transfer to glass of cellular sediment from a patient with metastatic pulmonary adenosarcoma by Friesh *et al* (1955) yielded a strain presumably malignant human epithelial cell (Maben)(Berman *et al.* 1957). Maben cells were resistant to low pH and sensitive to alkalinity and lipemic serum. This can be successfully preserved at -70^0 C derived from malignant human epithelial cells.
5. H. Ep#1 from epidermoid cervical carcinoma - a stable strain derived by Sabachewsky & Toolen (1955) and carried in irradiated and corticonized rats.
6. H. Ep#2 derived from plasma clot culture of pharyngeal epidermoid carcinoma by Fjeldo (1955).
7. H. Ep#3 derived from lymph node invaded by metastasis from buccal epidermoid carcinoma.
8. H. Emb. Rh#1 derived from embryonal Rhabdomyosarcoma.
9. Detroit cells:- Berman *et al.* (1955) isolated Detroit strains of human cells. These were Detroit 6, 32, 34, 52, 98, 30A, 56 A and 116P. The Detroit - 6 strain is epithelial like cells arose in bone marrow culture after 51 days of incubation as cellular plaque among proliferating fibroblasts. Detroit 32 and 34 from culture of sternal marrow of patients having primary and metastatic carcinoma. Detroit 52 and 98 from culture of sternal marrow from patients having malignant disease. Detroit 30A and 56A from direct culture of carcinomatosis peritoneal fluid. All eight cultures were used for growing viruses Adeno, ECM, ND and Polio viruses.
10. DMB and Dhov are stable cell strains from human nasal mucosa derived by Jordan (1956).
11. A. F1 Human fibrinosarcoma strain.

Use of Different Cell Culture for Vaccine Production:

In 1949 Enders *et al.* started new era when they showed that poliovirus could be grown in non-nervous cell with the production of cytopathic effect. First biotechnological application of animal cell culture started with the development of poliovirus vaccine in the Salk Institute in 1954, using mouse kidney cell culture. Hayflick and Moorehead (1961) opened new era for the production of mumps, measles and rubella vaccine by using human diploid cell. BHK-21 cell lines has been grown in suspension upto 1000 litre tanks resulting in higher yield of cells and viruses and thus is more economical for production of vaccines. Sharma *et al.* (1985) used BHK-21 cell line for isolation of FMD virus. Now a days cell culture is being used in recombinant DNA technology in vaccine development and use of naked DNA as viral vaccine. Dog kidney has been used for th reproduction of canine distemper vaccine whereas cow kidney cell culture has been used for FMD vaccine production. There are several cell lines which are being used for vaccine production. The details of various vaccines expressed in mammalian cell culture has been depicted in Table-7.

Table-7: Vaccine Expressed in Mammalian Cell Culture

Sl. No.	Gene Expressed	Cell Culture System	Expression Vector
1.	HBsAg	Mouse transformed cell	Bovine papilloma virus
2.	HSVgD & 9B	CHO cell line	DHFR amplified vector
3.	EBVgP40	C-127 cell line	Bovine papilloma virus
4.	HbsAg - PreS1	CHO cell line	DHFR amplified vector
5.	HIVgp160	- do -	- do -

Application of Large Scale Animal Cell Culture:-

The following mammalian protein currently in demand are not available in bulk to meet the requirement of suffering. For this relevant gene is selected and cloned in mammalian cell culture rather than the microbial cells. So, this is one of the important application of cell culture. The details of some useful proteins obtained through cell culture along with their application has been presented in Table-8.

Table-8: List of Proteins Produced in Cell Culture along with their Application.

Sl.No.	Protein	Application
1.	Human growth hormone (Somatotropin)	Pituitary dwarfism
2.	Human insulin	Diabetes
3.	Interferon α 2a	Hairy cell leukaemia, Kaposi's sarcoma
4.	Interferon α 2b	Hairy cell leukaemia & genital warts
5.	Erythropoitein	Anaemia associated with kidney dialysis.
6.	Tissue plasminogen activator	Myocardial infarction.
7.	Hepatitis B coat protein	Vaccination.
8.	Granulocyte colony stimulating Factor	Neutropoenia arising from cancer therapy.
9.	Interleukin-2	Cancer therapy.
10.	Interleukin β	Aids therapy.
11.	Interleukin γ	Rheumatoid arthritis & cancer therapy
12.	Superoxidase dismutase	Free radical damage or reperfusion renal transplant.
13.	Factor VIII	Haemophilia.
14.	Tumor necrosis factor	Cancer therapy.
15.	Lung surface protein	Respiratory distress syndrome
16.	Epidermal growth factor (EGF)	Healing of ulcer.
17.	Fibroblast growth factor (FGF)	Healing of ulcer.
18.	Relaxin	Facilitation of child birth.

Some animal cell cultures have served as surrogate models for whole organisms in identifying molecules that effect cell commitment to particular types of differentiation. Some of them are as follows:-

Chinese Hamster Ovary (CHO) Cells Line:- This has been derived from adult Chinese hamster ovary. The cell are fibroblastic, which are normal. These have diploid number of chromosomes. These have been used for the generation of Tissue Plasminogen activator (tPA) and Erythropoietin (EPO). Human blood clotting factor VIII has also been prepared in CHO cell line and are being presently used to cure specific cases of haemophilia.

BHK Cell Line:- Has been derived from embryonic Syrian hamster kidney cell. The cells are normal and show aneuploidey. The cells are fibroblastic cell in morphology. This has been used for the expression of hepatitis B surface antigen (HbsAg) and interleukin-2.

HeLa Cell Line:- Derived from adult human cervical tissue. These cells are neoplastic and show aneuploiedy. Gene expression by RNA viruses was studied in the polio infected Hela cell.

W138 :- This is an embryonic human fibroblast cell line derived from normal cells from lungs. These cells are diploid and used for surface adherence property of normal and malignant cell membrane.

There are many application of cell cultures, which are as follows:-

(i) The cell culture technique has provided us many viral vaccines.

(ii) It has provided greater insight into the understanding of neoplast and cancer research.

(iii) Human growth hormone, insulin, interferon and some recombinant viral vaccines can be produced by exploiting cell culture technology.

(iv) Cell culture has enhanced our understanding of cell interactions and intracellular control mechanism in cell differentiation and development.

(v) Cell culture can be used to study the toxic effects of pharmaceutical compounds and potential environmental pollutants and toxins.

(vi) It has become important tool to study apoptosis, location, trafficking and function of gene products.

(vii) The successful demonstration that cultured epidermal cells may form functionally differentiated sheets and endothelial cells may form capillaries has opened up new vistas in homografting and reconstruction surgery using an individual's own cells.

(viii)The somatic cell cloning has demonstrated the possibility of cloning the entire animal/human.

(ix) Expression of heterologous genes coding for proteins of interest in an appropriate system of expression has emerged as a powerful tool for large scale production of proteins for diagnostic, immuoprophylactics purposes. For biological and functional studies, large amounts of proteins are required, which is facilitated by bacterial expression system and *Escherichia coli* was found to be the most favoured organisms for expression of many

proteins because of ease of genetic manipulation, high rate of cell growth and cost effective cultural condition.

(x) Productive virus infection in cell culture is done as some viruses may cause transformation without giving productive infection.

MEL- Cell Line :- Derived from murine erythroleukaemia and is model system for erythroid differentiation.

K-562:- It is a human erythroleukaemia cell line used to express both erythroid or mega-karyocytes.

Pc-12 Cell Line:- It is useful model system to investigate the molecular mechanism underlying neuronal differentiation and has been extensively used for the study of molecular events that underline the biological actions of nerve growth factor.

Neuro 2-A Cell Line :- It has been derived from mouse neuroblastoma. Using this cell line Omura and his co-workers discovered lactacystin by cell basal screening. Lactacystin is a streptomyces metabolite that induces differentiation of Neuro 2-A cells. It causes a transient increase in the intracellular c AMP level and morphological changes including neuritogenesis in Neuro 2A cells (Tai *et al.* 1992).

Cells required for the large scale production of different preventive and therapeutics (Table. -7) has been mentioned below:-

Table. 9: Product and Cell Requirement.

Product	Cell Required/Dose	Culture Volume Required/Dose
Vaccine.		
Polio	$2x10^4$	0.0001
Rabies	$4x10^8$	0.005
FMD	$2x10^7$	0.01
HSV	$2x10^7$	0.03
Therapeutics:		
Interferon	10^5/day	0.1
Mab	10^{12}	100
Urokinase	10^{12}	500

Practice Assignment VIII

1. Cell lines are used for the:
   (a) Growth of different viruses (b) Production of vaccine
   (c) Gene expression study (d) All of the above.

2. CHO cell line:
   (a) Has been derived from adult Chinese hamster ovary
   (b) Used for the generation of tissue plasminogen activator (tpa)
   (c) Both of the above (d) None of the above.
3. All the following cell lines are of normal human origin, Except:
   (a) DNBE cell line (b) Hep3E cell line
   (c) Chang E cell line (d) U-12-F cell line.
4. All the following cell lines of normal animal origin, Except:
   (a) MDBF-F cell line (b) ND-E cell line
   (c) Walker 256 cell line (d) RB 1-E cell line.
5. Genetically homogenous cell strain is :
   (a) HeLa cell line (b) KB cell line
   (c) Both of the above (d) None of the above.
6. Maben cell line has been derived from:
   (a) Malignant human epithelial cell
   (b) Epidermoid cervical carcinoma
   (c) Both of the above
   (d) None of the above
7. Animal cell culture are used for large scale production of:
   (a) Different vaccines used in human and animals
   (b) Different therapeutics used for the treatment of human and animal diseases
   (c) Both of the above
   (d) None of the above.
8. H. Ep#1 cell line has been derived from :
   (a) Human epidermoid cervical carcinoma
   (b) Pharyngeal carcinoma
   (c) Human Rhabdosarcoma (d) Human buccal carcinoma.
9. DMD cell lines are stable cell strain derived from:
   (a) Human fibrinosarcoma (b) Human nasal mucosa
   (c) Uterine carcinoma (d) Carcinoma of tonsil.
10. All the cell lines mentioned below have been derived from human neoplastic cells, Except:
   (a) HEP-2E (b) LAC-E
   (c) MDBK-F (d) HB-E.

Answers:

1. -(d), 2. -(c), 3. - (b), 4. - (c), 5. -(c), 6. -(a), 7. -(c), 8. -(a), 9. -(b), 10.-(c).

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Chapter 9

Growth Kinetics of Cells in Culture

Growth of a cell is defined as an orderly increase in its all components, commonly associated with multiplication. It is the direct result of substrate uptake. Growth kinetics is the science that relates growth rates to the nutritional concentrations on which the cells depend. It can be divided into two processes, firstly, the kinetics of concentrated-limited accumulation of nutrients and secondly, the efficiency that those nutrients provide for growth. The determination of kinetics of growth cycle is important in designing routine subculture and various experimental protocols. At each stages of growth there are changes in the cell behaviour and biochemical constituents. The growth in close system is observed by the rate of proliferation of a population and in open system growth is observed by the rate at which a population can be removed without disturbing the steady state or by injected trace movement through it. The shape of the growth curve can also give information on the reproductive potential of the culture where differences in growth rate, adaptation or survival and density limitation of growth.

Population of animal cells cultured *in vitro* increase in number as the individual cell divides mitotically whether they are primary cells, cell strains or established cell lines. Multiplication starts only after a period of adjustment and stops when a number is reached that is saturating for the system. These phases are best studied by representing it graphically. If the logarithms (log) of the number of cells present at any time in the culture are plotted along the ordinate against the time interval in hours along the abscissas, a sigmoid growth curve characteristics of the culture system is obtained. (Fig. :-6) The growth curve of mammalian cell culture display the same type of growth pattern as microorganism and thus similarly the growth curve can be divided into four phases – Lag phase, Log phase or Exponential phase, Stationary phase or Plateau phase and Decline phase.

(a) Lag phase : Is the first stage of growth curve and is defined as the time taken by the cells to adopt itself to grow in fresh environment in the culture medium, till the onset of log phase. After culturing, the cell population decreases slightly probably because while attaching to the solid surface some cells are lost due to incapability of adherence or other factors. Later, the cells adapt itself in the new growth medium and cells try to adjust to new conditions like new medium, serum concentration, type of culture vessels/ surface and cell density etc. These factors determine the length of lag phase, however, it may vary from 24 to 48 hrs. There may be increase in cell size but practically there is no division of cells in this phase. If the cells are cultured initially at too low density these never enter into log phase. In general, the duration of this phase is long but vary according to the following conditions:-

(i) When the inoculum of the cells are small, duration is longer.

(ii) If the medium and temperature are unfavourable, generation time of lag phage prolongs.

(iii) If the inoculum is from lag, stationary or decline phase lag period is long. On the other hand, cells multiply without lag phase if taken from log phase. In general young culture shortens lag phase.

(b) Log phase or Exponential phase- Lag phase is followed by Log phase and it is a sort of rejuvenescence prior to multiplication, ordinarily $1^1/2$- 3 hours in bacterial cells and in fast growing population is doubled every 15 to 20 hours. During this period regular and maximum multiplication of cell occurs. Here, first cell is enlarged 2to 5 times than normal cell and divide fast. Number of cells increase exponentially and lasts for 2-8 days. Here change in biochemical and respiratory activity of cells also occur. In this phase the medium is rich in nutrients, space for growth is enough and thus there is neither competition for nutrients nor contact inhibition. The population doubling time for cultured cells ranges between 12 to 36 hrs. Since culture is started with 50, 000 to 200, 000 cells, 4 to 5 population doubling during the culture cycle. A proportion of cells stop dividing when the essential nutrient is depleted or an inhibiting substance is produced. At this stage cells enter into plateau phase.

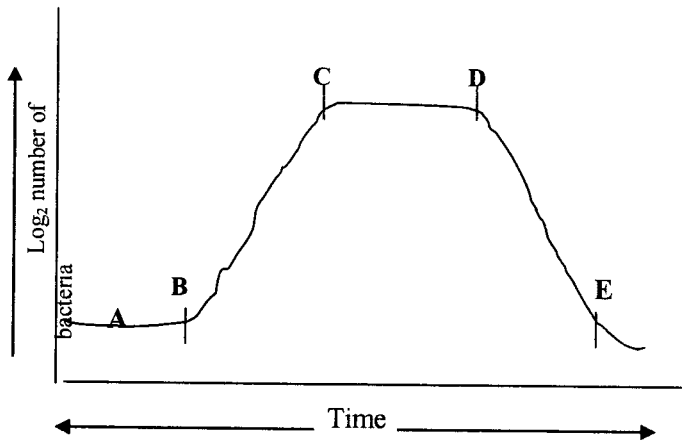


Fig. 6. Showing the Different Phases of Growth Curve.
A - B : Lag phase, B - C Log phase : C - D : Stationary phase: D - E : Decline phase.

Doubling Time :- As growth is constant the interval between the doubling the cells is known as doubling time and can be represented by (td). Thus, the amount of cell present after time t (X_t) can be related to the initial cells (X_0).

$$X_t = X_0 2^n \quad \text{(i)}$$

Where n= number of doubling after "t" time. This can be represented by the time t divided by the time it takes to double t_d, the doubling time.

$$N = t / t_d \quad \text{(ii)}$$

Taking logarithms:

$$\ln(X_t / X_0) = \ln 2 \frac{1}{t_d} \quad \text{(iii)}$$

$$\frac{\ln X_t / X_0}{t} = \frac{\ln 2}{t_d} = 1/t_d \cdot \frac{\log 10^2}{\log 10^e} \quad \text{(iv)}$$

$$1/t_d\, 2.303 \log 10^2 = 1/t_d\, 2.303 \times 0.302 = \frac{0.693}{t_d} \quad \text{(v)}$$

(c) Stationary phase or The Plateau phase :

In he plateau phase due to exhaustion of available nutrients and limiting of growing space as cells approach confluence, rate of division

slows down and thus this phase has a constant number of cells. At this stage the maximum number of cells that can be grown per unit volume of the medium can be determined. The saturation density represents the density at which the cells can no longer grow exponentially and it will vary depending upon the conditions used. This prevails for sometime because either cell division ceases in all cells or some cells degenerate and die and others continue to divide.

The phenomenon of cell growth is related to synthesis of macromolecules, i.e., DNA, RNA and proteins. DNA being genetic material must be duplicated before cell division. RNA(s) serve to read the message in the nucleus, translates it and enable protein synthesis in cytoplasm. Proteins make up more than 60% of cellular dry matter. They serve as structural material and also as enzymes to carry out cellular biosynthetic functions. Synthesis of RNA molecules has been studied in randomly growing log phase population. It has been found that enzyme RNA polymerize synthesize single stranded RNA or DNA template in the nucleus. Two types of RNA molecules called ribosomal RNA (rRNA) and heterogeneous nuclear RNA (HnRNA) are recognized as nuclear products. HnRNA has been termed as chromosomal RNA by Edstron & Davehod (1987). rRNA is synthesized in the nucleolus and is released in the cytoplasm. HnRNA is synthesized in the nucleoplasm. This RNA undergoes another kind of processing prior to release into the cytoplasm as mRNA.

Since it is not possible to analyze the sequential order in a single cell, an idealized objective would be to grow population of cells that are carrying out the same biosynthetic reaction in synchrony. In such a system every cell will divide at the same time and the event occurring at any time in a population would be an amplified version of the reaction occurring in the cell.

This ideal growth condition in animal cell population has not been achieved, but quasi-synchronous growth is possible by physical or chemical manipulation of a randomly growing population. Toyozo Terasima & Leonard Tolmach have developed a simple procedure for selectively isolating dividing cells. Advantage is taken that cells growing on a glass surface round up and are loosely attached during mitosis. Such cells are easily dislodged from the surface by slight agitation. If the dividing cells are collected and cultured they grow synchronously through one division cycle. This study indicates the DNA synthesis is discontinuous and can be divided up to cell cycle.

The mitotic (M) phase occupies 3. 0% of cycle, a post-mitotic phase (G1) follows up to 41% of the time, a DNA synthesis phase(s) occurring next take up to 46% of time and finally a pre-mitotic (G2) phase occupies 11% of the cell cycle.

Decline phase:- In this phase there is rapid decline in the number of cells as division of cells practically ceases as nutrients are completely exhausted and there is accumulation of metabolites.

Several workers have tried to find out the standard growth curve of cell in cell culture. In one of the study of standard growth curve of skin fibroblast in cell culture, growth curve was fitted to check the optimal cultural conditions. For this, 16 representative cultures were seeded into 60 mm^2 petriplates at a concentration at a concentration of 40, 000 cells in each for a period of 8 days. One petriplate from each set was harvested using normal procedure exactly after every 24 hours and cell count was made The data collected in 8 days on cell count was plotted as growth curve (Fig:- 6).

Metabolism, Metabolites during Growth Kinetics of Cell:

During the growth of cells there are also Trophophase and Idiophase. Trophase occurs during primary metabolism whereas Idiophase occurs during secondary metabolism.

Trophophase :- The term trophophase was used to describe the log or exponential phase of a culture growing on an artificial media in which all nutrients are provided in excess amount. Cells grow at an exponential rate. Cell will have optimum content of all various macromolecules of the cell such as DNA, RNA, proteins, lipids etc., but their proportion will change as growth processes and then slows down. During growth they break the high molecular carbon and energy sources through the action of catabolic enzymes. The end product of catabolism are reassembled to form primary metabolites. Even when growth ceases their metabolism does not ceases, it ceases only when cell dies. The sole products produced during metabolism are either essential for growth such as aminoacids, nucleotides, proteins, nucleic acid, lipids and carbohydrates or the byproducts or energy yielding catabolism such as ethanol, acetone or butanol. . The metabolites produced during trophophase is known as primary metabolites. Kinetics of growth of trophophase, idiophase and production of metabolites has been depicted in Fig. - 7.

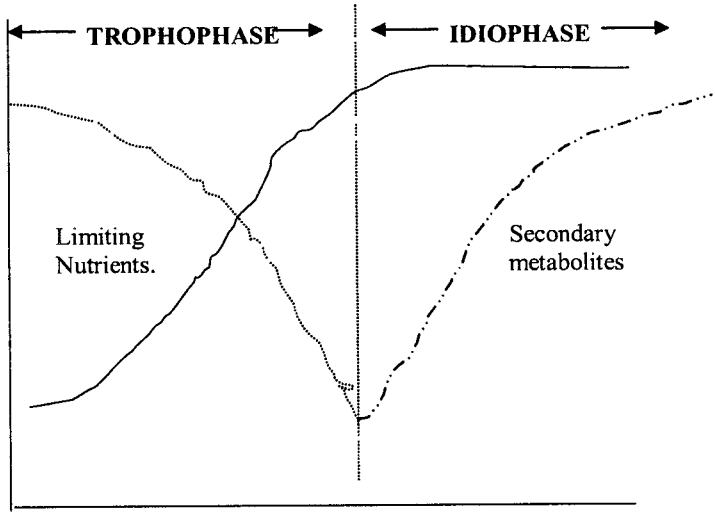


Fig:- 7. In the Initial phase (balanced growth = Trophophase) all Nutrients are in Excess, when Nutrients are Consumed (.....) Cell Growth (_____) Slows Down and Secondary Metabolites (— ·· — ·· —) are Formed in the Idiophase.

Idiophase:- This is the phase of the culture during which active multiplication has ceased, cell diverts its core metabolites into products other than the primary one which are not needed in abundance by the growing cell. These products are called secondary metabolites. The definition of secondary metabolites is rather vague in contrast to carbohydrate, lipid and protein or nucleic acid as these are not present in every cell. These are of no importance as a source of energy or as reserve substance and these are only slowly metabolized originating from sideways of the primary metabolism (Budzikiewicz 1993).

The biosynthesis of secondary metabolites is closely interrelated to the primary metabolism of the producing cells. These products are synthesized by slow growing or non-growing cells, which play no obvious role in cell growth and proceeds from the uptake of nutrients into the cell through intermediary metabolism to specific pathway for secondary metabolism. The various pathways involved in secondary metabolism are closely connected to the catabolism, amphibolic and anabolic pathway of primary metabolism. Precursor of secondary metabolites are normal and modified primary metabolites (Drew and Demain 1977). The products of secondary metabolites are taxonomically limited in their distribution. The various secondary metabolites are antibiotics (penicillin, cephalosporin,

tetracycline), anticancer agents (krestin and bestatin) and immunosuppressive agents (cyclosporin). Amylase produced by *Bacillus steriothermophillus* by idiophase type culture may be considered as equivalent to secondary metabolites (Manning and Campbell 1961). The secondary metabolite produced by neem (*Azadirachta indica*) is tetranortriterpenoids such as azadirachtin-A, nimbin and nimbidin. Different fluorescent *Pseudomonas* sp. produces variety of siderophore (meaning in Greek; Sideros = iron and Phore = bearing) and other antibiotics like phenazines (a heterocyclic compound with broad spectrum antibiotic properties), 2, 4-Diacetyl-phoroglucinol and pyrrlonitrin etc. So, we see that lot of useful secondary metabolites are produced by various organisms during idiophase.

Practice Assignment IX

1. The duration of the lag phase vary according to the following conditions:-
   (a) When the inoculum of the cells are small, duration is longer
   (b) If the medium and temperature are unfavourable, generation time of lag phage prolongs.
   (c) If the inoculum is from lag, stationary or decline phase lag period is long.
   (d) All of the above.
2. In lag phase:
   (a) There may be increase in the size of cell
   (b) There is no multiplication of cell
   (c) Lag phase varies from 24-48 hours
   (d) All of the above.
3. In plateau phase number of cells remain constant because:
   (a) The division of cells ceases
   (b) Some cell degenerate and die
   (c) Both of the above
   (d) None of the above.
4. After log phase population of cells stop dividing, because:
   (a) There is depletion of essential nutrients
   (b) Production of inhibitory substance
   (c) Both of the above
   (d) None of the above.
5. The secondary metabolites are synthesized
   (a) By slow growing or non-growing cells, which play no obvious role in cell growth

(b) It proceeds from the uptake of nutrients into the cell through intermediary metabolism
(c) Both of the above
(d) None of the above.

6. The determination of kinetics of growth cycle is important :
   (a) In designing routine subculture
   (b) In various experimental protocols.
   (c) To study the changes in the cell behaviour and biochemical constituents
   (d) All of the above.
7. During idiophase :
   (a) The active multiplication of culture ceases
   (b) Cell diverts its core metabolites into products
   (c) Both of the above
   (d) None of the above.
8. During trophophase:
   (a) Culture remains in log or exponential phase
   (b) All nutrients are provided in excess amount in culture media
   (c) Both of the above
   (d) None of the above.
9. During idiophase the various secondary metabolites produced are:
   (a) Antibiotics (penicillin, cephalosporin, tetracycline),
   (b) Anticancer agents (krestin and bestatin)
   (c) immunosuppressive agents (cyclosporin).
   (d) All of the above.
10. During trophophase the products synthesized are:
    (a) Used by the cell itself for its growth
    (b) Not used by the cells and remain accumulated in the medium
    (c) Both of the above
    (d) None of the above.

Answers:

1. - (d), 2. -(d), 3. -(c), 4. -(c), 5. -(c), 6. -(d), 7. -(c), 8. - (c), 9. - (d), 10.-(a).

Chapter 10

Gene Expression and Application of Animal Cell Culture for Its Studies

Gene and its Expression :

The term gene was coined by Wilhelm Johansen in 1909 to describe a heritable factor responsible for the transmission and expression of a given biological character, but without any reference to any particular theory of inheritance. The gene with which the cell is originally endowed with, carries the potentialities a cell possesses for developing into an organized functional organism. The aim of gene manipulation is to promote the expression of a gene that has been cloned so as to amplify the synthesis of a desirable gene product. The genes are expressed at different times during growth of the organism and to an extent depend upon the environment. Expression of transferred genes in different expression system is often a prerequisite for cell and molecular biological research. In important biotechnological applications, animal cells are necessary host for the expression of foreign protein, this is often the case where the protein product must be glycosylated in a characteristically mammalian way for therapeutic applications but the different expression system have some advantages and disadvantages. A single expression system cannot meet all the requirements of gene expression. The expression of genetic information of DNA genome has been the focal point of molecular biology. The different commonly used expression system used are:-

(i) Mammalian Cell Expression System
(ii) Bacterial Expression System
(iii) Insect Virus Expression System and
(iv) Yeast Expression System.

How genes express? The DNA of the gene translates its specificity into the specific protein-gene product. When the gene for alkaline phosphatase is introduced by conjugation, the enzyme synthesis begins upon lowering the phosphate concentration in the culture. When the gene is introduced by F-mediated transduction, enzyme synthesis begins immediately after mixing the parent culture. After introduction of the gene for galactose/urease or beta galactosidase by transduction, enzyme synthesis begins following 15 or 30 minutes lag respectively.

The expression of genetic information of DNA genome has been one of the focal points of molecular biology and the expression of heterologous gene coding for protein of interest in an appropriate system of expression has emerged as a powerful tool for large scale production of protein for diagnostic and prophylactic purposes. Gene can be subdivided in to smaller units. Seymour Benzeer introduced the term muton, recon and cistron to define individual unit of mutations recombination and function respectively. Gene controls a cell phenotype by determining which protein the cell can synthesize. Each gene is responsible for the synthesis of a specific polypeptide. Gene works by controlling the sequence of aminoacids in proteins. This is clearly shown by the discovery that many mutations cause the production of protein molecules that differ from normal protein by a single aminoacid replacement. These character can only be studied *in vitro* by gene expression, and thus there is need to study the gene expression. Gene expression can be defined as function of a gene which is expressed. Expression of heterologous gene coding for protein of interest is an appropriate system of expression has emerged as a powerful tool for large scale production of protein for diagnostic and immune prophylactic purpose. It is done by incorporating foreign gene in vector with suitable expression system and inserting it into the different expression vector system to obtain the product. The information contained in the gene is read by protein that attach the genome at the appropriate position and initiate a series of biochemical reaction known as gene expression.

Repressor Gene:- Repressor gene regulates the gene expression in eukaryotic cells. Like other prokaryotic counterparts, eukaryotic repressors bind to specific DNA sequence and inhibit transcription. In some cases, eukarytic repressors simply interfere with bindings of other transcription factor of DNA.

Regulator Gene- Genes are expressed in response to certain internal or external stimuli. Their products are needed only under certain

environmental conditions e.g., lactose grading enzyme.

Operon and Operator Gene - The operon is defined as the genetic unit whose phenotypic function is regulated by the action of specific repressor. Repression is thought to be effected by the action of the repressor substances on a sensitive site genetically specified by an operon gene.

The aim of gene manipulation is to promote the expression of a gene that has been cloned so as to amplify the synthesis of a desirable gene product.

The different gene expression models are as follows:-

(a) Operon model

(b) Tryptophane model

(c) Lac operon.

The gene expression can be used in the production of the cell commercially. The lac operon model has been proposed in this, there is a promoter gene, repressible gene, expressible gene and suppressor gene. Lac operon is the promoting operon model of gene where transcription takes place but tryptophane operon is the inhibitory operon model. The tryptophane operon inhibits the transcription of the protein whereas operon lac promote the transcription.

The ability to express cloned genes in animal cells has been essential for the studies of the regulation of eukaryotic genes. Expression of such eukaryotic genes in a homologous eukaryotic system has the advantage that the expressed protein has been correctly folded and glycosylated, something, which cannot be guaranteed in heterologous prokaryotic expression systems.

The ability to introduce specific fragments of DNA into cultured animal cells has been used:-

- to identify and analyze non-protein coding regulatory elements which control the expression of particular genes
- to analyze the role of specific gene products *in vitro*,
- for the *in vitro* production of proteins for biochemical analysis and
- to study the functionally important domains of many enzymes, proteins and cell receptors

Techniques used to introduce the cloned DNA fragments of interest into appropriate animal cells are:-

- Electroporation - cells are exposed to high voltage pulse for short duration which results in the temporary formation of pores in the membranes through which DNA may be transported into the cells.
- Microinjection of exogenous DNA directly into the cell nucleus (for details refer Chapter- 12).

Gene Regulation and Expression

According to Dr Syed Ehtesham Hasnain, an eminent scientist and recipient of Dr Bhatnagar award, gene expression typically implies the synthesis of the final functional molecule encoded by the corresponding gene. Usually the gene is first transcribed and then translated to produce a protein molecule, which may have a structural or functional role.

Regulation of gene expression ordinarily occurs at the transcription, post-transcription, translation or post-translation levels. Different genes in organism are meant for the synthesis of different proteins and specific proteins are needed at different times in the lifecycle. In other words differentiation of cells and tissues in an organism is the product of differential expression of genes at different stages of growth. All cells have identical complements of genes but the different genes are active in different development phases. The variable gene activity at different timings indicates a regulatory mechanism.

There are three types of genes, from the point of view of regulation of gene expression:

- **Constitutive Genes** - genes whose expression can not be regulated, are expressed continuously, their products are found in the cell almost in the same amount at all the time. e.g., RNA polymerase components
- **Housekeeping Genes** - are genes whose products are constantly needed for cellular activity
- **Regulated Genes** :- *Vide supra.*

Application of Animal Cell Culture in the Study of Gene Expression:

1. **Study of Gene Expression** - The ability to express cloned genes in animal cell has been essential to studies of the regulation of the eukaryotic genes. Expression of such eukaryotic genes in a homologous eukaryotic system has the advantage that the

expressed protein has been correctly folded and glycosylated, something, which cannot be guaranteed to heterologous prokaryotic expression system. The ability to introduce specific fragments of DNA into cultured animal cells has been used to identify and analyze non-protein coding regulatory elements which control the expression of particular genes, to analyze the role of specific gene products *in vitro*, for the *in vitro* production of proteins for biochemical analysis and to study the functionally important domains of many enzymes, proteins and cell receptors.

2. **Biotransformation:-**Biotransformation is generally done to achieve various desired proteins and biopharmaceutical products. So cell culture is one of the major application for the expression of gene.
3. **Production of Secondary Metabolites:-** In the production of secondary metabolites generally the suspension culture and immobilized plant cells are produced by the gene expression in the case of animal and plant cell culture. Various antibiotics, which are the genetic expression of different microorganism are the product of secondary metabolites, such as penicillin by *Penicillium chrysogenum*, purronitrin by *Pseudomonas aureofaciens*, gibberellin by *Gibberella fugileuroi*, β-lactam antibiotics by *Cephalosporium acremonium* and gramicidin by *Bacillus brevis*. Special secondary metabolites obtained due to the expression of gene in cell culture are Transplasminosen activator (tPA), Factor VIII and Insulin etc.
4. **Mutant Selection:-** Most mutations occurring in the cells consists of structural changes in a protein. These mutations can be observed directly by various physical changes of the protein, such as isoelectric point, electrophoretic mobility and heat stability etc. or directly by aminoacid changes. For selection of the such mutant genes animal cell culture may be used.
5. **Single Cell Protein (SCP) :-**Single cell protein (SCP) is a microbial biomass produced by the algae, fungi and yeasts. Protein occurs naturally both in vegetable and animal kingdom. Protein supply essential building blocks for the formation of tissue protein, blood protein, hormones, enzymes and antibodies. It is not the exaggaration of the fact that the protein requirement of the rising population will not be met with the available vegetable and animal protein in future and thus there is need for alternative source of protein to meet the protein hunger.

It is difficult for higher plants to use atmospheric nitrogen for the synthesis of proteins. On the other hand algae, fungi and yeasts utilize free nitrogen to build up the required protein. Yeasts may produce two and half times its weight of protein per day. Scientists have therefore turned their attention to this new area and they have found that new strains of microbial digestion of protein results into conversion into protein bodies which are nitrogen free. In other words petroleum distillates in presence of nutrients may be turned into what is known as single cell protein using certain firms of microbial yeast. About half of the petroleum is utilized in this method and the other half wasted into Co_2 and heat energy.

6. **Gene Therapy and Gene Cloning :-** Use of different cell lines for the expression of gene is the major tool of gene therapy and gene cloning. The general strategy of gene therapy is to introduce a normal Cdna allele into the affected cells of patients. In some cases gene can be introduced into the target cells (lymphocytes) that can be maintained in culture and then returned to the patient.
7. **Transgenic Animals and Plants:-** Various cell culture is required for the expression of genes in transgenic animals and plants. The ability to introduce genes into the gem line of mammals or plants is of the greatest technical advances in biotechnology. The result of gene manipulation are inherited by the offspring's. Transgenic mammals have provided a means for studying gene regulation during embryo genesis and in differentiation, for studying the action of oncogenes and for studying the intricate reactions of cells in the immune system.
8. **Biologically Active Useful Compounds:-** Certain biologically active compounds are produced in cell culture as a result of gene expression, e.g. Interferon (IFN).
9. **Study of Microbes:-** Different cell lines have been used to study gene expression, cell biology, virological and prion disease (Harris 1999) and at the same time to study the pattern of release of different micro-organisms (Wisseman *et al.,* 1976).
10. **Miscellaneous :-** Theses days recombinant genes are used for the production of various drugs and enzymes in cell culture for research and commercial purposes. These days gene expression is essential to measure the activity of gene in a cell or tissue. By comparing the activity of healthy and diseased tissues or by analyzing pattern of gene expression, researchers aim to identify

which genes may serve as markers or predictors of various disease, including cancer, or which may present potential targets for drug intervention.

Genetic Studies with Cultured Cells :

The Karyotype of Cultured Cells :- The analysis of chromosome constitution (karyotype) of tissue culture cells has gained paramount importance in genetic studies as it is clear that karyotype anomalies is associated with certain human diseases like Thalessamia (a globin gene disorder), night blindness, haemophilia, Lesch and Nythan syndrome and Xenoderma pigmentation (caused due to the faulty repair mechanism of DNA) etc. Karyotype gives indication of degree of abnormalities that cells have attained during cultivation *in vitro.*

The amnion cells of culture from the amniotic fluid of the placenta is used to study various expression of already identified gene which cause various birth defects. For this generally amniotic fluid is taken from mother's womb. The amniotic fluid contains many free cells which are cultured in various ways to find out their karyotypes, restriction site pattern analysis and many other informations regarding these cells.

Staining technique with Quinacrine mastered and examined under U-V fluorescent light determines the number of chromosomes and also helps the precise cytological identification.

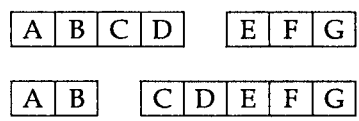
In young cell strains cells are mostly diploid (2n) chromosome number characteristic of the animals. Types of chromosomes are normal and cells are called Euploid. Cells of older strain and cell lines, especially of transformed cells, always deviate from the normal number and distribution (addition or loss of more than one set of chromosome) and is called Aneuploid cells. Number of chromosomes may be different from the diploid- called Heteroploid either higher (usually between 3n to 4n) called Hypertriploid (Trisomy of chromosome 4, seen in Down's syndrome - flat palm, mental retardation; Trisomy of chromosome 13-Edward syndrome; Trisomy of chromosome 18- Patus syndrome) or lower Hypodiploid. The haploid may be Monosomic (2n-1) e.g., Turners syndrome., or Nullisome (2n-2). Quasi-diploid has number of chromosomes 2n but their distribution is abnormal as a pair of chromosomes may be replaced by other. In addition, chromosomal aberrations (e.g., translocation and deletion often involve highly characteristic morphologic abnormalities in individual chromosome, which are also useful marker for cell identification. In

addition chromosomal aberration often involve highly characteristic morphological abnormalities in individual chromosomes which are useful as markers for cell identification.

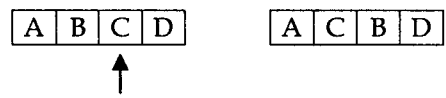
Detection of Fragile Sites:- These are those sites in chromosome which are prone to breakage and is characterized by breaks or gaps and is generally formed prior to death in the DNA (chromosome). These represent a site of high cytological disturbances e.g., Philadelphia chromosome. With the help of different enzymes we can identify these sites which are quite useful in recombinant DNA technology as we know that chromosome of different individual has different site of action for restriction enzyme (endonucleases). So, by cutting DNA in different length by restriction enzyme, separating by electrophoresis and by southern blotting, one can identify the cells of individual.

Deletion of Deficiency:- a portion of chromosome gets lost, e.g., Cats cry syndrome which occurs due to deletion of chromosome -5. Philadelphia chromosome occurs due to deletion of chromosome- 22 resulting into blood cancer where there is more synthesis of WBC and marked destruction of RBC. Retinoblatoma where detachment of retina takes place due to deletion of Chromosome- 13

Translocation :-Exchange of chromatid between non-homologous chromosome occurs, e.g.,



Inversion:- In this process a piece of chromosome is moved and reinserted in reverse order-i.e., 180 degree



Duplication :- It is opposite of deletion, i.e., addition of extra part of chromosome

Functional Chromosome Marker :-Chromosomes carrying genes specifying recognizable proteins with different electrophoretic mobility can be readily identified. In hybrid cells resulting from cell fusion, chromosomes with genes for oligomeric enzymes produce distinct hybrid oligomers so called isoenzymes.

Sister Chromatid Exchange :- During Sister chromatid exchange each DNA duplex doubles producing two sister chromatids, which at metaphase lie side by side in the same chromosome. Induction of chromatin exchange is a sensitive test for mutagenic activity of chemicals.

Expression of Certain Mutant Gene results into neoplasm. The p53 tumour suppression gene product is a phosphoprotein with a short half-life and low nuclear concentration. In most animal tissues the p53 protein nuclear concentration is below the threshold of detection by the usual immunohistochemical methods. The p53 protein is regarded as guardian of the genome (Lane 1992) with key function in regulating DNA repair and cell proliferation.

Mutation of p53 gene results in various human neoplasms, e.g., melanomas, squamous cell carcinoma of skin and basal cell carcinoma. The p53 protein accumulates in the nuleus when it can be demonstrated histochemically.

Practice Assignment X

1. Expression of transferred gene is essential to know;
   (a) The expression of foreign protein
   (b) Expression of protein product
   (c) Both of the above
   (d) None of the above.
2. Expression of eukaryotic gene in a homologous eukaryotic system has following advantages :
   (a) The expressed protein is properly folded
   (b) The expressed protein is properly glycosylated
   (c) Both of the above
   (d) None of the above.
3. Application of cell culture in gene expression is useful to study the:
   (a) Mutant selection
   (b) Production of secondary metabolites
   (c) Biotransformation
   (d) All of the above.
4. Expression of gene in heterologous expression system may result into:
   (a) Improper folding of protein
   (b) Improper glycosylation

(c) Improper amino-terminal modification of polypeptides
(d) All of the above.

5. Genetic studies in animal cultured cell include:
   (a) Karyotyping (b) Detection of fragile sites
   (c) Translocation (d) All of the above.
6. Karyotyping of cultured cell is done to know:
   (a) Exact karyotyes (b) Restriction site pattern analysis
   (c) Both of the above (d) None of the above.
7. The different expression system used to the expression of gene includes:
   (a) Mammalian celi expression system
   (b) Insect virus expression system
   (c) Bacterial expression system
   (d) All of the above.
8. In expression system the modification of aminoacid within protein include the following, Except :
   (a) Phosphorylation (b) Sensation
   (c) Acetylation (d) Sulphation.
9. Various cell culture system used for the expression of gene include:
   (a) HeLa cell line (b) W138 cell line
   (c) BHK cell. line (d) All of the above.
10. Gene is responsible for:
    (a) Synthesis of specific polypeptides
    (b) Controlling the sequence of amino acid in expressed protein
    (c) Both of the above
    (d) None of the above.

Answers :

1. - (c), 2. - (c), 3. -(d), 4. -(d), 5. -(d), 6. -(c), 7. -(d), 8. -(b), 9. -(d), 10. -(d).

Chapter 11

Organ Culture

An organ is a structure that has adopted to fulfill a specialized function in the body. Any modelling approach must take into account the fact that cells within the tissues and organs interact within each other in a variety of ways (i.e., by release of short range diffusible molecules or by contact dependent mechanism) and may be independent. Various small organs such as growing bones, oviduct, fallopian tube and endocrine glands etc., have been kept alive and sterilized under different experimental conditions (the small organs are affected by hormonal activity). Burrows (1910) cultivated chicken embryo outside the body using chicken plasma clot.

Organ culture refers to a three dimensional culture of a small fragment of tissue or whole embryonic organ that is explanted to retain some or all of the histological features of the tissues and cell interaction. Here the growth of tissues and organ fragments takes place *in vitro* in such a way that the physical disposition of the component parts relative to each other remains more or less stable and also retains histological and biochemical differentiation for longer duration but cannot be propagated and there is greater variations between replicates. Unlike monolayer culture, organ culture provides three dimensional structure which has many critical advantage with respect to maintenance of tissue structure with the anatomical relationship and functions including cell-cell interactions, gene expression and drug sensitivity. These depend on tissue architecture and cell shape which are preserved *in vivo* by the *in situ* so that the explanted tissues closely resemble the parent tissues *in vivo.*

In a successful organ culture the explants size is also important. It is better to use explants of 2mm diameter, otherwise the center of the explant will be degenerated and necrosis will take place due to inadequate oxygenation. Embryonic tissue survives well in the air but the explants from adult animal, generally requires pure oxygen or a mixture of 5% Co_2 and 95% O_2. Explants should be provided with the minimum of nutrition necessary to keep alive, but not

sufficient enough to stimulate frequent cell division. The conditions should be such that cells do not migrate from the explanted fragments. The outgrowth of isolated cells from the periphery of explants are rejected and is discouraged and minimized by suitable cultural conditions such that the new growth is composed of differentiated cells and thus they retain *in vitro* the same position in relation to neighboring cells that they had *in vivo*. So, new glandular structures are formed in glands. In lung tissues small new bronchi develop at the periphery of explants. They consist of alveoli lined with secretary, cuboidal, columnar, glandular or bronchial epithelium. In tissues lined with squamous epithelium such as skin, esophagus, bladder lined with transitional epithelium, follows similar pattern of differentiation as *in vivo*. Functionally, hormones dependent tissues remain hormone sensitive and responsive to hormones. Endocrine organs continue to secrete specific hormones and in case of foetal tissues. morphogenesis *in vitro* closely resembles foetal tissues *in vivo*. Organ culture provided the initial milestone to cell culture.

Whilhem Roux (1885) and Arnold (1887) for the first time demonstrated that explants (medullary plate) isolated from chick embryo can be maintained *in vitro* in warm saline for a short time. Jolly (1903) demonstrated that leukocytes from frog or Salamanders can grow in warm saline or serum and observed movement and division in living cell. Later, Loeb (1897) cultured the fragments of adult rabbit liver, kidney, thyroid and ovary on small plasma clot inside a test tube and observed that they retain their normal histological structure for three days. Loeb and Fischer showed that the tube must be filled with oxygen to prevent central necrosis of the explants. Medavaan grew slices of rabbit skin on a serum saline mixture in a flask filled with 70% oxygen. However, disadvantage of growing skin in a fluid medium is that the fragments tend to curve up and the epithelium migrates and covers the dermal surface. McGee and Woods (1987) stressed the need of organ culture in various microbial researches.

Organ Culture Technique:- The following methods were adopted from time to time by various workers for organ culture.

1. Hanging Drop Technique :

Ross Granivelle Harrison in 1907 described hanging drop technique for the cultivation of salamander leukocytes. For hanging drop preparation a cavity grease free clean sterilized slide is taken. On a

cover slip the explant of organ is kept and is covered with clotted lymph. The glass slide containing the organ explant and the lymph clot is inverted over the cavity of the slide in such a way that the preparation should hang in the cavity (Fig. -8). The all open margin of cover slip is sealed and is then incubated at 37^0C and examined daily for any growth.

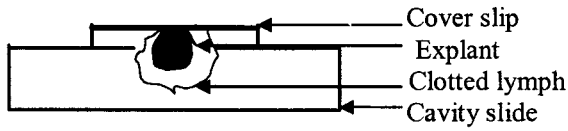


Fig. 8- Hanging Drop Preparation.

Harrison used this technique for the cultivation of amphibian spinal cord using frog lymph clot and demonstrated that axons are produced as extension of a single nerve cells and later he was in a position to cultivate nerve cells for several weeks by hanging drop method as done to demonstrate the motility of bacteria.

2. Watch Glass Technique:

Dame Honor Fell and Robinson introduced plasma clot substrate originally to study development of avian limb bone rudiment. It was also used to investigate growth and differentiation of other avian and mammalian tissues. The avian tissues are placed on a clot made up of chicken plasma (for plasma, cock is preferred than hen because of fluctuation in calcium content during egg production), and chick embryo extracts in equal proportion in a watch glass. Clot served two purposes, first it provides nutrient to the tissue and secondly clot produces a network of fibrin which gives physical support to the explant and to the cells which grow out from it, whereas chicken extracts provide a rich source of molecular nutrients such as, amino acids and nucleic acid derivatives. It also stimulates the migration and mitotic division of cell *in vitro.*

Gaillard (1951) described the technique of organ culture on watch glass. In this method the watch glass is enclosed in a Petri dish carpeted with moist cotton wool or filter paper to prevent evaporation of the clot and explants and incubated at 37. 5^0C. The clotted plasma substrate method has been modified to investigate the action of hormones, vitamins, and carcinogens. Watch glass containing plasma clots are closed with glass lid and sealed with paraffin wax (Fig. -9).

The clot contains two parts of human plasma, one part of human placental serum and one part of human baby brain extracts. The whole part is mixed with 6 parts of saline solution. Some workers used human plasma instead of chicken plasma. Other workers opined that clot formed from human plasma is not firm, so it may be detached from explants. It also forms a coarse fibrin network, impairing the optical clarity of the culture.

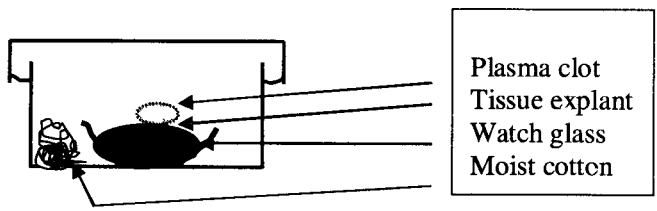


Fig. 9. Watch glass technique of organ culture.

Advantage of Watch Glass Technique:

(i) This technique is simple and cheaper.

(ii) Materials required can be easily available.

(iii) This is a well suited technique for growing embryonic organ.

Disadvantage of the Watch Glass Technique:

(i) Usually plasma clot is not a suitable substrate, as it liquefies in the neighbourhood of explant so that they come to lie in the rest of the medium;

(ii) Because of the simplicity of the medium no biochemical investigation was possible.

3. Agar Gel Substrate Technique:- Due to the liquefaction of plasma clot there is chances of sinking the organ in liquefied plasma, so it was suggested by Wolff and Wolff (1952) that agar gel should be mixed in an embryological watch glass to prevent liquefaction. It consists of 4% agar gel consisting of bicarbonate buffer saline, glucose, 11 amino acids, 10 vitamins and agar. Healthy and growing tissues usually appear translucent with shining surface. Opacity suggests loss of viability or the beginning of necrosis of explanted tissues. Agar is also used with the preparation of combination culture to study the interaction of various tissue components in particular that of mesenchyme and epithelium during the development of foetal organ. It has advantage that as agar does not liquefy so additional support is not required, but even though agar does not liquefy, it cannot be

added or analyzed without transplanting the culture. Hence, Raft and Grid method were developed to overcome this disadvantage of agar clot.

Collagen as an Alternative to Plasma Clot:

Recently collagen has been used as an alternative to plasma clot for cultivation of certain types of tissues or cells. Though collagen has practically no nutritive function, but it provides strong physical support for cells *in vitro* as it is not liquefied or digested by the growing living cells. It also provides congenial surface on which cell readily adhere or migrate. The most unique property of collagen is its ability to promote and maintain the differentiation of highly specialized cells like those of nervous system, muscle and liver.

4. Maximow Single Slide Technique:

Maximow (1925) adopted single slide technique for the developmental studies of foetal organs or organ rudiments. Any change can be observed by simple light microscopy or by polarized or U. V light microscopy. Generally tissues are too thick to be examined by phase contrast.

5. Combination of Clot and Raft Technique :

Shaffer (1956) described the the technique of organ culture by using cellulose acetate raft. Strips of lens paper or cellulose acetate is moistened with HBSS and these strips are placed on the clot. The explants are deposited on the top of the strips. Advantages are that the tissue is prevented from sinking into the pool of liquefied plasma and changing the medium becomes simple. Using the plasma clot technique, the period of cultivation can be restricted to within 4 weeks.

6. Grid Organ Culture Technique:

It combines the use of fully defined or semi defined media with a firm support of the explanted tissues which prevents them being submerged into the medium which thus becomes deprived of oxygen. Culture explanted on a grid is enclosed in a chamber under an atmosphere of CO_2 and O_2. This method is used for embryonic and adult tissue.

Procedure:

1. Remove about 100mg of foetal lung tissue from freshly aborted human foetus and transport in a 2 inch Petrie dish.

2. Wash tissue in HBSS with high concentration of antibodies
3. After last washing transfer the tissue in a Petri dish and reduce to fragments 1-2 cu. mm in size.
4. A stainless steel grid was prepared by folding it in such a way that it forms a raised platform (as shown in figure-10).
5. Prepare a number of 2 inches Petrie dish with stainless steel grid and superimpose circle of coarse mesh paper or millipore filter membrane in each. Add 10 ml of Eagles MEM taking care not to wet mesh paper, simply to keep mesh paper moist.

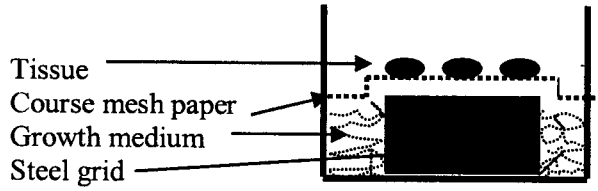


Fig. 10. Depicting the Structure of Grid Organ Culture

6. Place two to three fragments of chopped lung tissue evenly on the mesh paper circle. This constitutes the grid organ culture.
7. Incubate at 37^0C in a humidified atmosphere of 5% CO_2 and 95% air. It is important that the pH does not rise above 7. 4 during incubation.
8. Examine the culture under low power microscope objective at 2-3 days interval. After 8-10 days small monolayer islands predominately of fibroblast cells appear on the floor of the dish and increase in size thereafter. The islands have their origin in cells that migrate out from the original tissue fragments fall through open meshwork on paper and grid and attach themselves to glass. Some tissues may show no significant growth for about 3 weeks after incubation. With such slow proliferating tissue, the growth medium should be changed periodically. When the monolayer covers about 30% of the dish floor and appears to be growing actively, it is ready for subculturing.
9. Remove and discard the tissue fragments and supporting grid and aspirate the medium.

10. Add 5 ml. trypsin solution pre-warmed at 37^0C after 30 seconds, remove and discard fluid. Allow digestion to proceed at room temperature until the cells are detached.
11. Transfer entire suspension to a sterile fresh 2 inch Petrie dish incubate at 37^0C in a 5% CO_2 and 95% air. A confluent monolayer is produced in 48-72 hours.

Factors Involving Organ Culture:- These are the following factors on which the organ culture depends:-

1. Gas and Liquid Phase:- When an organ is taken as solid mass then diffusion of gases and exchange of nutrients are impaired. When this solid mass remains in monolayer then diffusion occurs rapidly. To get rid of this problem cell must be incubated at liquid- gas interface. While incubating in liquid-gas interface the following points should be kept in mind:-

- When cells are kept in liquid- gas interface at appropriate depth the cells tend to occupy special shape.
- If culture is done at deeper level then diffusion of gases do not occur.
- If culture is done in shallow water then due to more surface tension of water, outgrowth of cell will occur and no accuracy is maintained.

For better organ culture O_2 is supplied either as 95% pure O_2 or by passing hyperbaric O_2.

2. Structural Integrity:- For this culture must be interacted in such a way so that the combined effect is same as integrated organ and thus communication between cells take place.

3. Blocking of Differentiation:- When cells are differentiated then its regularity will be impaired. hence a system should be worked out which block the induction of differentiation.

Types of Organ Culture:

The organ culture has been divided into Histotypic organ culture and Organotypic organ culture:

Histotypic Organ Culture:- Culture of a characterized cell line when propagated at high density in the presence of appropriate environment is called histotypic culture i.e., if the explant of animal tissue maintains its structure and function in culture, it is described as histotypic culture. The term histotypic culture will employ that cells have been associated

in some way to recreate a 3-dimensional matrix, such as collagen gel.

Organotypic Organ Culture:- When the cells of different lineage are recombined in a specific proportion so that it seems like an organ it is called organotypic organ culture e.g., epidermal keratinocytes in combined reaggregated culture with dermal fibroblast. Here we can study the cell interaction which is not possible in histotypic culture.

Application of Organ Culture:

1. Organ culture can serve as an extremely useful and appropriate models, when the study is required on human and require species specificity and when ethical or practical consideration prelude using human as the experimental model.
2. It is most useful and better models when the infectious agents and the pathological events that occur in infection do not depend on host defense or serum factors, i.e., in infection when damage is due to macrophages and neutrophils, as these elaborate tissue destructive enzyme into the environment such as cavity formation in infection caused by *Mycobacterim tuberculosis.*
3. Organ culture was proved to be an important tool for studying immune responses at mucosal surface, as immune response of intestinal mucosa was studied by using organ culture of mucosal surface of intestine established after intestinal infection with enteroadherent *Escherichia coli.* (McQueen *et al.* 1986). Cooper *et al. (*1984) observed that organ culture from human fallopian tube infected with gonococci elaborated substantially more IgA than uninfected organ.
4. Organ culture of skin that contains keratinocytes at various stages of maturation is useful to study the pathogenesis of various micro-organisms that grow in native or maturing skin, such as herpes virus, dermatophytes and papilloma virus etc.
5. Organ culture of blood vessels could prove extremely useful in studying how micro-organism in the blood transgress endothelial barrier to enter the spinal fluid resulting into meningitis. (Ogawa *et al.* 1985).
6. Ramphal and Pyle (1983) used tracheal organ cultures to provide evidence that mucin and sialic acid served as receptors for attachment of *Pseudomonas aeruginosa* in the lower respiratory tract. This attachment is probably an antecedent step to pseudomonas pneumonia.

7. Organ cultures are ideal for investigation of the various components of the pathologic processes, when micro-organisms produce damage by using attachment to mucosal cells and elaboration of toxic moieties.
8. Organ culture has the potential to provide new information, critical for designing vaccines that can elicit an effective immune response at selected mucosal surfaces.
9. Organ culture can be used to study the morphology, physiochemical action of hormones and drugs.
10. In some cases organ culture can replace the use of experimental animals for drug testing and studying drug metabolism in tissues.

Advantage of Organ Culture:

(i) The explants remain comparable to the *in vivo* organs both in structure and function, which makes them suitable than cell culture for physiological studies.

(ii) The development of foetal organ is comparable to that *in vivo.*

(iii) In tissue explant culture hormone dependent organs remain so, while endocrine organ secrete specific hormone. Therefore, organ culture provides information on the pattern of growth, differentiation and development and on the influence of various factors on those features.

Limitations of Organ Culture:

1. It is very expensive.
2. In this yield of cell is very low which is not sufficient for molecular or biochemical assay.
3. Each processes require a new resource.
4. It is very complex process than replicating the culture.

Practice Assignment XI

1. All the statements about organ culture is true, Except:
   (a) In organ culture explants of tissue or organ is taken,
   (b) Organ culture maintains the histologic features of parent tissue or organ,
   (c) The anatomical relationship and functions are not maintained,
   (d) Explanted tissue closely resembles the parent tissue *in vivo.*

2. For the first time organ culture was done by:
   (a) Loeb (b) Enders
   (c) Joseph morgan (d) Raymond Parker.
3. Organ culture is generally done by:
   (a) Watch glass technique (b) Clot and Raft technique
   (c) Grid technique (d) All of the above.
4. Organ culture is extremely useful *in vitro* model as:
   (a) Whole animal are not required,
   (b) A piece of tissue or organ, both from human and animal can be obtained,
   (c) Both of the above
   (d) None of the above.
5. Watch glass technique for organ culture has all the advantages, Except :
   (a) Watch glass is easily available
   (b) Tissue fragments are kept on plasma clot,
   (c) Usually plasma clot substrate liquefies, resulting into dipping of explants,
   (d) None of the above.
6. Organ culture ;
   (a) Maintains the species specificity,
   (b) No ethical problem exists
   (c) Both of the above
   (d) None of the above.
7. The use of plasma clot substrate was originally introduced by:
   (a) McGee and Woods
   (b) Fell and Robinson
   (c) Morgan and Parker
   (d) Bailly and Darmon.
8. Organ culture also maintains the function of original organ as:
   (a) Glandular structures are observed in gland,
   (b) Alveoli are seen in lung explant
   (c) Both of the above
   (d) None of the above.
9. Organ culture may be an important tool for studying the:
   (a) Immune response at mucosal surfaces,
   (b) New information's critical for designing vaccine,
   (c) Both of the above
   (d) None of the above.

10. Tracheal organ culture to provide evidence that mucin served as receptors was given by:
    (a) Ramphal and Pyle
    (b) Morgan and Moorthy
    (c) Ogawa and Yurberg
    (d) Bernhard and Bailly.

Answers:

1-(c), 2- (a), 3- (d), 4-(d), 5-(c), 6-(c), 7-(b), 8-(c), 9- (c), 10- (a).

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Chapter 12

Transfection

Any heritable change in the genome of host by introduction of the foreign gene manually which express a new phenotype is known as transfection. Hershey and Chase demonstrated in 1952 that DNA is the essential component of a phage particle that involves *E. coli* cell upon infection with T2-phage. DNA alone can initiate and direct two major steps i.e., DNA replication and synthesis of phage specific protein confirming the role of nucleic acid in the infective process. Nucleic acid of viruses separated from virus was shown to be infective. Foldes *et al.* (1964) produced transfected complete virus particles by introducing isolated nucleic acid from virus particles into recipient cell. So, transfection system is distinct from the natural virus infection in the following ways:- (a) the isolated foreign DNA is introduced manually and (b) the infectivity is sensitive to nucleases but resistant to antiserum directed against the virus particles. Process of transfection has multiple obstacle in an eukaryotic cells as compared to a prokaryote because of numerous intracellular compartments, hostile and hydrolytic endo-lysosomal environment and presence of nuclear membrane etc.

Transfection is Interesting Because :

- Preparation of nucleic acid from infective virus particles is extremely homogenous population of molecules that can be subjected to physical and chemical alterations. This invites studies on the interrelationship between physical, chemical and biological properties of such nucleic acids.
- Transfection as a biological assay is the most stringent test for physical and informational integrity of a nucleic acid, e.g., the high fidelity of RNA and DNA polymerases in the in vitro replication of QB RNA or 174 DNA is demonstrable by transfection with newly synthesized material.
- Vegetative nucleic acid intermediates between infective particles and the progeny can be assayed and the biological properties of

such nucleic acids can be investigated through transfection.

- Virus nucleic acids are more useful agents in uptake studies than transforming DNA. Here the manifestation of the nucleic acid penetration is the production of the infectious virus particles. Whereas transformation in addition is dependent upon the complicated step of infection into the recipient genome.
- In case where the uptake of nucleic acid by component cells is not specific, transfection may be attempted with a wide range of virus nucleic acid. Transfection with nucleic acid from viruses that normally cannot infect such cells may yield information about mechanism of intracellular restrictions that determine virus/host specificity.
- In the course of investigation with bacteriophage it was learnt that phage infection via the normal route and transfection proceed along with different sites. Thus a comparison of these processes may yield information about the mechanism of infectious process.

Source of DNA for Transfection:

DNA which is used for transfection may be cDNA, genomic DNA, genomic DNA clones and retroviral vectors.

Clone :- It is a descendent of single parent cell derived non-sexually from a single parent and it is physically and genetically identical to its parent, unlike in the case of sexual reproduction where the progeny inherits one half of the genetic matter from the father and the other from the mother. The process of cloning involves the mechanical transfer of nucleus of a cell, along with all its genes and chromosomes, to an egg which has also had its nucleus removed. This enucleated egg is then "deceited" into thinking that it has been fertilized an all it takes is an electrical pulse to trigger off its division like a normal embryo. DNA used in the transfection is generally is a molecular clone of some type. A molecular clone is a replica of a specific stretch of DNA or RNA found in a cell. A cDNA clone is a piece of DNA that was originally synthesized *in vitro* using cellular RNA as a template. cDNA cloning usually utilizes the viral enzyme reverse transcriptase. cDNA is ligated with the enzyme ligase to form covalent bonds between two pieces of DNA and introduced in to a vector. Thus, the ligated cDNA into a vector allows it to be introduced into a host bacteria for its replication.

Methods of Transfection:

There are two main methods of transfection : (1) Vector mediated and (2) Non- vector mediated.

Vector Mediated Transfection:

Vector:- A variety of suitable vectors are available for transfer of foreign genes into the mammalian cells. The vectors commonly used for transfection processes are bacteriophage vectors, cosmid vectors, retroviral vectors, papilloma virus, SV40, Bacculovirusvectors, herpes virus, adenovirus and plasmid vectors. The suitability of vectors for the transfer of gene largely depends on the size and nature of the gene.

Bacteriophage Vectors :- It is commonly used for initial isolation of either genomic or cDNA clones from eukaryotic cells. This transfection process differs from the naturally occurring bacteriophage in the following way: Naturally occurring bacteriophage is engineered to include either cDNA or short piece of genomic DNA in addition to its original DNA

Retroviral Vectors:- Retroviruses are RNA virus which replicate via the synthesis of a DNA provirus, which is integrated into the chromosomal DNA of infected cells. A DNA copy of the viral RNA is synthesized by the viral enzyme called **reverse transcriptase**. This can be modified from original retrovirus. These can be propagated in bacteria as plasmids, have foreign DNA ligated into them, and be transfected into tissue culture cells.

Cosmic Vectors:- Cosmic vectors are modified plasmid that carry the DNA segments (cos segments) required for packaging DNA into bacteriophage particles, because cosmid carry an origin of replicon (Col E_1) and a very resistant, ampicillin marker (amp). Cosmid vector can be introduced into *Escherichia coli* by standard transformation procedure and propagated as plasmid. The major advantage of using cosmid vector is its high efficiency to produce a complete genome library of 10^6-10^7 clones from only one milligram of inserted DNA. However, it cannot accept more than 40-50 kb of DNA.

Bacculovirus Vector:- For many years the lower vertebrates and the vertebrates have been largely ignored though unique aspects of their development like tissue regeneration in amphibia and metamorphosis in insects make them attractive system for the study of molecular basis and development. It is an insect virus infecting insect larva of

Lepidoptera to which silkworm belongs. It secretes a protein called polyhydrin, which occurs as a nuclear vesicle. Foreign gene is inserted into this nuclear vesicle with polyhydrine gene and then transferred into the cell.

Plasmid Vectors:- Plasmids are small circular extrachromosomal DNA found in the cytoplasm of bacteria that can replicate independently without being associated with chromosomal DNA. Plasmid vectors usually consists of only 2 to 4 kb of DNA, in contrast to the 30 to 45 kb of phage DNA, facilitating the analysis of an inserted DNA fragment. Plasmid vector can only be used as vectors after ligating the DNA into plasmid and amplified in a bacterial culture.

Non-vector Mediated Transfection:

There are mainly of two types : (1) Direct and (2) Indirect.

Direct Method:-The following direct methods have been used for the gene transfer:-

Microinjection :

Lin (1966) described the technique of microinjection of mouse egg for the first time. It is a technique of delivering foreign DNA into a living cell/egg/oocyte/embryo of animals through microinjection (Graessman *et al.* 1980). For microinjection a glass micropipette is used. For preparing micropipette a glass tube is taken. Its one end is heated until the glass becomes somewhat liquefied. It is quickly stretched which forms a very fine tip at the heated end. The tip attains a diameter of 0. 5 μm. Process of delivering foreign DNA is done in a powerful microscope. Cells to be microinjected are placed in a container. Holding pipette holds a target cell at its tip when the cell is gently sucked. The tip of the micropipette is injected through the membrane of the cell. Contents of the needle are delivered into the cytoplasm and it is then taken out (Fig. -11). The injected DNA integrates randomly with nuclear DNA and its expression could be possible only when the foreign DNA is attached to a suitable promoter sequence.

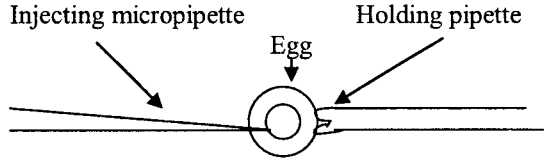

Fig. 11. Showing Microinjection technique.

Advantage of Microinjection

(i) Here when DNA is inoculated the 'hits' are certain but this cannot be applied to large number of cell (Kondoh *et al.* 1983), commonly used for animal cells and not for plant cells.

(ii) This is useful method for producing transgenic animal.

Disadvantase of Microinjection:-

(i) Only a very small amount (one picolitre) of transgene is inserted,

(ii) There is no control over place of insertion of transgene,

(iii) There is no control over the insertion of copies of transgene i.e., how many copies have been inserted.

(iv) Pronucleus in the fertilized oocyte is difficult to visualize. Mice and rabbit nucleus is easily visualized, however, the pronucleus in sheep, goat, pig, cattle and buffaloes are difficult to visualize by ordinary methods, it requires fluorescent or interface contrast microscope. In fluorescent microscope, the fluorescent and U-V ray can damage the ovum so interference contrast microscopy is preferred.

In order to select transformed cell several marker genes can be cloned with the gene of interest prior to the application into the test genome. Drug selection technique can be applied for the isolation of stable transfectants, one such gene is neomycin resistant gene which generally inhibit protein synthesis.

Disadvantage:

(i) Frequency of transfer is very low.

(ii) Integrated gene undergo substantial modification i.e., recombination, rearrangement and deletion etc.

(iii) Integration with host cell genome is random.

Electroporation :

Electroporation is defined as a physical process that transiently permeabilizes cell (both prokaryote and eukaryote) plasma membrane with an electrical pulse, permitting cell uptake of a variety of biological molecules (Chang 1989). It is a widely used method for efficient introduction of nucleotides, REA, protein, carbohydrates, dyes, virus particles and very large DNA molecules into prokaryotes or eukaryotic cells and the direct electrotransfer from donor to recipient cells (Chang *et al.* 1991). Electroporation was first described by Sendai and his co-

workers and later it was modified by Zimmermann Schewich. Here electric field is used to transfer foreign DNA into a fragile cell. It has been postulated that that short, high voltage DC electric field pulses applied to eukaryotic cell plasma membranes cause the destabilization of the phosphobilid layer which results in the formation of temporary pores in the plasma membrane, thus allowing an exchange of extracellular and intracellular ions and macromolecules (Zimmermann and Vienken 1982). Electroporation instruments consist of:

(i) Special chamber having suspended cells with two different commonly used electro pulse electrodes : Gene pulser X cell (Bio rad) and ECM 830 (BTX).

(ii) An effective buffer which provides optimized condition for transfecting primary and hard to transfect cell.

(iii) The pulse generator and

(iv) An oscilloscope to monitor the wave form and amplitude of generated pulse.

The cells to be fused are exposed to a weak AC field of about 200v/ cm^2 and 800kHz. This causes the cells to line up in a row, forming a **'pearl necklace'** chain. The number of cells within a pearl chain depends upon the population density of the cells and the distance between the electrodes and also on the pH of the medium. Passing of electrical pulse brings the membranes of cells in intimate contact. Exposure to a short electrical impulse (20 micron S, 2. 5 kv/cm^2) there is reversible breakdown of the cell membrane caused by a structural alteration in the lipid bilayer and then fusion occurs within minutes. Phosphatidyl bridges are formed at the zones of contact of the membranes and cell fusion takes place. The frequency of fusion is generally above 50% and is several order of magnitudes higher than one achieved with fusogens like viruses or chemicals. However, it may differ while using different micro-organisms, e.g., in *Escherichia coli* around 50µl of cells and DNA are placed in chamber filled with electrode and a single pulse of approx. 25 microfaradays 2. 5 kvolt and 200 ohm is administered for about 4. 5 milliseconds. Yield of transfection efficiency is 10^9 transformants per microgram of DNA for small plasmid (about 3kb) and 10^6 transformants for large plasmid (136 kb).

Electroporation is more successful with plant or yeast cells than with human cells. However, it has been successfully applied on hepatocytes, fibroblasts, epidermal cells and haematopoitic cells. It can be affected by-

- Electrical field strength,
- Pulse strength and
- number of field pulse.

A portion of cell is stably transformedand can be selected if a suitable marker gene is carried on the transforming DNA. The transformed protoplast is cultured for about a month. These develop microcalli, which are plated on solid medium containing selective marker (e.g., kanamycin). The cells are analysed after about 37-45 days for possessing the differences in transformed cells.

Advantages of Electroporation:

(i) Electroporation has been preferred over PEG as the apparatus delivers DNA effectively at appropriate electric pulse.

(ii) This process is cost effective and treatment is both reproducible and simple to apply.

(iii) It has proved to be broadly applicable gene transfer technique, facilitating foreign gene expression in protoplast as well as intact plant cells, both in suspension culture and as part of organised tissue.

(iv) Naked DNA may be used for gene therapy by applying electroporation device in animal cells.

(v) It provides a valuable alternative to chemical and other physical methods, that may be ineffective or toxic when transforming certain cell types.

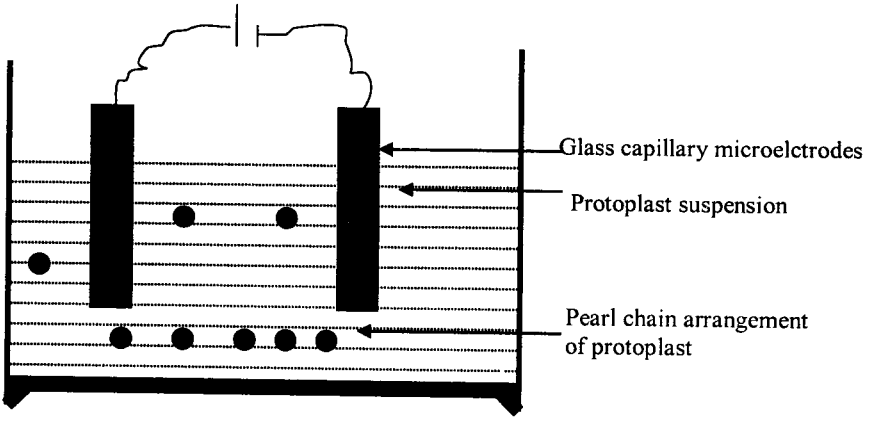

Fig. 12. Methods of Electroporation.

The disadvantage of electroporation:

(i) The disadvantage is same as that of PEG fusion as both methods rely mainly on protoplast for introduction of foreign gene.

(ii) Potter *et al.* (1984) showed that treating eukaryotic cells with Colemid before electropolation increases the transformation efficiency because this drug arrests cells in metaphase; the cells lack a nuclear membrane or have an unusually permeable membrane.

Particle Bombardment Gun :

Professors Stanford and coworkers of Cornell University (USA) developed this method in 1987. This method involves bombardment of particles carrying DNA (or RNA) of interest onto target cells using high velocity transfer mechanism. Other names of this method are Particle gun, Microprojectile bombardment, Particle acceleration. Later this method of gene transfer was used by several workers (Birch and Frank, 1991) and Klein *et al.* 1992).

The apparatus consists of a chamber connected to an outlet to create a vacuum. At the top, a cylinder is temporarily sealed off from the rest of the chamber with a plastic rupture disk. Rupture disk controls the helium pressure for nucleic acid/ microcarrier acceleration over a wide pressure range (450-2, 000 psi) inside an evacuated bombardment chamber. Helium gas flows into the cylinder. A plastic micro-carrier is placed close to rupture disk. It contains micrprojectile which are usually made up of gold or tungsten particles and are coated with DNA or RNA of interest, the microscopic pellets (i.e., coated micro-projectiles). At the time of working, the apparatus is placed in a Laminar flow to maintain sterile conditions. The target cells/tissues are placed in the apparatus. A stopping screen is put between the target cells/tissues and micro-carrier assembly. Helium gas is flown in the cylinder at high velocity (1400 ft/sec or 300-400 m/s). Here helium gas is used because of its unique electronic configuration, it is endowed with many extraordinary properties and defies combination with other elements. When pressure of cylinder exceeds the burstin point of plastic disk, it gets ruptured and propel the plastic micro-carrier containing DNA coated micro-pellets. The stopping screen allows only the DNA coated micro-pellets to pass and continues to accelerate towards target cell and strike it and eventually become integrated into the nuclear or organelle genome of the host cell by penetrating the cell wall (Fig. -13). This technique has been found

useful for many plants like maize, rice, wheat, sunflower, sugarcane and papaya etc. The transformed cells are regenerated onto nutrient medium. The regenerated plant tissues are selected over culture media containing either antibiotic or herbicide. The selected plants are then analyzed for expression of foreign DNA (Birch 1997).

Advantages:

(i) As the intact cell wall can be penetrated so there is no need of obtaining protoplast.

(ii) Genome of sub- cellular organelles can be manipulated.

(iii) This method eliminates the use of potentially harmful viruses or toxic chemical treatment as gene delivery vehicle.

(iv) It is simple and convenient as it includes coating DNA or RNA onto gold microcarrier, loading sample cartridges, pointing the nozzle and firing the device.

(v) This device offers to place DNA or RNA exactly where it is needed into virtually any organism.

Disadvantages:

(i) There is random integration.

(ii) Specialized equipment is needed.

Laser Method :- A brief pulse of finally focussed laser beam used for the transfection was described by Kurata *et al.* (1986). In this method DNA is mixed with the cells which are present in the culture and then a fine focus of laser beam is passed on the cell surface. Laser beam forms a small pore which is sufficient for up taking of DNA into the cells. The pore formed is transitory and soon it is repaired.

Indirect Method:- The indirect methods of transfection are as follows:

The calcium phosphate DNA- coprecipitate :- In 1973 Graham and van der Eb described the technique of calcium phosphate DNA - coprecipitate for the transfectionof mammalian cells. Successful DNA transfer depends upon the formation of insoluble co-precipitate of the DNA with calcium phosphate. For this solution containing DNA and $CaCl_2$ (2. 5 mM) is added to a buffer saline solution. The stock buffer used contains 2x HEPES buffer saline comprising 1. 5 mM Na_2HPO_4, 10mM Kcl. 280mM Nacl, 12 mM glucose and 50 mM HEPES [4-2(hydroxyethyl)-1 piperazine ethane sulfonic acid] at pH 7. 05 containing potassium phosphate. The mixture is incubated at room temperature 20-30 minutes which results into calcium phosphate

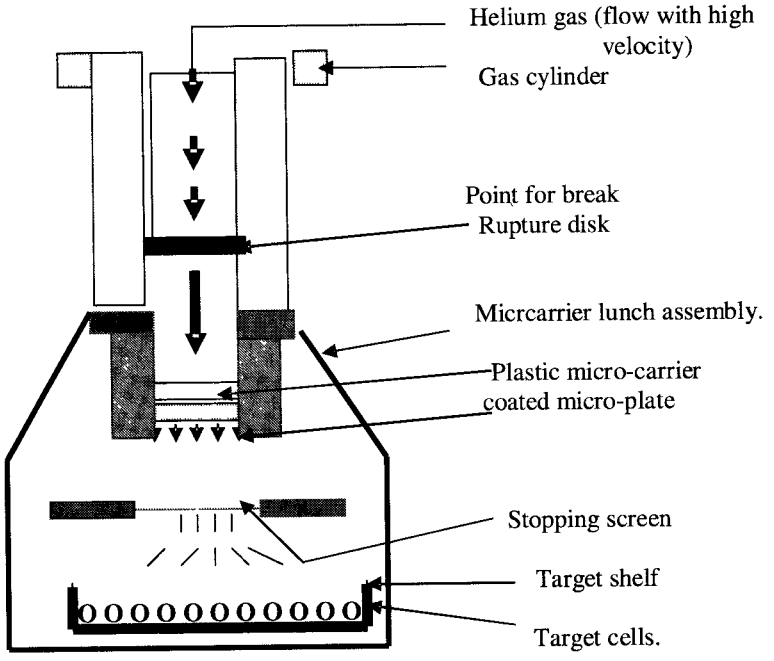

Fig. 13. Diagrammatic Representation of Particle Bombardment Gun.

DNA-coprecipitate. The ideal calcium phosphae DNA -coprecipitate is so fine that it resembles dilute milk rather than a precipitate and this does not sinks in the bottom of the tubes. After the formation of the precipitate, it is mixed to the culture cell in medium followed by incubation at 37^0C for 4-16 hours. Apparently the calcium phosphate DNA-coprecipitates are phagocytosed and the DNA is taken up by the cells. The role of ca-ion is to concentrate the DNA on the cell membrane which facilitates uptake of DNA by phagocytosis. The DNA new fragments get ligated to give a concatameric structure, which is then integrated into the nuclear DNA as one single piece. Here only 1-2% exogenous DNA is taken up. Chen and Ukayma (1987) described a modified calcium phosphate DNA-coprecipitate method of transfection in which there is 10-15% more uptake of genetic material. They observed that much finer precipitate is formed when the concentration of DNA is about 20-30 μg/ml and the circular DNA is more suitable than linear DNA.

In order to select transformed cell several marker genes can be cloned with the gene of interest prior to the application into the test genome. Drug selection technique can be applied for the isolation of stable transfectants, one such gene is neomycin resistant gene which generally inhibit protein synthesis.

Disadvantage:

(i) Frequency of transfer is very low.

(ii) Integrated gene undergo substantial modification i.e., recombination, rearrangement and deletion etc.

(iii) Integration with host cell genome is random.

DEAE-dextran mediated DNA transfection:- DEAE-dextran is soluble, polycationic having high molecular weight (MR 500, 000). In this method mixture of DNA with DEAE dextran may result in transfection. Here the above mixture is incubated in humid atmosphere for 1-8 hours, as the amount is very small. The excess DEAE-dextrane is removed by washing. DEAE-dextrane probably acts by mediating in some unknown way, probably the productive interaction between negatively charged DNA and components of cell surface resulting in endocytosis. In this method there is 100% uptake of DNA when fusion of cultured cells with bacterial protoplast containing the exogenous DNA is done (Schaffnor 1980). If DEAE-dextrane treatment is coupled with DMSO shock then 80% transformed cell can express the transferred gene.

DNA introduced by calcium phosphate DNA co-precipitate and DEAE- dextran method is subjected to high rate of mutation. (Ashman and Davidson 1985).

Advantage:

(ii) It is simple and cheap.

(iii) It can be used for cells which cannot tolerate Ca-phosphate exposure.

Disadvantage:

(i) Stable expression is difficult by this method.

(ii) As serum inhibits DEAE-dextrane so, cells are rigorously washed in order to make it free from serum.

Practice Assignment XII

1. In Ca-phosphate precipitate method for transfection:
   (a) DNA is mixed with buffer containing calcium chloride and phosphate
   (b) DNA forms the fine precipitate
   (c) Both of the above
   (d) None of the above.
2. High rate of mutation occurs, when :
   (a) DNA is introduced by calcium phosphate
   (b) DNA is introduced by DEAE-dextran
   (c) DNA is introduced by the mixture of calcium phosphate and DEAE-dextrin
   (d) None of the above.
3. Breakdown of cell membrane and formation of pore in electroporation is :
   (a) Irreversible (b) Reversible
   (c) Both of the above (d) None of the above.
4. In electroporation :
   (a) DNA is taken up into phagocytic vacuoles
   (b) DNA remains free in cytosol
   (c) Both of the above (d) None of the above.
5. Electroporation buffer contains all the following ingredients, Except :
   (a) Nacl (b) HEPES
   (c) Na_2HPO_4 (d) NaN_3.
6. The advantage of lipofection is :
   (a) Greater transfection efficiency is obtained
   (b) Incorporation of DNA in liposome is more in quantity
   (c) Both of the above (d) None of the above.
7. All the following statement regarding particle bombardment is true, Except :
   (a) This is more suitable method for transfecting maize
   (b) This is more suitable method for transfecting mammalian cell
   (c) Here there is no need to generate protoplast
   (d) Here DNA is adsorbed on tungstun particles.
8. In laser method of transfection:
   (a) A fine focus of laser beam is passed
   (b) Laser beam causes fine hole in the cell membrane of recipient cell

(c) The hole formed is repaired within seconds
(d) All of the above.

9. Microinjection methods have following disadvantages, Except :
   (a) Only a very small amount (one picolitre) of transgene is inserted,
   (b) There is no control over place of insertion of transgene,
   (c) There is no control over the insertion of copies of transgene i.e., how many copies have been inserted.
   (d) Here in insertion of DNA hit is certain.

10. The vectors used for tranfection processes are :
   (a) Bacteriophage vector (b) Cosmid vector
   (c) Retroviral vector (d) All of the above.

Answers:

1. -(c), 2. - (c), 3. - (b), 4. - (b), 5. - (d), 6. - (d), 7. - (b), 8. - (d), 9. - (d), 10. -(d).

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Chapter 13

Cell Fusion

Cell fusion is a biological process in which the plasma membranes of the two cells break down at the point of contact between them and the cytoplasm of the two mixes. The mammalian cell membrane contains phospholipid bilayer. The phospholipid bilayer of the cell membrane has property of enormous flexibility and the property of self closing when a transient pore is formed.

In the year 1957 Okada *et al.* (1957) for the first time observed that animal tumour cells in suspension can be fused under the influence of Haemagglutinating virus of Japan (HVJ) or Sendai virus. Later in 1960 Barski and his associates while conducting transformation experiment with the two morphologically and kinetically different cell line of mouse embryo cells, observed the unexpected result. They observed that the cells of two different origin can be efficiently fused by using inactivated Parainfluenza and Sendai viruses. They found the hybrid cells which contained the total number of chromosome equal to the sum total of chromosome in two parent individual cells and cell cytoplasm as well.

If the two cells have different origin, the resultant fused cell is called **heterokaryon,** and if of identical origin, it is called **synkaryon.** In the fused cells, there are cytoplasm of both the cells having two nuclei and there is mixing of the chromosomes from the two cells. There is genetic re-assortment resulting into a wide variety of combinations not attainable through conventional breeding. Such cells proceed to mitosis and produce hybrid cells. These can be cloned to produce hybrid cell line. Yet, these cells are unstable. Earlier the need for process of cell fusion was felt for two aspects i.e., The process of biological membrane function and the exocytosis and endocytosis, but recently the cell fusion is one of the important tool for getting a hybrid cell.

Types of Cell Fusion:

Spontaneous Cell Fusion:- Kuster for the first time observed

spontaneous fusion of mechanically isolated plant protoplasts. Protoplast of the cell lying in close proximity may fuse to produce homokaryons.

Induced Cell Fusion :-Cells can be induced to fuse by the following methods:

1. Virus e.g., Sendai virus, herpes, myxo, paramyxo viruses
2. Electrofusion and
3. Chemicals e.g., polyethylene glycol, $NaNO_3$, poly L-ornithine, dextran.

1. Viruses:

For the first time for cell fusion sendai virus was used by Okada *et al.* (1957). later, Harris and Watkins (1965) and Harris *et al.* (1966) by using ultraviolet inactivated sendai virus showed that wide variety of cells can be fused by this method resulting into production of heterokarynos, but the role of virion in cell fusion was not clear. In 1968 Hosaka and Koshi studied the role of HVJ virion (sendai virus) for cell fusion of Ehrlich ascitis tumour cell and observed electronmicroscopically that the fusion proceeds in the following sequences:-

(i) As sendai virus belong to *Paramyxoviridae* group and thus assembles at host membrane. First two cells come closer and agglutination of cells occur in cold by the virion.

(ii) Then close contact of adjacent cell membrane occurs near adsorbed virion of agglutinated cells.

(iii) There is degradation of the cell membrane in contact with virions and communication of the cytoplasm of adjacent cell through the break.

(iv) Then connection of broken cell membranes between adjacent cell and formation of cytoplasmic bridge.

(v) Enlargement of bridge and engulfing of virion within the vesicle.

(vi) Finally spherical fused cell is formed due to process of permeable osmotically induced cell. Alteration of virion within vesicle occurs followed by the appearance of free nucleocapsid in cytoplasm and disintegration of nucleocapsid.

For effective fusion healthy cells of high viability are taken as these give better yield. Before fusion the extreme pH of solution or media is avoided because this will require several times washing which will

kill the parental cell types. Fusion by Sendai virus is not in much use as it is difficult to get an effective preparation of the sendai virus.

Proteins of some of the other enveloped viruses also mediate fusion of cells, e.g., Herpes, myxo and parmyxo viruses. Sendai viruses bring about the best fusion. The frequency of fusion is about 10^{-6}.

2. Electrofusion :

Auer *et al.* (1976) for the first time described the methods of electrofusion. Adhesion of cells occurs in non-uniform electrical fields. Here electric field is used to transfer foreign DNA into a fragile cell. Electrofusion is a three step process:-

(i) Cells are first exposed to alternating electrical field (A. C.) i.e., brief pulses at high voltage (about 350 V) is applied to protoplast suspension containing naked or recombinant plasmid through two glass capillary microelectrodes which are placed in contact with isolated protoplast.

(ii) Due to application of electrical pulse there is combined action of dielectrophoresis and a transient change in membrane permeability. An electric field of low strength give rise to dielectrophoretic dipole generation within protoplast suspension.

(iii) Fusion occurs due to formation of transient pores of adjacent cell, when a short pulse of direct current is applied.

Electrofusion has so many advantages. As the electrofusion depends on the physical effects of the electrical current, so, it functions regardless of the genetic or biochemical predisposition of the cells. In hybridoma production it has the distinct advantage over the PEG fusion as it is less labour oriented and requires no chemical incubation and repeated washings. Electrofusion can be applied on cell types including human cell lines because PEG has cytotoxic effect on human cell lines.

3. Chemicals:

Polyethylene glycol (PEG):- In 1961 Protoconovo modified the process of cell fusion by using Polyethyleneglycol (PEG) as a mediator of cell fusion. PEG is a non-ionic water soluble surfactant which has strong dehydrating property. But due to this property only it cannot act as fusing agent because other chemical like dextran which is also a strong dehydrating property cannot act as fussogen. So, besides the dehydrating property PEG decreases the surface potential of the membrane by several hundreds of millivolts and it may induce

alterations in the orientation and hydration of the phospholipid head groups causing charge neutralization and segregation of the lipid. It acts as cationic glue to adhere the negatively charged cell membrane to one another. PEG may have toxic effect on some cell, so to prevent the toxic effect of PEG on cells a critical compromise must be made in terms of concentration of PEG and time of exposure between increased fusion and toxicity.

In the fusion mixture DMSO may be added as besides enhancing fusion (Norwood *et al.*, 1976) it facilitates the the removal of PEG after fusion and perhaps buffers the potentially damaging rapid osmotic changes in the actual fusion and subsequent steps.

These days PEG is commonly used in fusion of two cell lines or cells for the preparation of monoclonal antibodies. This substances enhances the fusion upto 90-95%. (for details of cell fusion by PEG refer the **chapter-** for monoclonal antibody).

Kao *et al.* (1974) showed that PEG can also be used for fusion of plant protoplast, like Nicotina, rice, maize etc., because it agglutinates protoplasts thus can subsequently be fused at high frequency. For fusion target protoplasts are kept in the PEG solution (mol. Wt. 8000 dalton, in 15% final concentration). $CaCl_2$ is then added, glucose or sucrose of the medium act as osmotic buffering agent. Addition of various lipid soluble substances like α-tocopherol, glycermonooleate and retinal can enhances the PEG-induced fusion. Intact surviving protoplasts are cultures to form cell wall and colonies in turn. After several passage in selection medium, frequency of transformation is calculated. Transformation frequency in excess of 5% normally achieved. Optimized PEG method is considered better by some workers than electroporation.

Fusion with $NaNO_3$:- Power *et al.* (1970) showed that high concentration of NaNO3 may be used to fuse the plant protoplasts and since then this method is mainly used for plants fusion. Here the isolated protoplasts are kept in the mixture of sodium nitrate (5. 5%) and sucrose solution (10%) and incubated in a water bath at 35^0C. Fusion takes place and this can be monitored under inverted microscope.

Fusion with Calcium Ion:- Calcium ion under alkaline condition and at high pH is a good fusion agent. In this method the protoplast is kept in a mixture containing 0. 05M $CaCl_2$, $2H_2O$ in 0. 4M mammitol at pH (10. 5) and centrifuged at 2, 000 rpm for 30 minutes. This mixture

is then further incubated at 37^0C in a water bath. Here the percentage of fusion is more as compared to the other methods (Graham and Vander 1973).

Cell Fusion with Polyvinyl Alcohol :- Nagata (1978) observed that polyvinyl alcohol (PVA) with an average polymerization degree of 500-1500 can also be used as fusogen. It is as effective as PEG as fusion agent.

Cell Fusion with Liposomes Containing DNA of Interest:- The delivery of DNA into cells using liposome is called lipofection. This was described by Fraley *et al.* (1980). On microscopic examination it was observed that liposomes are small sized particles and is made up of unilamellar phospholipid bilayer, which remains in concentric manner (liposomes). Liposomes enclose aquous chamber and can entrap water soluble molecules and thus called lipid bag. In this bag many plasmids may be entrapped which can be fused with protoplast by using PEG as fusogen. Due to endocytosis of liposomes, DNA enters the protoplast surface and get fused with the plasma membrane of recipient cells. Prevention of degradation of liposome is achieved by pre-treating cells with lycosomotropic agents like chloroquine, cytochalsin B or colchicin. Lipofection is used in many of the plants like carrot and tobacco etc. This technique has the following advantages:-

- Low toxicity,
- Protection of nucleic acid (DNA and RNA) from nucleases,
- Long stable storage of nucleic acid fragments in liposome
- Is applicable in various cell types and
- Has high level of reproducibility.

4. Cell Fusion with Red Blood Cells (RBC):- This method of fusion was described by Boogard and Dixon (1983). Here the cytoplasmic content of the RBC is removed and this empty space is refilled with different molecules called Hb-free ghost cell. This ghost cell is used with the target cell along with a fusogen like PEG, which permits the introduction of a variety of macromolecules in mammalian cells.

4. Protoplast Fusion:- Protoplasts are cells devoid of the cell wall by enzymic digestion. Protoplast fusion was first described by Schaffner (1980). He described the process of spontaneous (random) fusion in mechanically isolated protoplasm. Plant protoplast isolates can be induced to fuse with each other, even when derived from

different species. In protoplast fusion two basic methods are used. In first case co-incubation of protoplast with the nucleic acid (plasma DNA) with different fisogen like PEG or Poly-L-orniyhine is used, which leads to reversible permealization of the plasma membrane allowing nucleic acid to enter the protoplast (Krens *et al.* 1982). Secondly, electrofusion can be used (*vide supra*). When the two protoplast first fuses, a cell is produced, which contains nuclei and cytoplasm from both the cells. Depending on the heterologous or homologous protoplast heterokaryon or homokaryon respectively is produced. So far plastid (chloroplast or mitochondria) is concerned, plastid from both the cells do not stay together and finally plastid from only one plant predominates. After fusion the nuclear and cytoplasmic genome reassort and recombine resulting in a wide array of gene combination not obtainable through conventional breeding.

Applications of Cell Fusion:- Cell fusion technique is applied for:

1. Preparation of hubridoma for the production of monoclonal antibodies.
2. Gene mapping.
3. Source of human DNA for preparing chromosome-specific human DNA libraries.
4. Fusion to have required genotype of interest, as here process of combing is enhanced without involving mitosis and hence there is no chance of recombination or segregation, hence desired genes are not lost.
5. Study in the gene expression and differentiation.
6. Study the problem of malignancies.
7. Study of viral replication.

Practice Assignment XIII

1. $NaNO_3$ is used for the fusion of:
   (a) Plant protoplast of different taxa
   (b) Microbial protoplast
   (c) Both of the above
   (d) None of the above.
2. During cell fusion with NaNo3 better results are obtained:
   (a) When cells are treated with Calcium ion under alkaline condition
   (b) When cell are treated with calcium ion under acidic condition
   (c) When untreated cells are used (d) All of the above.

3. PEG is a:
   (a) Non-ionic water soluble surfactant
   (b) Efficient fusogen
   (c) Would efficiently agglutinate protoplast
   (d) All of the above.
4. PEG induced cell fusion can be enhanced by adding :
   (a) α-tocopherol (b) Glycermonooleate
   (c) Retinol (d) All of the above.
5. Electrofusion method of cell fusion is based on:
   (a) Transient change in membrane permeability
   (b) Action of dielectrophoresis
   (c) Both of the above
   (d) None of the above.
6. Liposome mediate cell fusion has the following advantages, Except:
   (a) Has lower level of reproducibility
   (b) Low toxicity
   (c) Long stable storage of DNA fragment
   (d) Protection of nucleic acid.
7. Liposomes are:
   (a) Small size particles containing phospholipids bilayer
   (b) These enclose aqueous chamber
   (c) These entrap water soluble molecules
   (d) All of the above.
8. Various chemicals used for the cell fusion are:
   (a) Poly ethylene glycol (b) Calcium chloride
   (c) Polyvinyl alcohol (d) All of the above.
9. In sendai virus fusion:
   (a) U-V inactivated viruses are used
   (b) Virus particles lies between two adjacent cells to be fused
   (c) Both of the above (d) None of the above.
10. The pearl chain formation on electrofusion, number of cells in pearl chain depends on :
   (a) Population density of the cell
   (b) The distance between the etctrodes
   (c) Both of the above (d) None of the above.

Answers:

1. - (a), 2. - (a), 3. - (d), 4. - (d), 5. - (c), 6. - (a) 7. - (d), 8. - (d), 9. - (c), 10, - (c).

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Chapter 14

Selectable Marker and Antibiotic Resistance

Selectable Markers :

Once the transfection has been done it is essential to know whether the transfected cells contain introduced gene segment or not ? In order to recognise the expression of genes in transfected cell, the transformation vector is designed to contain a marker gene which confers a selectable characteristic on the transformed cells. Selectable marker genes are used for recognition of external genome in transfected cells. These selectable marker genes select by allowing transformed cells to survive on media containing toxic level of the selective agents. It is also used to determine the lineage from which many of the cultures were derived. The unique character (marker gene) which may be able to select the desired character of transformed tissues or cells and is used to screen host to select specific fragment of DNA in gene cloning is known as selectable marker. The introduction of selectable marker gene i.e., screenable gene or scorable gene or reporter gene, allows the transformed cells to proliferate in the presence of selective agent while the non- transformed cells either do not grow or multiply at a slow rate. Efficient expression of selectable markers is judged by for high transfection efficiencies.

Plasmid as Selectable Marker:

Various plasmid vectors having specific genomic region can be used as selectable marker. One such vector is pUC. Its name has been derived from University of California,where it was initially prepared. This plasmid is 2700 bp long and possess ampicillin resistant gene and *lac* Z (β-galactosidase) gene derived from *Escherichia coli.* Within the lac region is also found a polylinker sequence having unique restriction sites. When DNA fragment is cloned in this region of pUC the *lac* Z gene is inactivated. The plasmid when transferred in an appropriate *Esch. coli* and grown in the presence of IPTG (isopropyl-

β-D- thiogalactopyranoside), which is a highly stable synthetic analogue of lactose and induces the synthesis of β-galactosidase enzyme that promotes *lac* utilization. It is used with X-gal to determine the *lac* phenotype in white/blue colony screening. X-gal (5-bromo-4-chloro-3 inodolyl-β-D-galacto pyranoside) is an inert chromogenic substrate for β-galactosidase. β-galactosidase hydrolyzes X-gal into a colourless galactose and 4-chromo-3-brom-indigo, which forms an intense blue precipitate. Induction of the *lac* Z gene with IPTG leads to the hydrolysis of X-Gal and to develop blue colour. It distinguishes recombinant colony (white colour or clear colony), on the other hand pUC having no inserts (non-recombinant ones) and transferred into bacteria will have an active *lacZ* gene and therefore will produce "blue colour colony"(Sambrook and Russell 2001). In this way it permits identification of colonies having pUC vector with DNA segments.

Use of Drug Resistant Gene on Plasmid as Selectable Marker:- Plasmid carry genes that confer resistance to antibiotics (e.g., ampicillin resistance), so bacteria carrying the plasmids can be selected. This can be engineered to have drug resistant genes in addition to other foreign DNA that they contain. Thus a cell with this gene integrated into its genome and correctly expressed as a protein product, can be resistant to drugs that would normally kill the untransfected cells, and can therefore be selected. Retroviral vectors are usually constructed in this way to contain a drug resistance gene and the DNA of interest.

Various chromosomal aberrations (structural rearrangement of chromosomes) result in the changes of phenotypic appearance that is very characteristic and may be useful marker for cell identification.

Functional Chromosome Marker :- 'One gene one enzyme theory', i.e., chromosome carries gene encoding recognizable proteins with different electrophoretic mobility can be readily identified. In hybrid cells resulting from cell fusion, chromosomes with genes for oligomeric enzyme produce distinct hybrid oligomers (so called isoenzyme).

Isotope Labelling – Thymidine labelled with H^3 and its uptake may be used as a selective maker.

Co-transfection :- It is the another way of identification of transfection. In Co-transfection cells can be simultaneously co- transfected by a mixture of two physically unlinked DNA in the calcium phosphate precipitate. It allows the stable introduction into the cultured mammalian cells of any cloned gene. To obtain co-transfectants,

cultured cells are exposed to thymidin kinase gene in the presence of a vast excess of a well-defined DNA such as bacteriophage QX 174 DNA. Tkf transfectant were selected and recovered by molecular hybridization for the unselected QX174 DNA.

For more specific identification of cell types there are a number of markers. These are, Intermediate filament proteins, Cell surface antigens, Expression of functional properties of matured cells and Analysis of isoenzymes.

Intermediate Filament Proteins: -Eukaryotic cells contain a number of proteins collectively known as Cytoskeleton which maintain cellular morphology and also play role in intracellular transport, mitosis and meiosis. Intermediate filament proteins form an essential part of cytoskeleton proteins and are frequently used in the identification of cell types. For example, cytokeratin are indicative of epithelial cells, viruentin of stromal cells, fibronectin of fibroblasts, demin of myogeneic cells, and neurofilaments of nerve and glial cells. The technique used is to enrich cell extracts for intermediate filament proteins and then use antibodies against various intermediate filament proteins to identify cell types. Alternatively, immunofluorescence can be used to directly stain the cell.

Cell Surface Antigens :- Lymphocytes contain various surface receptors. These receptors are useful in sorting out subspecies of lymphocytes. This has been expanded recently due to availability of monoclonal antibodies. With the different cluster of monoclonal antibodies receptors present on the cell are characterized and these are called CD markers. These selectable markers are useful in denoting the different function of the lymphocytes.

Expression of Functional Properties of Mature Cells :- Specialized function *in vivo* are often expressed in the activity of specific protein products, e.g., erythrocytes express Hb and mammary epithelial cells express milk proteins. Induction of these products requires specialized conditions such as addition of hormones and specific substance.

Analysis of Isoenzymes or Isozymes :- Isoenzymes or isozymes are enzymes which exists in animal tissues in multiple forms or in different molecular forms. These catalyse the same reactions. Some cell lines do not express any cell type specific enzymes but the pattern of isoenzymes for some house keeping enzymes may be characteristic for that cell type. Enzymes used to characterize human cell lines are phosphoglucomutase (PGM1 and 3), 6-phosphogluconate

dehydrogenase (PGD), malic enzyme (ME 2) and adenylate kinase (AK-1).

Some enzymes exist in animal tissues in multiple forms and different molecular forms and are enzymes catalyzing the same reaction, these are called isoenzymes or isozymes.

Other Selectable Markers:

Selectable markers other than those mentioned above include Dihydrofolate reductase and associated methotrexate resistance, Rodent CAD, Bacterial XGPRT and Bacterial neomycin phosphotransferase

Methotrexate (4-amino-10 methylfolic acid) (MTX) :

MTX is the analogue of folate and binds with Dihydrofolate reductase (DHFR) an essential enzyme in the synthesis of thymidine. It is a folic acid antagonist. MTX is actively transported in the cell and is converted to a high molecular weight polyglutamate metabolite by folypolyglutamate synthetase that in turn binds to DHFR and inhibits its activity. Cultured wide type cells are sensitive to even 0. 1 μg /ml concentration of the drug. MTX-resistant cell lines have been selected and have been found to fall into three categories i.e., Cells with decreased cellular uptake of MTX, Cells over-producing DHFR, and Cells having structural alterations in DHFR, lowering its affinity for MTX.

It has been shown that cells producing DHFR contain increased number of the gene. The Chinese hamster ovary 'A29' cell line is extremely resistant to MTX and has been shown to excrete increased amount of the altered DHFR. Wigler *et al.* (1980) used genomic DNA of the cell line A29 as a donor of the DHFR gene in co-transfection experiments with MTX-sensitive cells. This system has the advantage that the high gene copy number in donor DNA gives efficient transfection. Additionally the selection system is powerful. It can be applied to non-mutant MTX - sensitive cell lines. Cancer cells which has become resistant to MTX also produces increased level of the enzyme DHFR and thus there is a proportional increase in the DHFR gene copy number. Further highly resistant variants of the transfected cell line can be selected so as to give concomitant amplification of the unselected DNA.

Carbamoyl-Phosphate synthase-Aspartate transcarbamoylase-Dihydroorotase (CAD) : N-phosphonacetyl-L aspartate (PALA):

The CAD is a multifunctional protein complex having three enzymes namely Carbamoyl phosphate synthetase II, Acetyl transcarbamlase and Dihydroorotase. These three enzymes are involved in the synthesis of first three steps of *denovo* uridine biosynthesis. One of these activities i.e., activity of acetyl carmamylase is specifically blocked by the action of a PALA and thus can be used as selectable maker. PALA-resistant mammalian cells over produce CAD from highly amplified copies of the CAD gene in a manner to MTX resistance. The CAD gene of the Syrian hamster has been cloned on a cosmid vector in *Esch. coli* and has been shown to provide a dominant amplifiable genetic marker that can be selected in a non-mutant cell on the basis of resistance to high concentration of PALA.

XGPRT : Mycophenolic Acid Resistance :

The *Esch. coli* xanthine - guanine phosphoribosyl transferase (XGPRT) is a bacterial analogue of the mammalian HGPRT. However, by contrast with HGPRT, it has the additional ability to convert xanthine to XMP and hence ultimately to GMP. Only hyopxanthine and guanine are substrates of HGPRT. Mulligan and Berg (1980) have cloned the XGPRT gene and incorporated it into a variety of vectors in which transcription of the bacterial gene is directed by a SV 40 promoter. Such constructs are capable of transfecting $HGPRT^-$ cells to $HGPRT^+$, but more importantly provide a selectable marker for non-mutant cells in medium containing adenine, mycophenolic acid and xanthine. The selection can be made more effective by adding aminopterine which blocks endogenous purine biosynthesis.

Green Fluorescent Protein (GFP):

This is recently found marker from *Victoria aqueoria* and is tremendously employed as selectable marker since it is non-toxic, easily selectable by irradiation with U-V light and O_2 /ATP is not required. With tagged gene of interest, the GFP is also encoded and expressed in the infected cells and if the cultures cells have unable to incorporate the gene of interest with tagged GFP gene then no fluorescence will be observed after the cells will be irradiated.

Chimeric Selectable Marker:

Various chimeric selectable marker, such as 35S/NPH, NPII (neomycin phosphotransferase II gene) and NoS (Nopaline synthetase) are used

to characterize cells in eukaryotes when procaryotic resistant gene is used.

Table:-8: Amplifiable Selectable Markers for Animal Cells

Selection	Gene	Reference
Methotrexate	Dihydrofolate reductase	Kauffmen *et al.*, (1985)
Cadmium	Metallothinase	Beach & Palmike (1981)
PALA	Aspartate transcarbomylase	Wahl *et al.* (1984)
Adenosine, alanosine and 2'-deoxycoformycin	Adenosine deaminase	Kauffman *et al.* (1965)
Adenosine, azaserine and coformycin	Adenylate deaminase	Debatisse *et al.* (1981)
6-Azauridine and pyrozofuran	UMP synthesis	Kanalas & Sutts (1957)
Mycophenolic acid	Xanthine-guanine phosphoribosyl transferase	Chatma *et al.* (1983)
Multiple drugs	p-glucoprotein 170	Kane *et al.* (1980)
Methionine sulphoximine	Glutamine synthetase	Coe...*et al.* (1990)
B-aspartylhydroxamala or Albizziin	Asparagines synthetase	Cartier *et al.* (1969)
Alpha-difluoromethylornithin	Ornithin decarboxylase	Chian & McConlayes (1988)

Selectable Markers Used in the Plants:

Antibiotic resistant gene has also been employed in plants as selectable markers. The most commonly used selectable marker antibiotic resistant gene in legume is neomycin phosphotransferase (*npt* II), which imparts resistance to the antibiotic kanamycin or its analogues, genticin or paromomycin by inactivating them by phosphorylation. However, effective phosphorylation of tranformed cells with *npt* II was found difficult in some legumes due to their high level of inherent tolerance to kanamycin (Schroeder *et al.* 1993).

The hygromycin phosphotransferase (*hpt*) gene which confers resistance to hygromycin, also provides efficient selective markers in peas as the untransformed cells were killed faster and the plant regeneration from transformed cells was not inhibited. The successful

selection of transformed plants using hpt gene has been accomplished in pea (Lusdorf *et al.* 1991 and Dhier *et al.* 1991).

But recently antibiotic resistant gene as selectable markers both in legumes and cereals has been replaced with genes for herbicide tolerance for selecting transformed cells. The most widely used herbicide for selection is Basta (glufosinate or phosphinothricin). The *bar* gene encodes for phosphinothricin, the active ingredient in the herbicide Basta. Bialaphos and glufosinate the active ingradient of the herbicide Liberty have allowed efficient selection of transformants in pea, soyabeen, lupinus, *Madicago sativa* and *Triofolium subterraneum.* In pea, both the CaMV355 promoter as well as *nos*-promoter have been used to drive the bar gene.

Antibiotic Resistance and Antibiotic Resistant Gene as Selectable Marker :

Antibiotic resistance is the most widely method used to select stable transformant. Drug resistance gene which are mostly found on plasmid may be resessive or dominant. Gene conferring recessive drug resistance require a particular host which is deficient in the activity is being selected. Gene conferring dominant drug resistance can be used independently of the host. Drug resistance gene helps to select the drug resistant mutants from the sensitive population.

The discovery of penicillin by Alexander Flaming in 1928 was infact the greatest landmark in the history of antimicrobial therapy and this provided a potent weapon against many diseases. The apparent innocuous nature and low toxicity of this drug qualified it for the label "miracle" drug. The constant exposure of the body flora of man and animals to antibiotics has led to the wide dissemination in the human population of antibiotic-resistant strain of bacteria and the transfer of resistant genes from one bacterial stain to another has greatly widened the range of organism in which resistance are prevalent. Antibiotic resistance has two sorts of ill effect in human and veterinary medical practice :-

(i) Infections with important pathogens can no longer be treated sucessfully with the antibiotics of choice or, in the worst circumstances, with any available agent and

(ii) Giving an antibiotic to a patient may lead to the overgrowth of other resistant bacteria already present in the body flora or acquired by the patient from another person while treatment is in progress.

Microorganisms have developed various ways to resist the toxic effects of antibiotics and other drugs. (Neu 1997). Antibiotic resistance determined by plasmid was first discovered in Japan in 1959. Until then it was thought that the antibiotic resistance in bacteria was the result of selection of chromosomal mutants and multiple resistance was due to sequential accumulation of mutation. Drug resistance refers to acquired genotypic changes that persist during cultivation of all organisms in the absence of a drug and not the natural resistance of a species. Genes essential for bacterial growth is carried on chromosomes while plasmid genes are essential associated with specialized functions. Genes with independent evolutionary origin may be assimilated by plasmid that is widely disseminated among bacterial population. As consequence of such a genetic event, swift spread among bacteria population of plasmid borne resistance to antibiotics after their indiscriminate use has been observed.

Mechanism of Resistance:

Resistance of bacteria ageist commonly used antibiotics, whether inherent or acquired, can be ascribed to one of the four types of mechanisms:

- Production of drug-inactivating enzymes
- Reduced affinity for a target
- By passing of a sensitive target
- Impermeability to a drug
- Active extrusion of drug from the cell.

Production of Drug-inactivating Enzyme:- Some of the bacteria produce enzymes which inactivate the action of antibiotics by hydrolysis or formation of inactive derivatives. Well known example is the production of penicillnase enzyme, β-lactamase (Bush *et al.* 1995) by some of the strains of *Staphylococcus aureus* which inactivates the action of penicillin, enzyme that phosphorylate, adenylate or acetylate aminoglucoside antibiotics.

Reduced Affinity for Target:- In this type of resistance cellular target of antibiotic action is altered by mutation or enzymatic modification in such a way that the affinity of the antibiotics for the target is reduced.

By Passing of a Sensitive Target:- A third mechanism of the resistance is the by passing of antibiotics the sensitive target of the action thus antibiotics could no reach to the actual proper site resulting into the non-action of antibiotics.

Impermeability to a Drug:- Here there is inhibition of drug entry into the cell. Due to the low permeability of the outer membrane of Gram-negative bacteria and the exceptionally efficient barrier of the Gram-positive bacteria, drug diffusion across the cell envelop is reduced. The permeability of the outer membrane can be further decreased by the loss of porins. These barriers however, can't prevent the drugs from exerting their toxic action. Once they have entered the cell and the active efflux of drugs is essential to ensure significant level of drug resistance.

Plasmid and its Significance in Antibiotic Resistance:

The word "Plasmid" was introduced by Joshua in 1952 and was defined as an extra-chromosomal circular genetic element, which replicates independently to the bacterial chromosome. It was supplemented for a while by "Episome" a term proposed by Jacob and Wolfman in 1968. Later episome was suggested for the chromosomal material which remain either in free form or in combined form, but now for both the term the recommended terminology is plasmid.

Plasmids are extrachromosomal molecules of DNA that vary in size from 1kb to more than 200 kb. Most of them are double stranded covalently closed circular molecules acting as dispensable accessory source of DNA that provide unique function to bacteria e.g., R-factor and Resistance Transfer Factor (RTF) and several other things.

Some scientists are of opinion that Plasmids and viruses were derived ancestrally from cell chromosomes by cyclization of small excised segments of DNA including sites for autonomous replication. This primordial DNA could then have evolved by gene duplication and mutation plus incorporation of additional host DNA.

Plasmids are found in a variety of bacterial species, most plasmids have a narrow host range and can be maintained only in a limited set of closely related species. These are inherited independently of bacterial chromosome and replicate autonomously and are dependent on the enzyme and proteins of the host cell for their multiplication. Plasmids have evolved a variety of mechanism to maintain a stable copy number in the bacterial host and to partition plasmid molecules accurately to daughter cells

R -factors :

These are groups of conjugative plasmids of medical and veterinary interest. Antibiotics have not induced but have selected for resistance

plasmids.

The classic R-factors are large plasmids with two fundamentally distinct parts, viz., RTF (Resistance transfer factor) - 80 kb long, containing genes for autonomous replication and conjugation and R-determinants (resistance determinants) which are similar. It varies widely in size and in its content of genes for antibiotic resistance, i.e., R genes. R-plasmid possesses few distinct types of information, viz., gene for conjugal transfer and gene for antibiotic resistance.

Gene for Conjugal Transfer:-These genes dictate both the synthesis of pilli and the transfer of DNA.

Antibiotic Resistance Gene:

These genes in R-plasmid are symbolized differently than comparable genes in *E. coli* chromosomes. Thus, chromosomal streptomycin gene is denoted as str-r while the plasmid borne gene is written as Sm. Chromosomal and plasmid genes express the resistance in different ways. For chromosomal genes the resistance is typically the result of an alteration in ribosomal protein whereas plasmid gene typically dictate the synthesis of enzymes that inactivate the antibiotic as it enters the cell. e. g., Cm gene directs the synthesis of chloramphenicol acetyl transferase, an enzyme that adds an inactivating acetyl group to the choramphenicol molecule. Some R-plasmids carry only one resistant gene whereas others may carry more than one. The penicillinase plasmid of *Staph. aureus* encodes the inducible enzyme, β-lactamase. It is induced when cells are exposed to penicillin.

Transposons

Transposons are genetic elements that contain several kb of DNA including information for their migration from one genetic locus to another and persist because they insert themselves into preexisting chromosomes or plasmids and replicate along with the host DNA but lack ability to replicate themselves.

Simple and Complex transposons: Insertion sequence (Is) carry only the genetic information for insertion while the Complex transposon carry genes for specialized functions such as antibiotic resistance. Unlike plasmid, transposon (Tn) does not contain genetic information for their own replication. They move from one plasmid to another or from one site to another within same plasmid. A typical Tn carries one or more genes for antibiotic resistance and two inverted repeats

at its terminal and there appears to be essential for inserting the Tn into recipient replicon.

Transposon Plasmid Interaction

R-plasmid can pick up additional transposons. If a bacterium harbors an R plasmid carrying Tn10 which encodes for tetracycline-resistance and if it is co-infected by a plasmid carrying Tn3 which encodes for ampicillin- resistance, the two plasmids can interact in such a way that copy of ApTc transposon is inserted into Tc carrying plasmid. The bacterium will then carry and can transmit to its progenies a TcAp plasmid carrying simultaneous resistance to the two antibiotics.

The mobility of transposon and ability of a given R-plasmid to 'pile on' large number of resistance genes has major implication in both medical and veterinary medicine.

Indiscriminate use of antibiotics in medicine and agriculture has created a pool of drug resistant bacteria that can transmit their resistance genes to infecting pathogens and other bacteria in polluted environment.

Antibiotic Resistant Gene as Selectable Marker:- Various antibiotic resistant gene can be used as selectable marker as genetically modified cells can grow in the presence of such antibiotics. Some of them are as follows:-

β-lactamase Marker Gene : -It is being felt that the use of b-lactamase which is produced by many bacteria, may be useful as selective marker as this will not transfer antibiotic resistant gene to gut flora of the person consuming genetically modified food. b-lactamase hydrolyzes the β-lactam ring and this inactivate the antibiotic making them resistance. The expression of *Amp* C genes have significant role in the resistance phenomenon to b-lactams. Organism expressing *Amp* C b-lactamase are usually resistant to all the b-lactam drug except for cafepime, Cafpirome and the carapenems (Livermore 1995).

Kanamycin and Hygromycin Resistant Gene:- These resistant marker genes have been commonly used for the selection of genetically modified plants.

Ampicillin Resistant Marker Gene:- It is commonly used for the selection of genetically modified bacteria.

Neomycin Phosphotransferase : G 418 resistant marker gene:- Bacterial transposon (segment of DNA that can move from one position in genome to another) in Tn5 and Tn601 encode distinct

neomycin phosphotransferase whose expression confers resistance to aminoglycoside antibiotics (kanamycin, neomycin, G418) which are protein synthesis inhibitors, active in bacterial or eukaryotic cells. Berg (1981) incorporated neomycin phosphotransferase gene into construct analogous to those containing XGPRT. Other constructs have linked neomycin transferase gene to the Herpes simplex virus in an *E. coli* plasmid. Antibiotic resistance can select Transfectants of non-mutant mammalian cells, which contain such constructs. Colbere - Gaspien *et al.* (1981) demonstrated the application of their constructs to the co-transfection of a variety of cell lines for different mammalian species. Grosveldt *et al* (1982) have also constructed cosmid (DNA cloning vector used for cloning large DNA fragments containing plasmid and phage sequence, the genome library is constructed on cosmid). Cloning vectors which include selective markers for growth in the host bacterium (beta-lactamase) and animal cells. The markers of the animal cells were neomycin phosphotransferase, HSV thymidine kinase or XGPRT. Such cosmids can be used to construct libraries of eukaryotic genes from which a particular recombinant can be isolated. The recombinant cosmid DNA can be transfected into animal cells at high efficiency where transfectant in which the DNA has integrated into the nuclear genome can be readily selected. The process of at aminoglycoside antibiotic resistance as a selection system in eukaryotes is now very evident.

Practice Assignment XIV

1. Selectable markers are used:
   (a) To know whether the transfected genome has been expressed or not,
   (b) To recognise expressed gene in transfected cell
   (c) Both of the above
   (d) None of the above.
2. pUC plasmid vector :
   (a) Has specific genomic region(b) Can be used as selectable marker
   (c) It was named as it was prepared in the University of California
   (d) All of the above.
3. pUC having no inserts and transferred into bacteria will have an active lacZ gene and therefore will produce:
   (a) White colony (b) Blue colony
   (c) Both of the above (d) None of the above.

4. pUC plasmid;
   (a) Posses ampicillin resistant gene
   (b) Lacks lacZ gene
   (c) It is derived from *Escherichia coli*
   (d) All of the above.
5. For the identification of specific cell types markers used are:
   (a) Intermediate filament protein
   (b) Cell surface antigen
   (c) Analysis of isoenzyme
   (d) All of the above.
6. Viruentin is indicative of production of:
   (a) Myogenic cell type (b) Glial cell type
   (c) Somatic cell type (d) Fibroblast cell type.
7. MTX is the:
   (a) Analog of folate (b) Binds with DHFR
   (c) Both of the above (d) None of the above.
8. Dihydrofolate reductase is:
   (a) A folic acid antagonist
   (b) Is sensitive to methotrexate
   (c) Is used as selectable marker
   (d) All of the above.
9. The activity of the following enzyme is inhibited by PALA (N-phosphonacetyl-2-aspartate), Except:
   (a) Aspartate transcarmylase
   (b) Carbamyl phosphate synthetase
   (c) Dihydroreductase
   (d) None of the above.
10. Xanthine-guanine phosphoribosyl transferase (XGPRT):
   (a) Is a bacterial analogue to mammalian HGPRT
   (b) It can convert xanthin to XMP and GMP
   (c) Both of the above
   (d) None of the above.

Answers:

1. -(c), 2. -(d), 3. -(b), 4. -(d), 5. -(d), 6. - (c), 7. -(d), 8. -(a), 9. - (c), 10. -(c).

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Chapter 15

Hybridoma and Monoclonal Antibodies

Generally when an animal is immunized against any whole micro-organisms, antiserum obtained is of polyclonal nature because the antibody is produced against all the epitopes of the organism present on the microbial antigen. This polyclonal antisera due to its polyclonal nature are inherently heterogenous, so it may hinder the specificity of test when used as it is. Thus, a need for developing monoclonal antisera was felt. Some of the monoclonal antisera was obtained by selective adsorption. But it was felt that antibody producing cell should produce monoclonal antibody for a unlimited period of time and this was not possible by a normal antibody producing cells as these were having limited life span. Though the rate of antibody production by B-cells can be enhanced through molecular mechanisms, i.e., by artificially augmenting the promoter enhancer sequence (Gulzman and Shenk 1983) but this can also be achieved when an antibody secreting cells should be fused with the cancer cell, which has capacity for multiplication for unlimited period of time. This became possible only in 1975 when George Kohler and Ceaser Milstein succeeded in developing a hybrid cell by fusing antibody secreting cells with myeloma cell line. Thus, the advent of hybridoma technique whereby a cell line capable of secreting a single species of antibody has in fact led to a spurt in biotechnological application of cell culture system.

Three important steps in sequence are:

1. Immunization of appropriate animals with the antigen under study.
2. Fusion of the suitable drug-resistant myeloma cell line with the plasma cells obtained from the spleen of the immunized animal.
3. Selection and cloning of the hybridoma cells (cells that grow in culture and produce antibodies of desired class and specificity against the antigen of interest).

Preparation of Antigen - About 10-15 microgram of antigen is taken in 0. 1 ml of normal saline. Equal volume of Complete Freund's Adjuvant (CFA) is added. Emulsion is made using glass syringe and needle. If a small drop of the emulsion put over water remains as discrete globule, the emulsification is considered complete.

Immunization - Although different strains of mice have been used, BALB/c strain is the most commonly used as the most available myeloma cell lines for the splenocyte fusion partner are derived from a MOPC-21 (BALB/c) myeloma. So use of BALA/c mice will maintain the compatibility with the myeloma cell. Administering the antigen of interest stimulates the immune system of the mice. BALB/c mice is immunized with 0. 1 ml of the emulsion. The route of administration may be intraperitoneal (lower part of abdomen) / subcutaneous (hind flap or around neck) / intramuscular (in thigh) / intravenous (caudal vein). Booster dose is given subcutaneously with the same dose of the antigen emulsified with Incomplete Freund's Adjuvant (IFA) after an interval of three weeks. If the cell fusion is carried out shortly after primary immunization without a boost, one gets primarily an IgM antibody response. Repeated immunization will give rise to hybridoma that make IgG antibodies. Test bleeding is done after two weeks. Mice can be bled from heart/ retro-orbital plexus of eye /tip of tail. The titer is checked by ELISA. Four days before fusion another booster of 10. 0 μg of antigen diluted in normal saline is given intravenously.

Preparation of Antibody Secreting Cells:

- The immunized mouse is sacrificed with ether.
- Spleen is removed aseptically in a Petrie dish containing 10. 0 ml of RPMI-1640*. The best result is obtained when a pool of 3 to 4 spleens are used for fusion.
- The spleen is teased with sterile forceps to release cells. Using a 10. 0 ml syringe and 21 gauge needle, the cell suspension is drawn and ejected 2-3 times to obtain single cells. The process is repeated 2-3 times using 23gauge needle.
- The cell suspension is taken into a 10ml centrifuge tube and centrifuged at 200xg for 5 minutes.
- The pellet is resuspended in RPMI. 50 micro liter is taken with a pipette and mixed with 450 micro liter of 0. 9% ammonium chloride to lyse red blood cells.

- The cell suspension is centrifuged again at 200x g for 5 minutes. The Lymphocytes in the pellet are counted with haemocytometer.
- The pellet is resuspended in 10ml of RPMI. For use as control, 0. 5ml of the suspension is taken out in a tube.

Myeloma Cell Line and its Preparation:

There are several murine cell lines available for use as the fusion partner in hybridoma production. The fusion partner should be of a transformed (tumour) cell line if it is to confer immortality to the hybrid and should be of similar lineage. In the original hybridoma production, Kohler and Milstein (1975) used P_3 myeloma cell line which used to produced antibody itself but later they used NS-1 myeloma cell line that secreted only endogenous ê-light chain. These cell lines also resulted into contamination of the original antibody for which B-cells were primed. Now a days there are several HGPRT (Hypoxanthine-Guanine phosphoribosyl transferase) lacking mutant of myeloma cell lines are available, which are non-secretory. These are sp2/0-Ag14 (sp2), NSO, P3-X63-Ag8. 653 (653) (Kearney *et al.* 1979). MPC11-45. 6TG1. 7 (45. 6) and FOX-NY etc.

- The myeloma cell lines are obtained from any of the virus reference laboratory. Myeloma cell line used in the fusion should be healthy and for this after obtaining cell line from reference laboratory this should be passaged daily at an optimal concentration of 4. $5x10^5$ commencing 3-4 days prior to fusion to give enhanced viability, fusibility and clonability. After that these are harvested and centrifuged at 200x g for 5 minutes and suspended in 10. 0ml of RPMI.
- The myeloma cells are so diluted in RPMI as to contain one-fourth the number of spleen cells. Some myeloma cells are kept aside for use as control.

Cheking the Viability of Splenocytes and Myeloma Cell Line:

- The above mentioned splenocytes are examined for their viability by trypan blue dye exclusion test (Patterson 1979). Trypan blue is the most commonly used dye as it can be used with both living material and also material fixed with glutaraldehyde. It also gives reproducible results both before and after fixation. The viability must be 95. 0% for proceeding with the hybridization.

Fusion :

Fusion can be induced either chemically, virally or electrically. Kohler and Milstein (1975) reported fusion of antibody producing mouse spleen cells with mouse myeloma cells. For fusion most commonly fusogen used is Polyethylene glycol (PEG) (Pontecorvo, 1975). Different lots of PEG differ in terms of toxicity and thus to prevent toxic effect of PEG on cells a critical compromise must be made in terms of concentration/time of exposure between increased fusion and toxicity. In a 50. 0ml centrifuge tube, the suspensions of myeloma cells and the spleen cells are mixed in a ratio of 5-10:1 (i.e. 0. 5-1x10^8 splenocyte per 1x10^7 myeloma cells). The mixture is centrifuged at 200x g for 5 minutes. The supernatant is discarded and the pellet is gently broken. The procedure in brief is as follows:

- PEG (mol. wt 1500) (50% W/V)- 1. 0 gm in a screw capped glass tube is autoclaved at 121^{0}C or 15 lbs/in^2 for 20 minutes to sterilize and liquefy. While PEG is still hot, 1. 0ml of RPMI is added to it.
- The pH of PEG is adjusted to alkaline (pH 7. 6 to 7. 8) by adding few drops of sterile 0. 1N NaOH. The colour attained is light pink.
- 5 ml of PEG solution is added drop by drop to the cell pellet over a period of 30 seconds. The cells in the pellet are gently mixed with a pipette for 30 seconds and left undisturbed for another 30 seconds.
- A tube containing 10. 0ml of RPMI is taken. 5. 0ml of it are slowly added into the cell pellet over a period of 90 seconds. The tube is gently rocked during this period. Then all the remaining 5. 0 ml of RPMI is added at a time.
- The cell mixture is incubated at room temperature for 5 minutes.
- In a 50. 0ml centrifuge tube, the suspensions of myeloma cells and the spleen cells are mixed. The mixture is centrifuged at 200x g for 5 minutes. The supernatant is discarded and the pellet is gently broken.

N. B. :- DMSO may be included as it besides enhancing fusion (Norwood *et al.* 1976) it facilitates the removal of PEG after fusion and perhaps buffers the potentially damaging rapid osmotic changes in the actual fusion and subsequent steps.

Selection of Fused (hybrid) Cells:

The fused cells are selected by culturing the hybrid cells on

HAT**(Hypoxanthine Aminopterin and Thymidine) medium, called **HAT selection** which can be done by the following methods:

Nucleic acid synthesis in a cell follows two pathways. Cells in normal situations follow *de novo*-pathway. In case this pathway is blocked, the cells have to follow *Salvage pathway* in order to remain alive. Here the cells use hypoxanthine and guanidine as substrate for purine synthesis and form inosinic ribose phosphate and guanine acid ribose phosphate respectively with the help of HGPRT (Hypoxanthine-guanine phosphoribosyl transferase). HGPRT allows the cells to take up hypoxanthine, a nucleotide precursor, from the culture medium through transmembrane transport and converts into guanine needed for DNA synthesis in Salvage pathway. For the synthesis of pyrimidine, the *salvage pathway* uses thymidine to form thymidine monophosphate with the help of thymidine kinase. If both these are blocked, cells cannot survive.

For selection of the hybrid cells HAT (Hypoxanthine, aminopterine and thymidine) medium is used. The aminopterin of HAT medium is a folate antagonist, inhibits dihydrofolate reductase required for *de novo* purine (pyramidine and glycine) systhesis. The mutant cells (Sp/2) and spleen cells (lymphocytes) from immunized mouse are sensitive to HAT medium because these are TK^+ HGPRT -and TK^- $HGPRT^+$ respectively. However, the hybrid cells are TK^+HGPRT^+ and thus insensitive and so grow selectively in the HAT medium. In the presence of aminopterin normal cells survive if supplied with hypoxanthine and thymidine but the mutants die since these are HGPRT- and cannot synthesize nucleotide from hypoxanthine and the TK-cells cannot use thymidine. The hybrid cells only grow because of these have the genes for both HGPRT and TK.

The culture plates are examined daily for growth of colony and the colour of the medium indicate condition of the culture medium. The hybrids are fed for four to five days by changing the medium of each well (removing 0. 5ml of the medium and adding fresh medium HAT gently). The control myeloma cells by now have mostly died.

The plates are examined further for next five to seven days. When colonies are macroscopic and the colour of the medium has changed to yellow, the hybrids are considered ready for screening and selection. This timing is critical. Samples taken early are associated with risks of missing antibody-producing hybrids as the concentration in the supernatant would be low. Delay is associated with risk of overgrowth.

- Supernatant is taken from wells containing hybrids and is tested for antibody activity by ELISA.
- Fresh medium is added to wells to replenish.
- This is repeated after 3-4 days. Sometimes the antibodies produced by the surviving spleen cells may affect the result.
- Colonies producing the desired antibodies are expanded, by withdrawing cells and medium into a sterile pipette and transferring to a well of a 24-well plate containing 1-2ml of HAT medium. When there are sufficient number of cells, these are frozen.
- The pellet is suspended in HAT medium* at a spleen cells concentration of 10^7/ml.
- A 24 well plate is taken and 1. 0ml of HAT medium* is dispensed in each of the wells.
- From the fused cells suspension 50 micro liter is added to each of the 22 wells.
- In the remaining two wells add 50 micro liter myeloma cells and spleen cells respectively as controls.
- Plate is incubated in humid, 5. 0% CO_2 at 37^0C.

The hybrid secreted antibodies of the specificity dictated by the spleen cells and retained the growth characteristics of a myeloma.

Feeder Cell Layer and Myeloma Conditioned Media:

The three cell populations commonly used as feeder layer are splenocytes, peritoneal exudates cells and thymocytes. Many scientists believe that it is the macrophage contaminating the splenocyte and thymocyte feeder layers which exert the major beneficial effect in conditioning the medium and in ingesting the debris and dead cells.

Spleen Cells :- Splenocytes may be used as feeder cells. The procedure for harvesting non-immune spleen cells to be used as feeder layer is identical to that for the immune splenocyte to be used in the fusion. Spleen cells are used in the density range of 2-5x10^5/ml. At the higher doses stromal fibroblasts may overgrow the bottom requiring additional feedings for assay. Though splenocytes do not multiply in culture yet some worker is of opinion that splenocytes to be used as feeder layer be irradiated at 2000-2500 rad. before use.

Peritoneal Exudate Cells (PEC):- Cold tissue culture medium at 4-10^0C is injected intraperitoneally in a mouse and drawn back into a

syringe. The medium would contain PEC. Yield of PEC is increased from a single mouse upto 2x-10x by priming the mice with pristane (4, 8, 12, 16-tetramethylpentadecane).

- 100 micro liters of spleen cells ($5x10^4$/ml) or PECs (10^4/ml) are dispensed into each well of a 96-well plate.
- Hybrid cells are counted and diluted in HT medium to a concentration of 10 cells/ml.
- 100 micro liter of diluted hybrid cell suspension is added to the wells containing feeder cells.
- The plate is incubated in humid, CO_2 incubator at 37^0C.
- In 7-10 days colonies are visible. The supernatant is tested for the specific antibodies.
- This process is repeated at least three times to ensure that antibodies are monoclonal. Whenever a positive clone is expanded, a part is cryopreserved.

Thymocyte Feeder Layer:- Thymocytes are supposed to contribute unique factors supporting hybridoma cells. It is generally added in high densities. It is less contaminated than spleen cells with stromal fibroblasts, which may overgrow certain wells requiring more frequent feedings. Thymocytes are obtained from the thymus. Thymus gland which is just situated above the heart is removed by taking all aseptic precautions and then this is minced with plunger of 3-10 ml. glass syringe by keeping it on a sterilized stainless steel strainer having fine mesh and using any tissue culture medium (HBSS). The whole content is taken in a centrifuge tube and centrifuges at room temperature for 10 min. at 1200 rpm. After centrifugation supernatant is discarded and the cells are resuspended in HBSS.

The hybrid secreted antibodies of the specificity dictated by the spleen cells and retained the growth characteristics of a myeloma.

Cloning of Hybrid Cells:

In order to avoid growth of unwanted cells, first cloning should be done as soon as possible. Cloning may be done by (a) limiting dilution method or (b) by soft agar cloning.

Limiting Dilution Method : The cloning is performed by limiting dilution method in 96-well culture plates. The principles of cloning by limiting dilution is intuitively obvious: the more wells positive for growth, the more clones present on average per well. So, dilution of cells is done up to a level where only one viable cell per well is expected

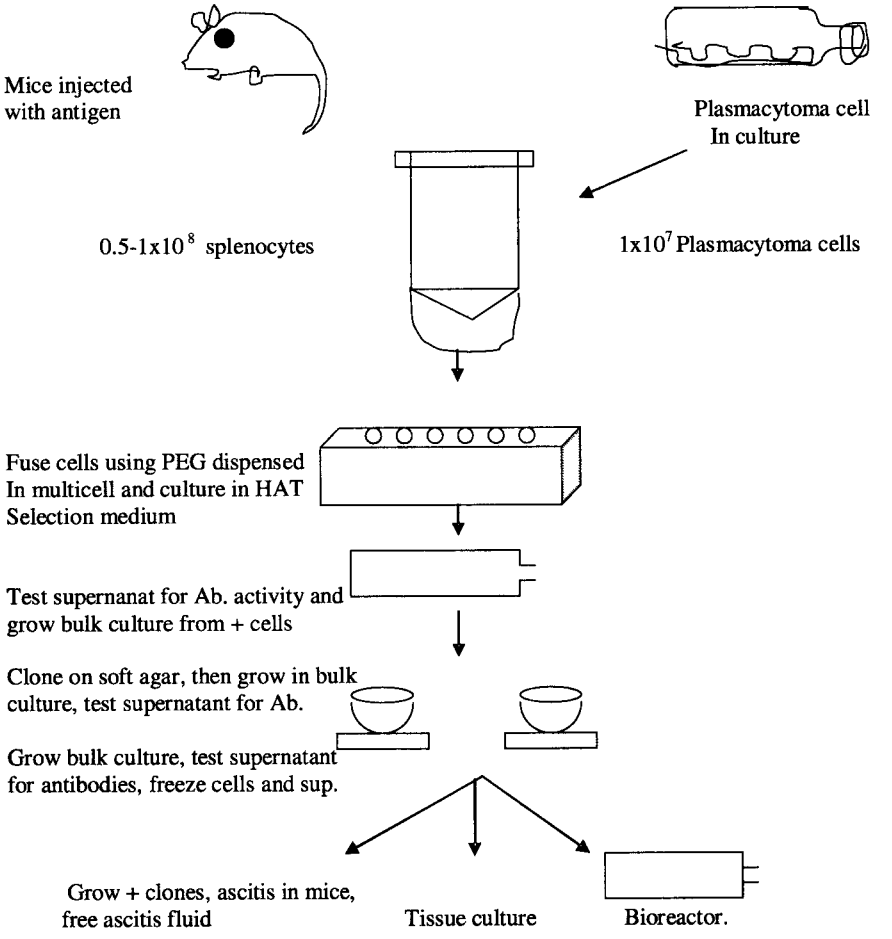


Fig. 14. Protocol for the Production of Mab.

in a culture plate and this cell is expanded into a colony (i.e., colony derived from single cell). It is advisable to put up several wells and use different cell concentrations, usually one cell or less per well for cloning. Thus it is possible to generate antibodies derived from a single clone, which are known as monoclonal antibody. Monoclonal antibody from one clone is identical in its antigen specificity and affinity. The percentage of wells with macroscopically visible growth indicates the probability of monoclonality after cloning (Deblas *et al.* 1983).

(b) Soft Agar Cloning:- In this technique to hold the colony together and prevent mixing, the cells are suspended in agar or methocel or plated out over on agar underlay or cnto non-tissue culture grade dishes. Here each cell line reacts in a unique way. This technique is successfully used to select and clone cells which have been transfected are differentiation-resistant and retrovirally infected and those which are metabolically deficient. This technique allows the workers to be confident that cell colonies are cloned while simultaneously enabling the cells to be plated out at a reasonably high density.

Media Used in Hybridoma:

1. RPMI-1640 (Rosewell Park Memorial Institute) or DME (high glucose) :- These media are customarily used. These media should contain glutamine along with buffering system i.e., bicarbonate/CO_2 The pH may be held more constant by addition of 10-20 mM HEPES [4-2(hydroxyethyl)-1piperazine ethane sulfonic acid]. It should be noted that HEPES is toxic to cells permeabilized by PEG in the fusion step. The solution is sterilized by membrane filtration of 0. 2μ and stored at 4^0C

2. HAT Medium :-100x HAT stock containing hypoxanthine 13. 6 mg/ml; aminopterine 0. 019mg/ml; and thymidine 0. 388mg/ml is prepared and stored at -20^0C in dark. For use, 1. 0ml of 100x stock is added to 100. 0ml of 20. 0% Fetal Calf Serum (FCS)-supplemented medium.

3. HT Medium :- 100x HT medium is prepared in the same way as 100x HAT medium without adding aminopterine. It should be diluted 1 in 100 before use.

4. Sterile NaOH (0. 1 N) :- Four grams of NaOH dissolved in 100 ml of distilled water and sterilized by filtration through 0. 22 μ filter.

Scaling up of monoclonal antibodies :-

The scaling of monoclonal antibodies can be done by:

(a) Growing hybridoma in tissue culture :- The hybridoma is cultured in the growth medium at a concentration of 1-2x10^6/ml in 75 cm^2 flasks at 37^0C in 5. 0% CO_2 incubator for 24 hours. The cells are harvested and supernatant is collected and can be stored at -20^0C. About 5. 0 microgram/ml of Mab is the yield. Though through this method less amount of monoclonal antibody is obtained but the products are free from contamination with endogenous immunoglobulin.

(b)Scaling up of Mab in Ascites Fluid of Mouse :-

This is the *in vitro* method of scaling up of the monoclonal antibody. For this inbred Balb/c mouse (NII, Jackson Laboratories) is primed by injecting 0. 5ml of pristane (2, 6, 10, 14-tetramethylpentadecane, (Aldrich Chemical Co.) intraperitoneally. After one week to two months, mouse is ready for use. For production of the hybrid cells. Hybrid cells in the concentration of 5-10x10^6 are suspended in 1. 0ml of RPMI-1640 and injected intraperitoneally into the priatane primed primed mice. After about 10-14 days there is bulging of the mouse peritoneal cavity. The ascitis fluid is drained using 18G needle in a 10. 0ml sterile tube. The ascitic fluid is centrifuged at 200xg for ten minutes. The clear fluid (supernatant) is aspirated in a 10. 0ml sterile tube. The pelleted cells are suspended in 2-3. 0 ml of PBS (50mM, pH 7. 4). A drop from this is examined under microscope. If healthy hybrid cells are seen, the suspension is injected in pristane primed mouse. The antibody activity in ascitic fluid is checked, small aliquots are made and stored at -20^0C.

(c)Scaling up by Using Different Bioreactors:- Various bioreactors can be used for scaling up of monoclonal antibodies. For other methods of scaling up refer Chapter 6.

Cryopreservation and Maintenance of Hybridoma :

Hybridoma cell lines and other human and animal cell lines grown in culture are generally stored frozen. Freezing protect the cell line from changes due to genetic drift and minimize the risk of contamination. Liquid nitrogen used in conjunction with a cryopreservation agent such as DMSO has become a widely used method for preserving cells. Without the use of cryopreservation agent, freezing is lethal to most cultured cells. Generally damage is caused by mechanical injury by ice crystals, concentration of electrolytes, dehydration, pH change and denaturation of protein. These lethal effects are minimized by adding DMSO which lowers the freezing point and allows for a slower cooling rate. The following protocols are followed for cryopreservation:.

- Healthy cells are harvested. Adherent cells are harvested by using trypsin or other appropriate means.
- Cells are suspended in a concentration of 2 to 5x10^6 per ml in freezing medium. Freezing medium contains 70% culture medium (RPMI-1640 or DME), 10 to 20. 0% FCS, 5 to10. 0% DMSO and

this is distributed as 1. 0 ml in each of a 2. 0ml sterile labeled cryovials (plastic) and then capped tightly.

- Slow freezing is achieved by wrapping the cryovials in cotton and then placing in polystyrene foam or thermocool box and storing overnight at -70^{0}C in deep freezer.
- After 24 hours these cryovials are transferred to liquid nitrogen.
- To maintain high viability never allow frozen cells to warm above -40^{0}C.
- Subsequent use can be made after thawing. Cryotubes taken out from liquid nitrogen are cleaned with 70. 0% alcohol.
- Quick thawing is achieved by adding 1-2ml of warm medium (37^{0}C).
- Transfer the cell suspension immediately after thawing into a 10. 0ml tube containing 5-6 ml. of growth medium.
- The cells are then centrifuged at 200xg, the pellet is resuspended in fresh growth medium and then cultured in 25-cm^2 flask.

Use of Monoclonal Antibody:

1. Diagnostic in identification of pathogenic strains. Non-specific reaction is reduced.
2. Mabs can be tagged with fluro isothiocyanate(FITC) and Peroxidase or radio isotopes for better use in precise and quick diagnostics.
3. Tissue typing for organ transplant, also blood grouping - thus useful in transplant surgery and transfusion.
4. Therapeutic application is selective elimination of undesired cells, tumour cells or activated T-lymphocytes in transplantation patients.
5. Potential use in radiological searching for localisation of tumour cells.
6. Counting of and identification of lymphocyte-subsets.
7. Depletion of a particular type of T cell subsets from a mixed population of bone marrow cells to prevent graft vs host reaction.

Advantages of Monoclonal Antibody:

1. Monoclonal antibody has a unique advantage in that the antibody molecules are homogeneous and recognizes only a single antigenic determinant, thereby providing a highly specific immunological reagents.

2. Since the hybrid lines are practically immortal, the amount of antibodies produced are thus unlimited and can be scaled up to meet the demand at any further date.
3. Purified antigen is not required in either the immunization or screening of hybridoma so, impure antigen can be used to raise pure specific antibody.
4. Standardized antibody is produced, the properties from batch to batch are the same. Antibodies produced in animals are quite different because of genetic and physiological variations.

Disadvantages of Monoclonal Antibody :

1. Production of Mab is time consuming, requires intensive labour.
2. Technical expertise is required.
3. There are situations where high degree of specificity compromises sensitivity in a reaction, e.g., many viruses alter their specific antigens continuously in order to escape the host's immune response. This is antigenic shift or immunological drift. The host produces a new antibody responding to each variant as it arises so the serum of the host will contain antibodies to a number of variants of the same virus. Such a polyclonal serum is more useful for detecting the presence of virus in other patients during virus epidemic. The Mab can detect only one variant, which may or may not be present in other patients.

Mitotic Index :- is an estimate of the number of cells of the population in the mitotic stages, (viz., prophase, anaphase, telophase and finally cell division)

$$\text{Mitotic Index} = \frac{\text{Number of nuclei in mitosis x 100}}{\text{Total number of nuclei scored}}$$

MI is directly proportional to the duration of mitosis/duration of average cell cycle, provided all the cells in culture are going through cell cycle. Thus a peak mitotic index indicates an initial rapid shortening of the mean cell cycle (i.e., mean generation time).

Practice Assignment XV

1. Hybridoma is used to describe a hybrid cell produced by:
   (a) Fusing antibody producing cells with cancer cells
   (b) Fusing antibody producing cells with normal epithelial cells
   (c) Fusing antibody producing cellswith normal fibroblast cells
   (d) All of the above.

2. Cloning in hybriboma technology means :
   (a) Getting clone from parent cells as descendents
   (b) Limiting dilution insuch a way in order to have one cell per well
   (c) Both of the above
   (d) None of the above.
3. In hybridoma production hybrids cell are selected by
   (a) MAT selection (b) HAT selection
   (c) RAT selection (d) CAT selection.
4. Non-antibody secreting cells used in hybridoma preparations are:
   (a) P_3 cell line & NS_1 cell line
   (b) NSO cell line & FOX-NY cell line
   (c) Both of the above
   (d) None of the above.
5. Common used fusogen used in the fusion of splenocytes with myeloma cell line is:
   (a) Sendai virus (b) Polyethylene glycol
   (c) Electrofusion (d) None of the above.
6. Monoclonal antiboby can be used for:
   (a) Analysis of different functional subsets of lymphocytes
   (b) Diagnostic, imaging and therapeutic uses
   (c) Recognizing an epitope on a complex antigen
   (d) All of the above.
7. As monoclonal antibody is a single entity, so
   (a) Any physical treatment like freeing and thawing may destroy all the activity
   (b) It is not useful in detecting the various cross-reactions
   (c) Both of the above (d) None of the above.
8. *In vivo* propagation of monoclonal antibody is done by:
   (a) Tissue culture technique (b) Different bioreactors
   (c) Mouse ascitic fluid (d) All of the above.
9. To prevent water of crystallization during cryopreservation antibody the chemical added is;
   (a) DMSO (b) PQSO
   (c) $KMNO_4$ (d) $K_2Cr_2O_7$.
10. In monoclonal antibody formation, cloning denotes:
    (a) Descendents of cells (b) Limiting dilution
    (c) Both of the above (d) None of the above.

11. HAT media is generally used during production of monoclonal antibody. The "A" in HAT stands for:
    (a) Ammonia (b) Adenosine
    (c) Aminopterin (d) ATP.

Answers:

1. - (a), 2. - (b), 3. - (b), 4. - (a), 5. - (b), 6. - (b), 7. - (d), 8. - (c), 9. - (c), 10. - (b), 11- (c).

Chapter 16

Transplantation of Cultured Cell

Transplantation of cultured cell is one of the most fascinating areas of biotechnology. Transplantation method assumes that the next best alternative to a tissues normal environment is a similar environment in another host. The cell base strategies to treat diseases is often referred to as regenerative or reparative medicine. Where a large part of skin or organ have been damaged due to injury, the required cells can be grown and transplanted on the affected organs, giving relief to the patients. With the advancement of tissue culture, it has become possible to culture different cells *in vitro* and these cultured cells can be transplanted wherever desired and thus a long standing dream of surgeons i.e., **"organ without donors"** was fulfilled. Various human tissues for direct cell culture is available as surgical and obstetrical material, particular attention has been paid to cells obtained from human amnion (Ziteer *et al.* 1955), amnion and chorion (Lahelle, 1956) and fallopian tubes (Pizam *et al.* 1956). The relationship involved in the rejection of graft in higher animals are now clearly recognized. Medawar (1958) classified the different graft reaction which are as follows:

Isograft:-When graft is transferred between genetically identical (syngenic) individual.

Autograft:- Graft obtained from one part of the body and transplanted on the other part of the body of same individual.

Allograft:- This involves the transfer of graft between different individual of same species.

Xenograft:- Transplantation of tissues between two zoological classes i.e. from pig to man (Albrink and Greene 1953).

Allograft and xenograft have clinical advantage over non-biological synthetic biomaterials. Since allografts are rarely available,

dependence of xenograft as implants during surgery has grown exponentially over the years. Heart valve made up of xenograft like porcine valve tissue or bovine pericardium have the advantage of low incidence of thrombogenecity without anti-coagulation (Cary *et al.* 1998). Though these valves have near normal functional accuracy like the native valves but have a limited service time as they undergo generative changes and short life span discourage their use (Frederick and Robent 2005). These degeneration may occur due to several deficiencies in processing and also due to *in vivo* enzymatic digestion and immune response. Guhathakurta *et al.* (2006) have described the technique to process xenogenic tissues for cardiovascular implantation. These processes include decellularlization of xenograft tissues and thus preserve their architecture. Decellularization of xenograft tissues make them durable and sturdy to withstand physiological stress due to circulation during cardio vascular surguries.

1. Cultured Stem Cells and Bone Marrow Transplantation:

Stem cell culture is providing promising area of biotechnology to investigate the possibility of cell-based therapies to treat a disease, often referred to as regenerative or reparative medicine. Stem cells are pluripotent cells which retain the potential of differentiation in other cell types and can form many different cell types of body. This is an uncommitted cell that remains uncommitted unless it receives signal and to develop into a specialized cells/tissues in the body. There are two types of stem cell i.e. adult stem cells and embryonic stem cells. **Adult stem cells** resides in the adult tissues and are either unipotent or multipotent. The primary role of adult stem cells in a living organisms are to maintain and repair the tissues in which they are found. These cells have more restricted option, often able to select a differentiation programme from only a few possible pathway. The **embryonic stem cells** are derived from the inner cell mass of the blastocytes and in a few instances from cleavage stage of embryo. The promises of embryonic stem cell lies in their ability to pluripotent and develop into any cell/tissue type in the body. Evans and Kauffman (1981) was the first to describe the method for growing stem cells from mouse embryos, while Thompson *et al.* (1988) developed the first human embryonic stem cell line from *in vitro* prodermal embryo. This ignited the imagination for stem cell application in therapeutics, while at the same time stirring the political attention on ethical vs scientific issues. The main reason for ethical issue was due to the fact as it was observed that generally embryonic stem cell derivation

deprives embryo of any further potential to develop into a complete human being, but Chung *et al.* (2006) demonstrated that a few embryonic cells could be taken from a live embryo (while in uterus) for the development of stem cell line, leaving the embryo to survive and develop normally. So, now there will not be any ethical, legal or economical issues that have been clouding this field of research for a long time. This was considered to be a milestone in medical sciences and a major turning point, paving the way for beneficial gains in treating or curing disease such as diabetes, spinal cord injury and Alzheimer's diseases etc.

Recently, Prof. Esmail of Zanjani of the University of Neveda has created a chimera sheep, which has two or more different population of genetically distinct cells that originated in different zygotes has the body of sheep and half human organ (liver, heart and lung). For this stem cell from bone marrow isolated from the patient is injected into the peritoneum of sheep foetus. Then the cells would get distributed throughout the metabolic system of all the organs in the body. This will solve the problem of scarcity of organ for transplantation in human beings.

Bone marrow transplantation :

For bone marrow transplantation the stem cells used are called haematopoetic stem cells. These stem cells first appear in the yolk sac and then migrate to the liver as the fetus develops. In the fetus the blood cells are created in the liver but soon after birth; they are produced exclusively in bone marrow. These stem cells can be isolated stored and cultured.

Recently, peripheral blood and cord blood stem cells have been commonly employed. Peripheral blood stem cells or cord blood is collected separately by a leukapheresis machine and remaining blood portion is returned to the donor. Most recently, column separation methods are employed to select stem cells (CD 34 $^{+}$ blood cells) for engraftment and to remove contaminating donor T-lymphocytes which eventually mediate isograft *vs* host reaction. These are the main ingredients of the bone marrow transplant. If marrow is transplanted from same individual it is called autotransplant, if donated by other individual it is called allogenic transplant. Haematopoetic stem cells produce over 3-10 billion platelets, red cells, neutrophiles, lymphocytes and other cells of immune system.

During last two decades bone marrow transplantation has been extensively used to cure many haematological and immunological disorders as well as therapy of refractory cases of body tumours. These cells are also used in transplantation in patients whose blood forming system has been devastated by leukaemia, cancer, chemotherapy or other causes. It is also used to cure Adenosine deaminase deficiency which is one of the cause of severe combined immunodeficiency (SCTD), a group of heriditary disease in which lymphocyte fails to develop normally. In this case bone marrow transplantation only becomes successful when compatible bone marrow can be obtained from an antigenically matches sibling.

Though, the most common source of nesenchymal stem cells (MSC) has been bone marrow, but aspirating bone marrow from the patient is an invasive and painful procedure. In addition, it has been demonstrated that the number of bone marrow MSCs and their ability to differentiate increases with cell age (D'Jppolito *et al.* 1994). Despite the fact that bone marrow represents the main available source of mesenchymal stem cells, the use of bone marrow cell has the following disadvantages:-

- It may have high degree of viral infection.
- There may be significant drop in cell number and proliferative/ differentiation capacity when the cells age.

Recently, the blood present in the umbilical cord following birth contain haematopoietic precursors and this has become an important alternative source for haematopoietc stem cell (Lee *et al.* 2004). However, there is controversy as to whether umbilical cord blood contains mesenchymal stem cells that are capable of differentiating into cells of different connective tissue lineage such as bone, cartilage and adipose tissues and these cells are the best candidate for tissue engineering of musculoskeletal tissues (Romanov *et al.* 2003). Because of the immaturity of new born cells compared with adult cells umbilical cord blood has the following advantages:-

- It can be collected without any harm to the newborn infant.
- It provides no ethical problems for basic studies and clinical application.

Non-myeloblative Allogenic Stem Cell Transplantation :- It is also known as minitransplantation of reduced intensity conditioning transplantation. It is a major advance in the field of haematopoletic transplantation. This approach uses non-cytotoxic of reduced intensity

cytotoxic therapy to prepare patients for allografting of haematopoietic stem cell and lymphocytes. It has the potential to deliver the potent anti-tumour immunotherapy and bone marrow replacement capacity of allogenic stem cell transplantation to patients with reduced treatment related morbidity and mortality. It may also enable allogenic transplantation in patients who would be considered intelligible for conventional transplants because of co-morbidity or advanced age. However, this approach may necessitate more careful monitoring of post-transplant chimerism and malignant disease status than is usual with conventional allografting. In general $200x10^6$ bone marrow cells are needed for 15 kg body weight. There are two major problems associated with bone marrow transplantation.

Rejection by Host (patient) of Cells Donated :-Use of monoclonal antibodies will permit better typing of donor and recipients' lymphocytes to enable better cross matching. In the meantime immunosuppressive therapy is needed. There is rise in mortality due to various gram negative bacteria. The problem stems from the fact that antibodies successfully combating infection, leads to release of endotoxin, which causes death. Use of anti-endotoxin antibodies has been produced in human beings and shown to be effective in reducing death.

(b)Graft vs Host Reaction :-T cells in the bone marrow can recognize grafted cells in the new hosts as being foreign and start destroying them. This produces clinical syndrome which can lead to death of grafted material. This is known as graft vs host reaction. This can be avoided by removing T-cells from the donor's marrow by treating with anti-T cell monoclonal antibody prior to transplant.

2. Transplantation of Cloned Tissues:

Advances in mammalian cloning have proved that somatic cell nucleus can be reprogrammed to a state of totipotency by transferring it into oocyte. This technique is popularly known as somatic cell nuclear transfer (SCNT). Though SCNT has been successfully applied to produce clone animals in a wide range of species including sheep, cattle, mice, goats and pigs (Polejaeva *et al.* 2000) yet cloned tissues are also used for the transplantation purposes. Cloning has been allowed strictly for medical reasons and not for reproductive reasons, which remains banned. The idea is to clone on the basis of patient's DNA, and for this cell nuclei from the patient is taken and fused with the human egg cell to make artificial embryo, which is allowed to

grow in artificial media and then transplanted in them after 14 days of fertilization. At this stage, the embryo is mainly a cluster of so-called stem cells that have the ability to grow into any part of the body. The process of reprogramming whereby a somatic nucleus acquire new development potential, is thought to occur through epigenetic mechanism since gene expression is reset to an early embryonic state without alteration of DNA sequence. These cells would be cultivated in laboratory into purpose-built tissue, which in turn could be transplanted into the patient to replace the sick/ damaged tissue. The advantage of the technique is that the cloned tissue is considered by the immune system of the recipient to be friendly and is less likely to be rejected.

Briefly, the steps of process of cloning is as follows (Fig. -15):-

1. The nucleus is removed from an unfertilized human donor egg. The enucleation may be confirmed by epifluorescent microscope.
2. The enucleated oocyte is then laid side-by-side with a cell of patient that contains genetic material and then fused by any fussogen which activates the nucleus to enable the reconstructed eggs to start dividing.
3. The reconstructed egg is placed in a Petrie dish and grown into an embryo.
4. In three to four days the embryo reaches the blastocyst stage where stem cells can be seen. These stem cells are the parent cells that specialize and develop into every type of cell in the body.
5. In three or four days the embryo reaches the blastocyst stage where stem cells can be seen. These stem cells are the parent cells that specialize and develop into every type of cells in the body.

The stem cells are extracted to grow into the tissues needed by the patient whose cell was cloned.

Potential Uses of the Cloned Cells :

Brain cells can be used in patients having strokes and Parkinsons's disease, cells from eyes are used for degenerating sight, cells of spinal cord is used to treat spinal injury, myocardial cells for heart diseases, hepatic cells for the correction of liver cirrhosis, hepatitis, Pancreatic cells to treat diabetes, muscular cells for muscular injuries, keratinocytes to treat patients of burns injury and wound healing,

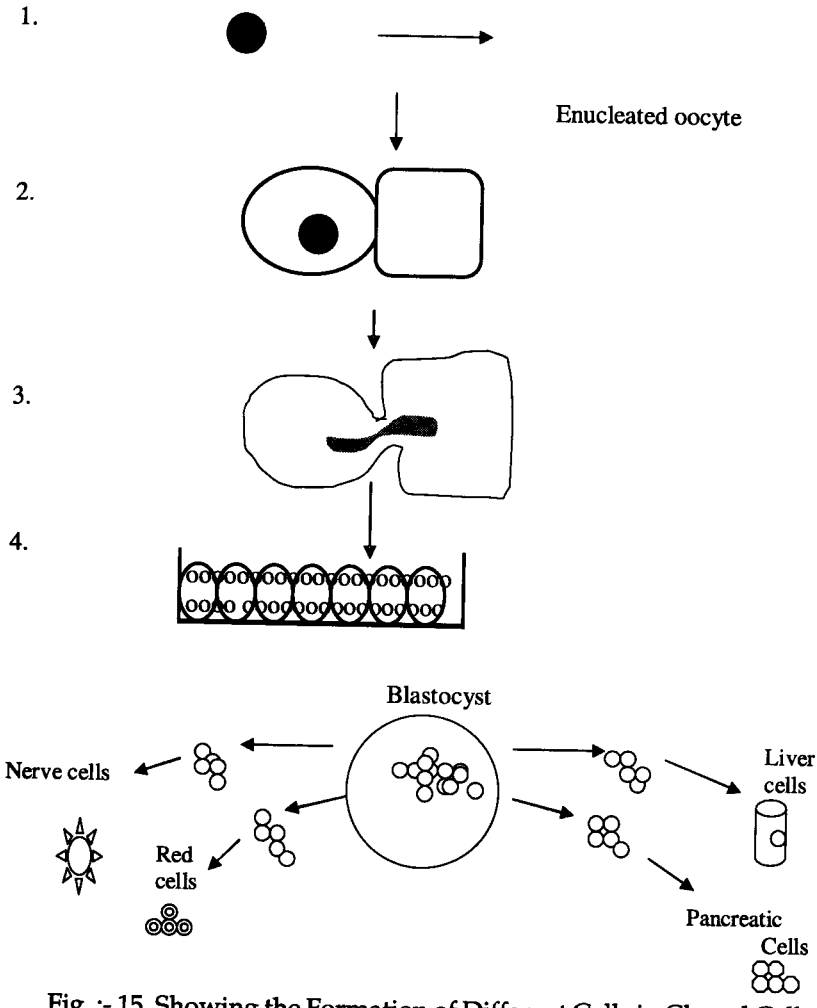


Fig. :- 15. Showing the Formation of Different Cells in Cloned Cells.

chondrocytes for rheumatoid arthritis and osteoarthritis, injuries; blood cells for leukaemia, sickle cell anaemia and various immunodeficiencies.

Disadvantage of Cloned Tissues:

The success rate of mammalian cloning by somatic cell nuclear transfer is very low, as majority of clones die *in utero* or neonatally, often in conjunction with developmental problem such as large offspring syndrome.

3. Grafting of Artificial Skin

Before the advent of culture of keratinocytes, in burn case skin was transplanted by taking the small portion of skin (generally 0. 3 mm thick) from unburnt part of the body of the same patient (autograft). This autograft was called **split thickness graft**. But during severe burns, where major part of the skin of the body has been destroyed large portion of skin was needed for grafting, which was not possible. So, to circumvent the problems surrounding cadaveric dermal allografts, a number of system for the creation of a totally synthetic skin have been proposed to cover the large burnt area of the patient. In one system autologous cultured keratinocytes, instead of being cultivated as isolated epidermal sheets, are seeded as a single cell suspension on a dermal substitute. This system is already in wide clinical use. Of the skin cells, which can be cultured, are keratinocytes and these make about 90% of the skin. It is important to culture keratinocytes used to generate epidermis from the unburned portion of the skin of patient himself otherwise there will be rejection (Davidson *et al.* 1963).

Rheinwald and Green (1977) described the method of cultivation of epithelial autografts which has received the widest use. Briefly, a small sample (2 to 4 cm^2) of the patient's skin, harvested as either full thickness excised biopsy specimen or as a split thickness keratome sample is taken in sterile Dulbecco's modified Eagle's medium (DMEM) containing antibiotics and amphotericin B (Fungizone). All dermis, if present is trimmed from the biopsy specimen and the trimmed skin is floated overnight at 4^0C on a calcium free buffer containing 0. 25% trypsin. The following day the epidermis easily separate from the dermis, which is minced and agitated to have single-cell suspensin. Released keratinocytes are collected by centrifugation and plated in DMEM supplemented with 10% fetal calf serum, cholera toxin $10^{-10,}$ hydrocortisone and feeder layers and growth factors. Commomly used feeder layer in keratinocyte culture is $3T_3$ cell line derived from murine fibroblast cells that were previously treated with mitomycin C[66] to prevent their own multiplication. These are added to cover the bottom of the vessel and then added epidermal cells for culturing them. Only 1-10% of the epidermal cells proliferated. Keratinocytes attach and began to form colonies on the plate that push the $3T_3$ feeder cells into a rim at the periphery. It is not clear why culturing cells on top of feeder layer of cells can enhance growth and differentiation of kertinocytes. It may be postulated that feeder

layer may provide paracrine factors, extracellular matrix or cell surface molecules for the appropriate interaction necessary for the differentiation of other cell type. After the 3rd day epidermal growth factor (10 ng/ml) is added. When there is confluent sheet of culture the remaining $3T_3$ is removed from the culture dish by spraying with the buffered 2-mM ethylenediamine tetraacetic acid (EDTA) solution. When the culture becomes totally confluent, usually 10 days after initial inoculation, the plates are treated with trypsin to release the keratinocytes. The developed sheet of keratinocytes are separated and transferred to fresh culture for better growth. The process of separating cells from colonies and reculturing them is a cumbersome process. To discourage stratification of cell layer and allow the cell colonies to become confluent forming a sheet of epidermis the cells of this sheet of epithelium are linked by desmosomes. The cultured epithelium can be detached from the vessel using the enzyme dispase, washed free of extraneous proteins attached to a bedding of gauze (basal cells facing upward) and brought to the operation theatre to be used for grafting on the patient having severe burn. To maintain appropriate pH of the cultured keratinocytes, the epidermal sheets are transported to the operating room in small portable incubator equilibrated with 5% CO_2. A meticulous preparation of wound is required since these are contaminated and complete elimination of microorganism is essential to prevent the patient from pathogenic effect of contaminating microorganisms.

A variety of diseases have been treated using cultured keratinocytes, such as:

- Scar on skin.
- To regenerate epithelium of the mouth.
- To repair congenital penile defects.
- Middle ear disease with troublesome discharge.
- Chronic skin ulcer.
- To treat non-functional tissue.

Transplantation of Other Organs:

Treatment of Defective Liver:- In the case of dejected liver, scaffold bead tissue can be prepared by the cell culture and then it is introduced into the abdomen of patient. The scaffold bead tissue can perform the function of liver. Recently, it has been found that pigs organs have been transplanted to human several times in the last few years

as here are considerable similarities between the procine and human anatomy. But pig liver transplants remain highly unusual and experimental, according to the US Food and Drug Administration's (FDA) website. Transplanting animal organs to human remain risky and controversial because of the possible spread of animal diseases to humans and ethical concerns but scientists are experimenting with genetically altering pig livers by inserting human gene that will produce human proteins to fight organ rejection. The severe shortage of human donor has stimulated interest in the potential use of animal organ for transplantation in human (Evans *et al.* 1992). A recent reports by the British Biopharmaceutical company research scientists claim that they have identified a gene in the human system which is responsible for rejection of the pig organ in the human body during organ transplant. They are planning to inactivate the above mentioned gene so that the genetic problem of human rejection of pig organs during transplantation could be overcome. One essential question arises in the mind of scientists whether in xenotransplantation animal organ or tissues can provide a physiologic replacement for a human organ or tissues as it is assumed that foreign organ or tissues might function poorly compared with their human counterpart.

Transplantation Experiments are Being Carried to Cure the Various Diseases:-

Parkinson's Disease and Neural Graft:- Parkinson's disease is characterised by the disturbance in the posture which is due to the defect in spinal cord. So, this can be treated by the adrenal medulla cells cultured *in vitro* in suitable tissue culture media.

Treatment of Immunosupressed Patients:- Generally the various drugs used in cancer patients have immunosuppressive effects. Thus patients become prone to infection not only with the established pathogens but with the so called opportunistic micro-organisms also. So in these case cytotoxic T-lymphocytes can be produced by cell culture and these can be transplanted to the immunosuppressed patients. After transplantation the immunosuppressed patients can be prevented from the attack of infecting micro-organisms especially cytomegalovirus.

Transplantation of Recombinant Proteins:- Various recombinant proteins can be synthesized and cultured in cell culture which can be transplanted to human beings for various therapeutic purposes.

Chondroplast Transplantation:- Scientists have also succeeded to culture chondrocytes from the same patients by taking unused cartilage and transplant to patients having various types of arthritis.

Recently Lee *et al.* (2007) have suggested for the production of miniature animals for use in organ transplantation.

Future Needs of Cell Culture for Transplantation :

- Vascular prosthesis (Artificial limbs).
- For repair of urinary bladder.
- For pancreatic islets to treat diabetes.
- For heapatofunction.
- For treatment of muscle disease.

Practice Assignment XVI

1. Transplantation of tissue from one place to other place of the same individual is called:
   (a) Xenograft (b) Allograft
   (c) Autograft (d) All of the above.
2. In embryonic stem cell transplantation, cells are taken from:
   (a) Blastocyte (b) Bone marrow
   (c) Spleen (d) Liver.
3. Bone marrow transplantation is needed for curing:
   (a) Haematological disorder (b) Immunological disorder
   (c) Both of the above (d) None of the above.
4. Transplantation of cultured keratinocytes is done to repair :
   (a) Extensive burn site (b) Congenital penile defects
   (c) Non-functional tissues (d) All of the above.
5. In future cell culture transplantation will be of great use for:
   (a) Pancreatic islets for treatment of diabetes
   (b) For treatment of various muscular disorders
   (c) Both of the above (d) None of the above.
6. In split thickness graft:
   (a) A small portion of skin is transplanted from one part of body to other part
   (b) Cultured keratinocytes are cultured
   (c) Both of the above
   (d) None of the above.

7. In somatic cell nuclear transfer (SCNT) :
   (a) Somatic cell nucleus is taken
   (b) This is injected in enucleated oocyte
   (c) Both of the above
   (d) None of the above.
8. Advantages of transplantation of cloned tissue includes:
   (a) It is friendly to immune system
   (b) Less likely to be rejected
   (c) Both of the above
   (d) None of the above.
9. Culture of keratinocytes requires:
   (a) Some feeder layer (b) Epidermal growth factor
   (c) Both of the above (d) None of the above.
10. Potential use of bone marrow transplantation is in :
   (a) Leukemia (b) Cirrhosis of the liver
   (c) Parkinson's disease (d) All of the above.

Answers:

1. -(c), 2. - (a), 3. -(c), 4. -(d), 5. -(c), 6. -(a), 7. -(c), 8. -(c), 9. -(c), 10. -(d).

Chapter 17

Differentiation of Cells

The progressive specialization in structure and function of a cell arising from the common progenitor cells leading to a production of unique protein pattern is termed as the cell differentiation e.g., Neurons, Epithelial cells. It is a unique process in the development of all living system and is generally used for the phenotypic expression of characteristics matured cell. One of the characteristic of cell differentiation is its stability i.e., a neuron cell persists as such throughout life. The nematode *Caenorhabditis elegans*, a simple multicellular organism possesses several notable features that make it one of the most widely used models of cell differentiation. The simplicity of *C. elegans* has enabled the course of its development to be studied in detail by microscopic observation. Such analysis have successfully traced the embryonic origin and lineage of all the cells in the adult worm.

There are Three Type of Cells:

1. Stem Cells:- These are pluripotent, proliferative, undifferentiated cells which can give rise to any type of cells on differentiation. These cells have high proliferative capacity, e.g., Haematopoietic stem cell –these can give rise to any type of blood cells.

2. Precursor Cells:- These are cells which are derived from stem cells. These are proliferative and are committed to differentiation into functional matured cells and these are controlled by several hormones and cytokines. Some of the examples of differentiated cells from precursor cells are epithelial cells, muscle cells, hepatic cells, osteoblasts and blood cells etc.

3. Differentiated Cells:- These are derived from precursor cells and are non-replicative with specialized function, e.g., Erythrocytes, sperm and ovum etc.

All higher plant and animal cells are constructed from a large variety of different cell types such as cells of vascular system, nervous system and urinogenital system etc. Here cells and cytoplasm content may

become so organized as to perform specialized function that the same type of function is not performed by the other cell. The changes by which such specialization is achieved is termed as cell differentiation. Cell differentiation results into a larger number of morphologically different progeny cell types. Differentiation of cell is for performing special function. Cell differentiation must be differentiated from cell proliferation. Nutritional factors such as serum, calcium ions, hormones, cell matrix interactions, cytokines, cell density in culture can affect cell differentiation and cell proliferation. Therefore the culture condition that favours maximum cell proliferation are not often conducive to cell differentiation. e.g., low cell density favours cell proliferation whereas high cell density favours cell differentiation. An embryo is a totipotent and it gives rise to the cells of body and its cellular differentiation is due to commitment of cells.

Bacterial Sporulation as Model for Cell Differentiation:- It is one of the simple model which explains the cell differentiation. Generally sporulation takes place in the adverse circumstances of growth, such as depletion of nutrients and accumulation of waste products etc. Cell differentiation of vegetative cell into a spore, is now available for successful analysis. One copy of the genome is segregated as a forespore and encased in a spore coat while the other remains in the mother cell, which is eventually destroyed. And thus sporulation involves the production of many new structures, enzymes and metabolites along with the disappearance of many vegetative cell components. Tough cell outside the spore has completely different composition from the surface of the corresponding vegetative forms. There is a morphological transformation and also changes in enzymatic composition of many cytochrome molecules responsible for aerobic metabolism which completely disappear and instead a new electron transport system occurs. Ribosome content is decreased and there is also decrease in the number of mRNA and increase in the appearance of a new RNA. Composition of many cytochromes molecule responsible for aerobic metabolism disappears completely, instead a new electron transport system occurs. The energy for germination is stored as 3-phosphoglycerate rather than as ATP. There is increase in number of vegetative cell enzymes such as alanine racemase and a unique enzyme formed is dipicolinic acid synthetase, which are responsible for calcium dipicolinate from an intermediate of the lysine biosynthetic pathway as mentioned below. This provides resistance against heat to the spore. The above mentioned changes involves alteration in the transcriptional specificity of RNA polymerase, which

is determined by the association of polymerase core protein with one or another promoter specific protein called a Sigma (ó) factor. Different factors are produced during vegetative growth and sporulation. An unique enzyme formed is dipicolinic synthetase which are responsible for the formation of calcium-dipicolinate (Pyridine 2, 6-dicarboxylic acid) from an intermediate of the lysine synthesis pathway (Fig. -16), which is not present in the vegetative forms. L-lysine synthesis is catalyzed by aspartate kinase and the further steps which occurs in the synthesis of dipicolinic acid is as follows:-

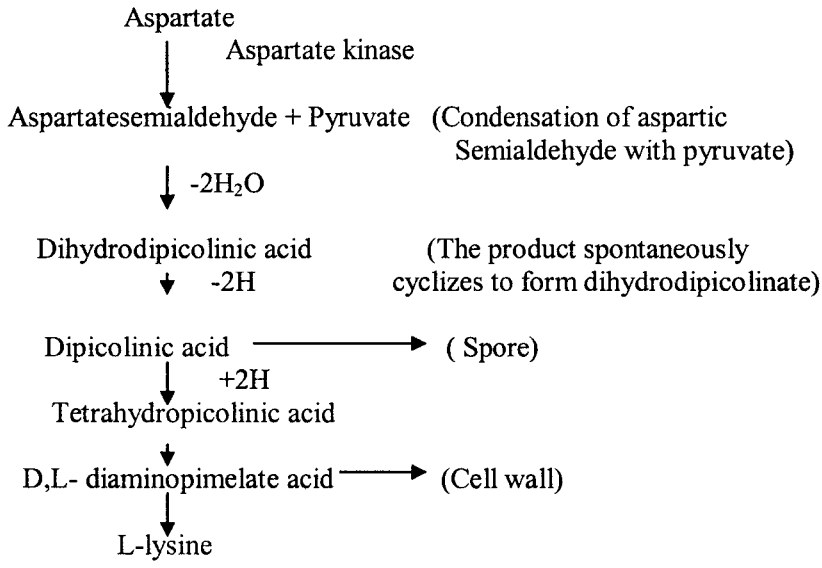


Fig. 16 : Intermediate of Lysine Synthesis Pathway.

Dipicolinic acid is responsible for the unusual resistance of spore towards the adverse circumstances.

Differentiation is an irreversible process e.g., a mature sperm do not revert back to spermatocyte, similarly an erythrocyte or leukocyte cannot revert to original stem cells.

Types of Differentiation:

Based on structure, function and chemical nature, differentiation is of four types:-

(i) Morphological differentiation

(ii) Physiological differentiation

(iii) Chemo-differentiation

(iv) Cyto -differentiation.

(i) Morphological Differentiation :- The differentiation which occurs on the basis of shape of the cell is called as morphological differentiation, such as, neurons are divided into axon and cytons. Epithelial cells are provided with cilia like structure lining the respiratory tract

(ii) Physiological Differentiation:- The differentiation which occurs on the basis of function is termed as physiological differentiation, such as, optic cells are specialized for vision, neurons for carrying impulses.

(iii) Chemo-Differentiation:- The differentiation which occurs on the basis of chemical constituents is called chemo-differentiation., such as erythrocytes have hemoglobin, chloroplast cells have chlorophyll and hairs, skin and nail have keratin.

(iv) Cyto-Differentiation :- The evolution of a particular cell type such as shape and structure is called cell differentiation, i.e., shape of hepatocytes, transitional epithelium and neurons etc.

Commitment:- Commitment is point between stem cell stage and precursor cell stage from where the cell will no longer transfer to other lineage. Destiny of each cell is fixed from the time of evolution.

Markers of differentiation:- Marker is secretion of cell or enzyme that is used in performing product e.g., Heme in erythrocytes.

Factors that Influences Cell Differentiation:

There are mainly two factors which directly or indirectly control the process of cell differentiation:

1. External factors and
2. Internal factors.

1. External Factors:- Various external factors present outside the cell influences the differentiation of cell, which can be exemplified by the following examples:-

(i) External Forces:- Various external force which are present outside the cell influences cell differentiation. External forces acting upon the originally undifferentiated cell might initiate a chain of events resulting in two progeny cells of different constitution. Such as, due to gravitational forces on the yolk of a fertilized hen's egg, some of the progeny cells may have more yolk in the bottom than others.

(ii) Chemicals:- Chemicals present in the environment also influences cell differentiation as cells respond in a variety of ways to the chemical present in the environment. Some basal cell differentiate into keratinized cells and get separated from the remaining layer but it has been observed that on exposure to vitamin-A, cell differentiate into mucus secreting cells.

(iii) Synergistic Action:- Wilde (1961) reported that a single isolated cell when kept in nutrient medium did not differentiate, when two cells are kept in medium one of them went under differentiation. This clearly shows that a neighbouring cells provide a micronutrient to initiate cell differentiation. The metabolic exchange between the cell and the medium result in altering the medium for differentiation of isolated group of cells.

(iv) Cellular Environment:- In an experiments a part of the embryonic tissue was transplanted on to another in different location. It was observed that transplanted tissue at the new location did not confirm to the normal pattern but developed into a pattern that was directed by the new location. This suggests that cellular environment is one of the deciding factor in differentiation. Significantly, after sometime during the course of the development, the transplanted cells change their behavior and proceed on their predetermined path of the differentiation and then environment do not play role any more. This clearly explain that phenotypic expression depends on environment and hereditary character.

2. Internal Factors:-

Internal factors can be further subdivided into Hormone-endocrine signaling, Paracrine signaling, Cell-cell interaction, protein molecules, Gene and Cytoplasmic controlling differentiation.

(i) Hormone-endocrine Signalling:- These are soluble inducers which include several hormones paracrine secretions. Hormones which stimulates various organs are released in the body at distant places without any duct, such as Thyroid stimulating hormone (TSH), Follicular stimulating hormone (FSH) and Interstitial cell stimulating hormone (ICSH) etc. These hormones acts on the cells and support the cells to differentiate into a particular organ.

(ii) Paracrine Signalling :- The paracrine factors are molecules, which do not travel via the blood stream but have their effect on target cells by cell-cell signaling, which are in close proximity to the signalling cells. Paracrine secretions, such as IL-6 and KGF play a vital role in

development and differentiation of cells *in vivo* leading into a particular organ.

(iii) Cell-Cell Interaction:- The process by which the cell interact to signals from another cell lying closer to it or being in far away with the help of various agents such gap junction and activities of protein molecules present in the cell. This may be (a) homologous and (b) heterologous interaction.

Homologous Interaction:- This includes Gap junction which allows the material to be exchanged from one cell to another and eventually the material could enter in the nucleus of receiving cell undergoing differentiation. Specific protein receptors on two neighboring cells can interact directly or a secreted substance from one cell can occupy receptors on other interacting cell such as Ectodermal and mesenchymal cells and formation of epidermis by dermis. Many other structures and organs of adult animal are constructed from ectodermally derived cells that have received and interpreted developmental signals from the cells of mesodermal origin. Endodermal and mesodermal interaction for the formation of internal organs is well established.

Heterologous Interaction :- The cells of different germ line are induced by these heterologous interaction. This differentiation proceeds following gastrulation or after organogenesis.

Protein Molecules:- Various protein molecules involved in cell-cell interaction are Integrin, Cadhesin, Adhesin and Selectin etc. All these molecules communicate with one another and help the cell to assemble with one another and finally undergo differentiation. (For details of cell-cell interaction refer chapter 2, page no 23). The changing pattern of individual gene activation and deactivation is the basis of differentiation of tissues. The development of each of specific cell types involves the specific interaction of at least two types of cells, possibly cell-cell interaction explains this.

Gene Controlling :- The differentiated cells undergo some genetic modification which is controlled by gene. Each cell has specific protein and enzymes which are produced by gene. Though gene directly do not produce protein and enzyme these are produced through an intermediate i.e., mRNA. Thus the process of cell differentiation is also due to the ability of cells to synthesize different kinds of protein.

Cytoplasmic Factors :- Cytoplasmic factors controlling cell differentiation can be proved with the nuclear transplantation

experiment in the frog. Nucleus of a specialized intestinal epithelial cells of a tadpole was transplanted into an enucleated eggs of frog and subsequently a mature frog was obtained. This shows that genome remains constant during cell differentiation and oocyte cytoplasm is able to reprogramme the expression of gene in the transplanted nuclei.

3. Soluble Factors:- The soluble factors which are responsible for cell differentiation are high calcium concentration (300-1500 mM) and certain polar compounds, such as dimethyle sulfoxide (DMSO).

4. Non-physiological Factors :- This factor includes transforming growth factor β (TGF-β) secreted by platelets and this causes differentiation of squamous epithelium of bronchial mucosa.

Differentiation is often irreversible. Now, with the advent of tissue culture large variety of differentiated cells can be grown *in vitro* maintaining the same morphological features as found *in vivo.* The nerve cell will have the same morphological appearance as found *in vitro* even when growing in tissue culture. Complete plant can often be generated starting from either highly differentiated root or epidermal cells.

Differentiated cells may have molecular differentiation which may be peculiar to that cell type. So, a complete description of the differentiated cell at the molecular level is a most formidable task.

Differentiation of Cells *in vitro* and *in vivo:*

Differentiation of cells have been recognized as four stage process:-

1. Non-differentiated stage of the stem cells represent a condition in which the cells produce specific protein prior to morphological change.
2. Pre-differentiated cells represent extended proliferation stage to produce vast population. Some are differentiated as specialized cells whereas others remain as memory cells with arrested growth.
3. The differentiation stage is distinct from other cells. In this the cells have cell-specific proteins.
4. In the differentiated cells modulation may take place in response to cellular factor viz hormone, pH and temperature.

Mechanism of Differentiation:

Constant Genetic Information:- Cells do not loose their potential

after differentiation, as large number of cells can be grown with the same morphology from differentiated *in vitro* as grown *in vivo*. A nerve cell produced in tissue culture is same as produced *in vivo*, similarly a single cell derived from pith callus of carrot grow into whole plant. The cultured cells continue to perform their specialized function in the cell culture.

Partial Shielding of Genome to Transcription:- In some *in vitro* experiment with athymus DNA, in which RNA polymerase was added to synthesize RNA or DNA. The template analysis proved that RNA produced *in vitro* represent only 10% of the transcribed DNA. This reveals that the transcription is restricted to some parts of the genome and the kinds of mRNA produced by a cell are regulated by the amount of DNA available for transcription.

Selective Amplification of mRNA :- Not all the mRNA molecules of the cytoplasm are utilized for protein synthesis rather certain region of mRNA is translated into protein. This selection mechanism of mRNA is need oriented and thus facilitates the process of cell differentiation.

Amplification of Gene in Differentiation:- During the process of cell differentiation a part of genome is amplified by concanavalin-A (Con-A) which also support the process of cell differentistion. Recent knowledge of transcriptional regulation in the liver has been derived primarily from the analysis of promoter and enhancer elements of genes selectively expressed in hepatocytes.

(A) Differentiation of Cells *in vitro* (in cell culture):

Animal tissues are classified as Epithelial, Connective, Muscular, Nervous, Blood and Lymphatics. It is essential to confirm that the differentiated characters of the cells are still maintained in the *in vitro* culture.

Epithelial Cells- Epithelial cells, like fibroblasts are grown on plastic dishes to which they adhere through their extracellular matrix protein, such as laminine secreted by them. Early studies with epithelial cell culture were disappointing because the important characteristics of epithelial cells were lost in culture. It was known that the level of expression of many differentiated cells was under hormonal control. Supplementation with specific hormones started the differentiation and proliferation of cells. Fully differentiated epithelial cells still lost their tissue-specific functions in culture within three days even in the presence of appropriate hormones and soluble factors. It appears now

that the establishment of correct cell polarity and cell shape in epithelial cell culture was still wanting.

Connective Tissue :- Cultured fibroblasts have the morphology of tissue fibroblasts although they tend to be less well differentiated and appear to be precursors of fibroblasts. They can be differentiated into say, adipose tissue or connective tissues if appropriate stimulants are added to culture medium.

Muscular Tissue :- Permanent tissue of muscle precursor cells is called myoblasts. When cells develop and reach certain density, they carry out functions similar to the functions of muscle cells *in vivo*. They fuse with each other and form cross-striated multinucleated muscle fibers (myotubes). A genetically homogenous clone of rat myoblast cells called L6 has been isolated and has been used to analyse the biochemical changes, which take place during differentiation *in vitro*.

Nervous Tissue :- In 1907 Ross Harrison cultured piece of tissue from medullary tube region of frog embryo in clots of frog lymph. The neural cells maintained their typical neural morphology *in vitro* and axons grew out from some of the cells. Recently, neonatal mouse dorsal root ganglia from the spinal cord have been used to obtain pure culture of Schwann cells and pure culture of neurons. Once isolated the pure cultures have been used to study the effects of different growth factors on neuronal differentiation.

While the majority of progenitor cells exhibit a limited proliferative potential in culture, under specific conditions, certain mammalian cells can be induced to exhibit and extend proliferative potential and hence generate a larger number of progeny-one of the characteristic of stem cell.

Blood and Lymphatic Tissues:- Isolation and culture of human peripheral blood leukocytes became standard practice in 1960s and many of the precursors of leukocytes (lymphocytes, monocytes, MQs) can carry out at least part of their differentiated functions in culture. The stem cell factor (Broudy, 1997) and erythropoietin (Krantz, 1991) are the important cytokines that regulate erythropoiesis. The clonal growth of these progenitor cells in culture was key to sorting out the haemopoetic lineages and the discovery of polypeptide hormones which regulate haemopoiesis.

Erythroleukaemic cells (abnormal precursors of RBC) can be induced to produce Hb and undergo structural changes in the cell membrane associated with normal red blood cell maturation.

De-differentiation :-

There are evidences that a differentiated cell under some stress can be dedifferentiated into a proliferative cell which can divide and give rise to many cells, however, the process of dedifferentiation is not fully understood. De- differentiation is important in tissues where occasional renewal of cells is required, e.g., during wound repair in skin. Some of the already differentiated cells may lose some of their differentiated property and re-enter the cell-cycle The cells proliferate until the tissue has gained the appropriate cell density at which point proliferation stops and differentiation is renewed.

De-differentiated cells could be returned to their normal state in many cases by growing them on a characteristic substrate or in the presence of another cell type. For example, mammary epithelial cells will show normal ductal morphogenesis when grown on a fat pad or on collagen type 1 gels. They are able to express and secrete milk proteins when the appropriate hormones are added. The culture of mammary epithelial cells demonstrates the importance of considering cell-cell and cell-extracellular matrix interaction as well as soluble factors, which induce differentiation.

In vitro, in a culture a differentiated cell can be made to proliferate and which can give rise to its specific cell type. Skin cell in culture can be induced to differentiate by adding certain growth factors, such as EGF and growing the cells on 'feeder layers' or other cell types. The skin cells will grow and divide and differentiate into cornified cells or keratinocytes that make up the outer surface of skin. It is not clear why culturing cell on top of feeder layers of cells enhances growth and/or differentiation of those cells. It may be that the feeder layer provides *paracrine* factors, extra cellular matrix or cell surface molecules which provide the appropriate interaction necessary for differentiation of the other cell type.

(B) Cell Division and Differentiation *in vivo*

The differentiation of a determined cell is the process by which genes are selectively expressed and gene products act to produce a cell with a specialized phenotype. When cells become differentiated, they stop growing and there are changes in their physiological and metabolic capabilities. Differentiated cells can be recognised morphologically through biochemical or staining methods. Cell types are connective tissue, epithelial tissue, muscular tissue, nervous tissue, blood and lymphatic tissue.

All the cell types develop from a single cell, formed due to fusion of two gametes, the fertilized ovum by the process of cell division and differentiation. The ovum is totipotent, which means that it is irreversibly committed to any developmental fate and is able to give rise to any cell type.

Cells may be Unipotent, Pluripotent and Totipotent.

Unipotent Stem Cells :- These cells give rise to one end stage differentiated cells, a cornified epidermal cells. The cornified cell is thin, non-living plate composed mainly of tough fibrous protein termed as keratin. For this, the skin cells have to go through several stages as these migrate through different epidermal layers (Fig. -17). Example: cells in the basal layer of epidermis.

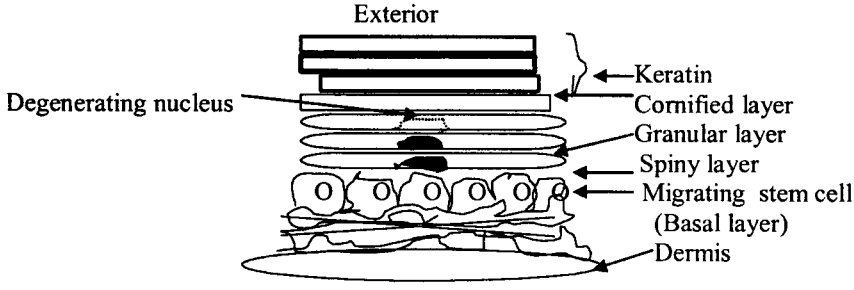
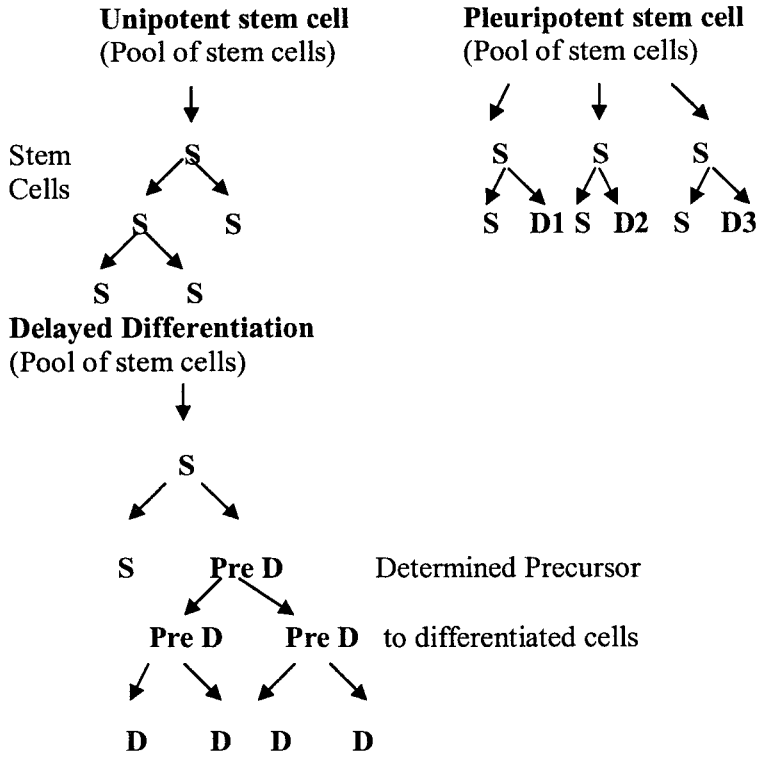


Fig. :- 17. Cells of Differentiated Skin Layers

Pluripotent Cells :

Those cells that may produce two or more independent types of differentiated cells e.g., haematopoetic cells. Stem cells are pleuripotent + unipotent.

Growth of stem cells occurs in two ways. Either the cell divides symmetrically to produce two identical stem cells or asymmetrically to produce one determined or fully differentiated cell and the other new stem cell. The determined cell from an asymmetric division may undergo several divisions on the way to reaching its final differentiated form. Therefore the final number of differentiated cells produced in a tissue is an outcome of the control of the stem cell population and the control of the number of divisions that the determined cells undergo before differentiating fully. Delayed differentiation is a mechanism by which the production of differentiated cells is controlled.



For the differentiation of the stem cells of the bone marrow specific protein factors trigger the growth and perhaps also control the growth rate of precursors to the final differentiated cells as observed in the lineage haematopoietic stem cell (Fig. - 18)

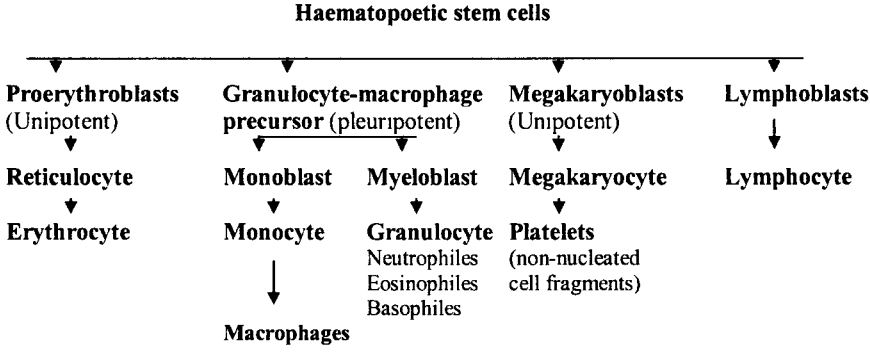


Fig. 18. Lineage of Haematopoietic Stem Cell.

Totipotent Cells :-are those cells that can give rise to a whole organism by division and cell specialization, e.g., fertilized egg. All the cell types develop from a single fertilized ovum by the process of cell division and cell differentiation. The ovum is totipotent. This means that it is not irreversibly committed to any developmental fate and is able to give rise to any cell type. First few cleavage products of a fertilized egg such as blastocytes are capable of forming whole animal, i.e., they are totipotent. However soon their topipotency is lost, but regeneration does take place in vertebrates. For example, making of extensive neural connections after birth in vertebrate nervous system. Moreover some regeneration process need to take place throughout life such as skin, blood, intestinal epithelium.

Totipotency in Ovum:- Eggs of animals contain yolk, some other macromolecules such as ribosomes and mRNA. Eggs have fixed structure and regularity of cleavage. The cells resulting from initial cleavage are 8-16 cells, are totipotent. When blastomeres are cultured they can form normal blastocytes. Such a blastocyte can be implanted into the uterus of foster mother appropriately treated with hormones and the normal animal will be born (Fig. -19).

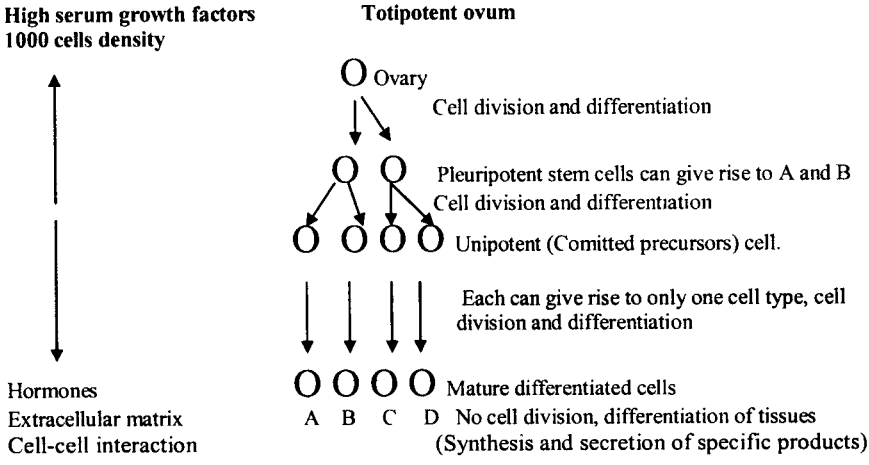


Fig. 19. Differentiation of Totipotent Ovum.

Reason for Totipotency:- Relative homogeneity of the egg cytoplasm and the consequent uniform distribution of cytoplasm to the initial cells.

At totipotent stage there are requirement of high serum and high growth factors. Low cell density is also required.

Extracellular matrix between two cell layers of different origins referred to as basal lamina. It consists of material that is secreted by one or both of the cells in the space in between. This is the mode of communication between two cells.

Extracellular matrix consists of Collagen and non-collagen proteins such as laminin and fibronectin.

Differentiation of Cell in Unicellular Organism:

To understand the differentiation of cell in unicellular organism, the example of acetabularia (a green algae) can be cited. The body of acetabularia can be divided in to three parts i.e. cap, stem and rhizoids. The large nucleus of acetabularia remains in rhizoids. Upon maturity nucleus divides into thousands of small nuclei which migrate to cap. When these are encircled by a thin cytoplasmic membrane enclosing a small mass of cytoplasm. Thus small cysts are formed. Gradually the cap degenerates and the cysts are released which gives rise to motile haploid gametes through nuclear division. These haploid gametes unite to pairs to form zygotes which are diploid and give rise to filamentous structure supporting in some substratum. A new individual is formed with a well grown cap, stem and rhizoids.

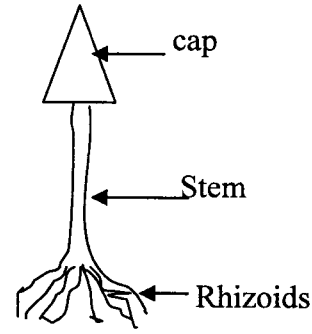


Fig. 20. Differentiation of Unicellular organism.

Differentiation of cells are not only important for production of various lineage of cells but it may be of importance in cancer therapy. Paran *et al.* (1970) observed that some cytokines may induce terminal differentiation of human and mice leukemic cells into macrophages or granulocytes, and this may serve as valuable tool for differential therapy in leukemia.

Practice Assignment XVII

1. Totipotent cell:
   (a) Is not irreversibly committed to any developmental factor
   (b) Is able to give rise to any cell type
   (c) Single fertilized cell is an example of totipotent cell
   (d) All of the above.

2. Factors responsible for cell differentiation include:
   (a) Hormones (b) Cell-cell interaction
   (c) Paracrine signalling (d) All of the above.
3. Basis of cell differentiation also includes:
   (a) Pattern of individual gene activation
   (b) Pattern of deactivation of gene
   (c) Both of the above (d) None of the above.
4. The differentiation of a determined cell is the process by which:
   (a) Genes are selectively expressed
   (b) Gene products act to produce a cell with a specialized phenotype
   (c) Both of the above
   (d) None of the above.
5. Differential of cell is an important tool for :
   (a) Production of various lineage of cell
   (b) Differential therapy in leukemia.
   (c) Both of the above
   (d) None of the above.
6. Growth of stem cells occurs :
   (a) Either the cell divides symmetrically
   (b) to produce two identical stem cells or asymmetrically to produce one determined or fully differentiated cell
   (c) Both of the above
   (d) None of the above.
7. Skin cell in vitro culture can be induced to differentiate by adding:
   (a) Certain growth factors like EGF
   (b) Some feeder layer
   (c) Other cell types
   (d) All of the above.
8. De-differentiation of cells are needed, when :
   (a) Occasional renewal of cell is required
   (b) During wound repairing of skin
   (c) Both of the above
   (d) None of the above.
9. Differentiation of cells in vitro i.e., in cell culture may occurs in :
   (a) Epithelial cells (b) Muscle cells
   (c) Connective tissue (d) All of the above.
10. Mechanism of differentiation include:
   (a) Partial shielding of genome to transcription

(b) Selective amplification of mRNA
(c) Constant genetic information
(d) All of the above.

Answers:-

1. - (d), 2. -(d), 3. -(c), 4. - (c), 5. -(c), 6. -(c), 7 - (d), 8. - (d), 9. - (d), 10 - (d).

Chapter 18

Transgenesis and Transgenic Animals

Transgenesis means transfer of gene. Generation of transgenic multicellular organisms, using standard molecular genetic technique, has been a major application of genetic engineering. The first example of transgenic animal came in 1982 when Palmitter and co-workers produced a transgenic mouse in which rat growth hormone gene fused to the promoter for mouse metalothionin-I gene was introduced. The transgene was expressed 8-9 times and these mice showed dramatic growth in their body weight leading to gigantism. Technical developments in the field of mouse transgenics in the early 1990 led to the development of mice strains that are capable of producing fully antibody responses. The first application of this technology to farm animals was reported in 1985, when pigs and sheep were made transgenic for the human growth hormone gene. In 1985, the first transgenic farm mammals was produced, a sheep called "Tracy". Tracy had a human gene that expressed high level of the human protein alpha-1-antitrypsin. The protein, when missing in human, can lead to rare form of emphysema.

The success of modern plants and animals is largely a result of many centuries of selection of desired genotype. In plants selection procedure by natural breeding takes less time but in animals it is a time consuming process due to long gestation periods in different animals. So, much time is required to acquire full genetic changes within a population. With the advancement of different biotechnological technique such as recombinant DNA technology to design, construct clone and express recombinant gene of desired protein in eukaryotic cells paved the way to produce protein with post translational modifications. Currently various therapeutic proteins are produced in transfected mammalian cell lines such as CHO or NSO and a wide field of research has built up around optimising these systems, either by changing the vectors, cell lines growth media or process (Chadd and Chamow 2001).

Gene transfer in animals has been used for modifying the fat or protein synthesis in mammary glands (Vilotte *et al.* 1997), transfer of growth hormone gene in pigs (Pursel and Rexroad 1993), transferring cysteine synthesis gene into sheep for enhanced wool production (Powell *et al.* 1994), imparting resistance to influenza virus in pigs (Muller *et al.* 1992), human alfa-trypsin in sheep (Carver *et al.* 1993), alfa-lactalbumin in cow (Colmon 1996), tissue plasminogen activator and antithrombin III in goat (Ziomec 1998) etc.

Transgene:- Is a cloned gene with appropriate promoter producing, what is called a gene construct.

Transgenesis:- Is the process of inserting a foreign gene (i.e., gene construct) into DNA of a living animals. The tools of molecular genetics coupled with reproductive biology have made it possible to transfer genes from one species, genera and even kingdom to another species, genera and kingdom. Such transferred genes are not only integrated into the host genome but are also expressed in the desired tissue, provided these are suitably modified before transfer. This approach of inserting foreign construct gene into DNA of a living animals is known as transgenesis.

Transgenic Animals:- Transgenic animals are animals which have been genetically transformed by splicing and inserting foreign animal or human gene into their chromosome. So, transgenic animals harbour unrelated genes in its genome. With the emergence of the advance biotechnological technology it became possible to accelerate the pace of genetic process including modifying the genome of animals in ways that possibly cannot be done by classical methods.

Methods of Producing Transgenic Animals:

To make a transgenic animal, it is essential to have large number of ovum from a mature female. This is obtained by super-ovulation induced by some drug. The oocytes are observed under stereomicroscope and only healthy oocyte (i.e., only cumulus oocyte complexes with more than three layers of compact cumulus cells) are selected. Then the oocyte is fertilized with sperm *in vitro*. Now a recombinant DNA carrying a recognizable foreign gene under the control of a regulator promoter is introduced into either the male or female pronucleus of a newly fertilized one cell ovum (the male pronucleus is the haploid nucleus of the sperm cell and the female pronucleus is also the haploid nucleus of the ovum) through microinjection (Brinster *et al.* 1985). Comparative opacity of ova of

cow and pig has made microinjection difficult for visualization of pronuclei. The male pronucleus is larger than female pronucleus, thus it is chosen for microinjection. Typically about 2pl of DNA containing solution is introduced and thus the two pronucleus is subsequently fuse to form zygote nucleus of fertilized eggs. Preparation of oocyte is one of the critical factor that determine the developmental competence of embryo produced by *in vitro* fertilization. Moore and Dai (2001) observed that oocytes are known to have a lower developmental competence as compared to *in vitro* derived oocyte. The fertilized egg is cultured *in vitro* to develop from 8-cell morula stage to blastocyst stage and then it is transferred into a suitable prepared recipient female (foster mother), which carries the resultant embryo till the birth in the usual manner. About one half of the transgenic animals actually express (i.e., produce polypeptide) from the transgene. Flowchart for the generation of transgenic animal showing the stages where transgene construct can be introduced using different methods has been shown in Fig. -21

Construction of Transgene:

The transgene is designed keeping in mind that magnitude of its regulation is under control both in space and time. Metallothionein-1 (MT-1) promoter controls the protein expressed in liver e.g., rat growth hormones (rGH) gene in transgene mouse was kept under the control of MT-1 promoter for subsequent expression of rGH in the liver under the signal of heavy metal ions. Complexed with regulatory protein one can induce the expression of rGH maintaining mice on a heavy metal ion containing diet (Palmitter *et al.* 1982). Similarly, cloned bovine metallothionein-buffalo growth hormone transgene construct (Fig. 22) may be used for producing transgenic cattle for buffalo growth hormone, which may provide an excellent model to study growth hormone.

Metallohionein gene encodes a metal binding protein called metallothionein. Mammalian metallothionein is a 61-or 62 aminoacid peptide containing 20 cysteins, 6-8 lysine, 7-10 serine, and a single acetylated methionine at the amino terminus and no aromatics or histidine. It also occurs naturally in many species and its role is to bind to heavy metals. Most cultured mammalian cells synthesize metallothionein and are resistant to moderate levels of heavy metal ions except W7 line of mice thymoma cell and CHO cells. In normal animals (non-transgenics), if the concentration of heavy metal becomes

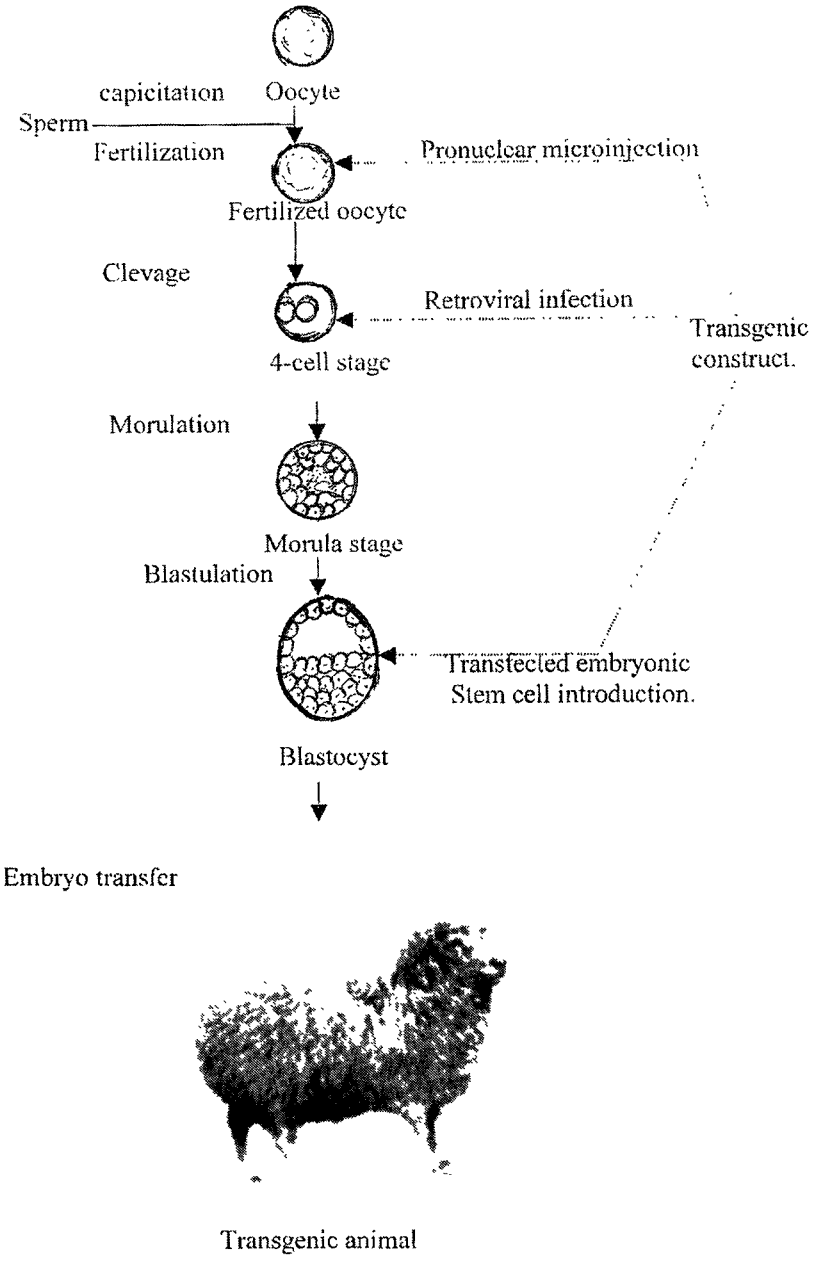


Fig. :- 21. Flowchart for the Generation of Transgenic Animal.

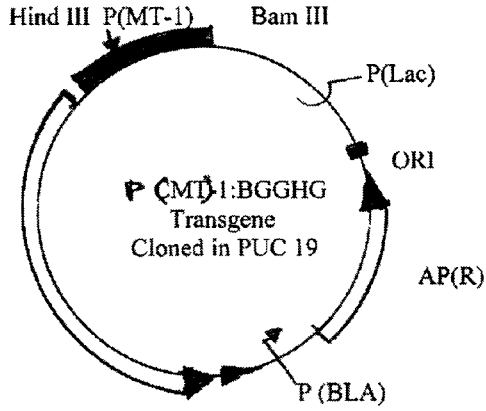


Fig. :-22. Potential Transgene Construct with Regulators Elements [prompter P(MT-1)] from cattle metallothionein and structural gene buffalo genomic growth hormone gene (BGGHG) cloned in PUC19. Restriction Site Enzyme Sites Involved in Cloning are Shown.

too high, the metallothionein gene produce metallothionein which binds to the excess heavy metals, thereby removing the threat of toxicity. If the promoter from a metallothionein gene is attached to the coding sequence of, say, a growth hormone gene, the resultant transgene can be switched on simply by adding heavy metals to the drinking water of the transgenic animals. This means that the production of growth hormone from the transgene can be turned on and off by a simple manipulation of the drinking water. Alternatively, if the aim to have a transgene expressed in just one tissue, a promoter is chosen from a gene that is naturally expressed only in that tissue. For example, a transgene containing the promoter froma milk protein gene will be expressed only in mammary gland of the transgenic animal.

Pre-implantation Analysis:

Reliable screening of the embryo for transgene integration prior to transfer is essential because of very low transgenesis rate in farm animals, such as, in pigs the frequency of developing transgenic pig is as low as 0. 6% even when as many as 7000 eggs were injected. As the biopsy of *in vitro* produced embryo shows decrease in embryonic viability to some extent, the low viability can be compensated by transferring multiple embryos to each recipient.

Sperm Sexing :

The selection of a particular sex is used to produce offspring of the desired sex. It also offers a way of avoiding the expression of the disease in the offspring (Fugger 1999). Sperm and ova which are produced by testis and ovary respectively contain half of the chromosome than somatic cells. Sperm posses autosomes and one Y-chromosome whereas ova contains autosomes and one X-chromosome. After fertilization union of XX results into female offspring whereas XY chromosome results into male offspring. The sex can be determined with 90% accuracy following the use of sexed sperm (Siedel 2003). The technique of sperm sexing relies on the fact that DNA content of X-chromosome sperm is 3. 8% more as compared to the Y-chromosome sperm (Johnson and Welch 1999). When a fluorescent dye (Hoechst 33342) is used it stains both X and Y chromosome with different intensity using an instrument called Fluorescent Activator cell Sorter (FACS) which causes flow cytrometric analysis and cell sorting (Welch and Jhonson, 1999). Generally sperms remain in form of suspension. The FACS converts the suspension of sperm into microdroplets. Each droplets consist of a single sperm cell. Individual microdroplet is passed through a lesser beam and each sperm is identified on the basis of the amount of DNA when it passes through a laser beam. Microdroplet of different intensities are deflected into separate collecting tubes, one tube for X-chromosome sperm whereas other tube for Y-chromosome sperm. The sperms are differentiated according to the how much light is deflected from the fluorescent dye, which is proportionately bound to the sperm DNA. Consequently X-chromosome bearing sperm with more total DNA glow brighter than the Y-chromosome containing sperm when hit by the laser beam. The sperm separated by FACS has recently been used and pre-sexed calves have been produced through *in vitro* fertilization. FACS is very expensive and slow.

It is also essential to sex embryo and only transfer the embryo of same sex to one recipient in order to avoid free martinism. Bovine embryo sexing using PCR has been reported by Sood and Madan (1998). Also, since for mammary gland transgenesis females are to be produced, the sexing of the pre implantation embryo is highly desirable. After pre implantation analysis the female embryo which harbor transgene are transferred to the foster mother and allowed to complete the term.

Pre-implantation embryo Nutrition:

The most important nutrients required by the developing mammalian preimplantation embryo are carbohydrate, amino acid which provide energy. It also prevents cellular stress. Quantification of embryo energy metabolism is thus necessary. An attempt in this direction has been made.

The 8-cell embryo block in development is overcomes by various growth factors incorporated in cellular media. The HB-EGF (heparin binding epidermal growth factor) improves the development of 8-cell embryo to the blastocyst stage. This factor is helpful in preimplantation embryo development (Tamada *et al.* 1999). The method of assessing preimplanation embryo viability by the self-referencing electrophysiochemical technique is possible. This is an non-invasive measurement. The technique measures the gradients of calcium, potassium, oxygen and hydrogen peroxide (Gardner 1998).

Choice of the Host:

Before selecting host one question need to be asked, whether the transgenic product is useful to the animal itself or it is to be expressed in host for commercial purposes. If the product is useful to the animal itself, then one can go straightway to the animal of choice. This has been demonstrated for the production of large number of animals harboring growth hormone transgene. If the transgenic animal is to be used as a bioreactor for the production of heterologous protein in some extracellular fluid, then the following criteria may be taken into consideration:-

- Animal should produce large volume of extracellular fluids,
- Purification of heterologous protein from extracellular fluids should be relatively easy,
- Have gestation and development periods of moderate length,
- There should not be adverse effects on the physiology of the transgenic animals.

There is no one suitable host which meets all the above criteria. Cattle and buffalo produce large volume of milk whereas their gestation and development period is long.

Postbirth Analysis :

Neonates are analyzed for transgene and expression of the transgene. The new born transgenic animals are then screened for the presence

of transgene (i.e., Ear, tail and blood DNA) using southern blotting of the pure genomic DNA with subsequent probing using DNA probes from transgene construct. They are then mated with their transgene sibling to obtain homozygous transgenic animal.

Application of Transgenic livestock:

(i) Transgenic animals can be prepared by keeping in view the economically significant traits, such as efficient feed utilization, faster growth rate, production of lean meat, increased production and enhanced immunocompetence,

(ii) Attempts have been made for exploiting the mammary gland to produce pharmaceuticals proteins in milk. The production of valuable pharmaceutical proteins using transgenic animal as mammary bioreactor is a major achievement in the field of live bioreactor.

(iii) This technology has been exploited to make insights into the functioning of promoter and other regulatory regions in gene expression in the mammalian system.

(iv) Transgenic sheep armed with genes involved in cystein synthesis from dietary serine exhibited significant positive effect on wool production,

(v) Various heterologous protein has been produced in the milk of pigs using cDNA encoding human protein (Velander *et al.* 1992) for production of functional human haemoglobulin (Swanson *et al.* 1992). Similarly a heterologous protein, such as a variant of human tissue plaminogen activator has also been produced in goat which is being used in patient suffering from thrombosis, for dissolving blood clots,

(vi) The long term goal of companies developing transgenic animals is to create a new generation of medicines based on gene product, rather than on drugs engineered by chemicals,

(vii) Transgenic processes have been adopted to increase the disease resistance in animals such as, genetic immunization with recombinant plasmid containing gene for antigen of interest has been attempted for prevention of infectious diseases, e.g., rabies, pseudorabies virus etc.

(viii) Another mechanism to increase resistance against various diseases in transgenic animal is the congenital immunization involving transmission of gene coding for immunoglobulin, MHC, T-cell receptor or lymphokine specific for the particular

pathogen, thus providing congenital immunity or inherited immunological protection without prior exposure to pathogen. This would prohibit the need for immunization by vaccination.

(ix) Breeding of pigs with humanized organs for use in xenotransplantation has become an important application of transgenic approach.

(x) The gene construct used to create the transgenic pigs containing the human beta-globin locus control gene may be suitable substitute for obtaining blood.

(xi) Transgenic animals are also used for testing of drugs or to undertake studies which is always not possible on human beings.

(xii) Transgenic animals have also been produced to serve as model to understand particular disease like cystic fibrosis. This is caused by a defective gene which can be mimicked in a transgenic mouse. Such transgenic animals allow the testing of drugs or to undertake studies not always possible on human. Another example of transgenic animals produced is used to understand a particular process of Transgenic Zebra-fish, which are important tool to help in understanding gene action during embryo development.

Advantage of Transgenic Animals:-

(i) Gene require certain cellular mechanism to help for the production of protein. The animals used for transgenic purpose naturally carry the mechanism needed to produce complex protein. Theses mechanism is absent in cell culture.

(ii) Expression through cell culture or bacterial culture requires constant monitoring and sampling.

(iii) The isolation and purification of expressed protein in conventional method is more difficult than purifying proteins from an animal's milk or body fluid.

(iv) It is more cost effective as the product is efficiently passed through milk with an average yield of 53% and with 99% purity.

(v) It has been estimated that transgenic animal can produce in its lifetime $100 to $200 million worth of pharmaceuticals.

Disadvantage of Transgenic Animals:-

(i) Sue Mayer of Genewatch, a UK group that campaign against the spread of genetic engineering has argued that setting up of transgenic animal project is extremely expensive.

(ii) Generation of transgenic animals are also expensive, because of long gestation period, litter size and higher maintenance cost of the recipient animals.

(iii) There may be high mortality rate and other deleterious effects on animals used by researchers to create transgenic breeds. It has been observed that transgenic pigs having enhanced growth rate and efficient feed conversion exhibit reduced reproductive performance and may suffer from arthritis and dermatitis etc. Similarly, transgenic sheep expressing growth hormone may show diabetic like conditions. Mayer argues that we do not understand the long term effects of genetic engineering on animals.

(iv) Large number of recipients is required for embryo transfer because of low transgenesis rate.

(v) Transgenic foods have been produced and offer better productivity in terms of both yield and quantity. However, there are some apprehension about the safety of transgenic foods (Berkowitz and Sorenson 1994).

Exploration of Animal Pharming through Transgenic Animals:

The creation of transgenic animals for mass production of heterologous proteins and human medicine is known as animal pharming. Through transgenesis attempts were made to use animals as bioreactor to synthesize pharmaceutically important proteins. Using animals as bioreactors are more cost-effective because of their inherent capabilities to effect post-translation modification (e.g., glycosylation single peptide cleavage, sulfuration, phosphorylation, amidation etc.) of complex mammalian proteins maintaining functional integrity in large unlimited amount, the transgenic animals function as ideal living fermenters for production of certain biomedically useful proteins. The most successful of this approach has been to exploit the properties of milk as a renewable resource to produce functional enzyme, antibodies and structural protein using existing dairy manufacturing processes. The strategy has been to use transcriptional promoters of specific mammary genes to direct the expression of soluble transgenic protein.

A milk borne protein do not cross into the blood or lymphatic fluid of the transgenic animal, there is less danger of producing undesirable side effects in the animal owing to the species or biochemical function of the transgenic animal to synthesize pharmaceutical proteins in milk,

cell specific mammary gland expression can also be exploited to produce nutrients enriched dairy products.

Automated gene sequencing and the biological advantages of animals, when compared to more traditional methods of recombinant protein production, have combined to make pharming a preferred alternative. Traditional method of recombinant protein production use laboratory cell culture of transgenic bacteria, yeast or animal cells to produce protein. Inherent disadvantages in traditional methods, when compared to using animals as bioreactors include the followings:-

- Cell and bacterial culture require constant monitoring and sampling,
- expansion is more costly, because substantial plant machinery must be purchased and maintained and
- isolating and purifying protein is more difficult than purifying from animal's milk or body fluid.

Several pharmaceutical companies are exploring to produce various heterologous proteins, for use in human and livestock for therapeutic purposes.

It is known the Omega-3 fatty acids is responsible for lowering the triglyceride and LDL level, as their high level are responsible for blockage of coronary artery resulting into heart attack. Omega-6 fatty acid is present in all mammals but it does not have genetic mechanism to convert it into omega-3 fatty acids. Recently, Kang *et al.* (2004) have engineered a mouse strain to produce omega-3 fatty acids, compound known to prevent heart disease in human beings. A gene called fat-1 from the roundworm *Caenorhabdis elegans* was used to convert omega-6 fatty acids into the healthier omega-3 version. This may offer a way of bumping up peoples omega-3 intake without a lifestyle overhaul. So, pharming of this mice will be useful in future to prevent the heart attack in human beings.

Some of the important biological active expression of transgenic protein through the mammary glands in livestock, attempted for clinical use has been depicted in Table-9.

Transgenic Plants:

The first transgenic plant developed was **"Flavr-Savr"** tomato, which has more self life as it remained firm and maintained the right texture for longer period of time. Later in several species of plant transgenic plant was developed including BT cotton. For making transgenic

Table. 9: Transgenic Proteins in Transgenic Animals.

Animals used	Drugs/protein	Uses
1. Sheep	Alpha 1-anti trypsin (Carver *et al.*,1993).	Treatment of emphysema
2. Sheep & goat	Human tissue plasminogen activator (Ebert *et al.*,1994)	Treatment of thrombosis
3. Sheep & pig	Human blood clotting factor VIII and IX.	Treatment of hemophilia
4. Sheep	Fibrinogen	Treatment of wound healing.
5. Goat	Human protein C	Treatment of thrombosis.
6. Goat	Antithrombin 3	- do -
7. Goat	Glutamic acid decarboxylase	Treatment of type-1 diabetes.
8. Goat	Pro 542	Treatment of HIV.
9. Cow	Lactoferrin	Treatment of G.I tract infection & infectious arthritis.
10 Cow	Human serum albumin	Maintaining blood flow.
11. Cow	Collagen I and II	Tissue repair & treatment of rheumatoid arthritis.
12. Cow	Fibrinogen	Wound healing.
13. Cow	Factor VIII	Hemophilia treatment.
14. Cow	Alpha-lactalbumin	Against infection.
15. Chicken, cow And goat.	Monoclonal antibody	For vaccine production.

plants any cell from the plant or protoplast is transferred, which becomes transformed in transgenic plant as undifferentiated plant cells as these are totipotent and has capacity to develop in whole plant through differentiation and growth., here separate sperm or ova, like livestock is not required. The production of transgenic plants includes the control of transcription of the foreign gene by an efficient plant promoter. In most cases the gene coding for the foreign antigen is put under the control of the cauliflower mosaic virus (CaMV) 35/2 promoter which ensures its constitutive transcription in different parts of the plant body. It has a limitation that in most cases the amount of the foreign gene expressed may vary. Genetic transformation of crop plants could be mediated by a plant infecting bacterium, *Agrobacterium tumifaciens* which contain a low copy number of Tumour inducing

(Ti) plasmid. When agrobacterium appears in conjuction with a wounded plant cell, the Ti plasmid transfers part of its DNA (T-DNA) into the plant genome. The T-DNA is flanked by repeated sequences (left and right borders) which are required for integration into the plant genome. Thus, when the foreign gene is incorporated between left and right borders, it can be transformed into the plant cell followed by regeneration to obtain transgenic plants. This is an efficient and cost effective method for production of transgenic plants.

Earlier monocotyledon plants, including important cereals were thought to be recalcitrant to *Agrobacterium* sp., but later agrobacterium mediated cereal transformation was found to be effective (Mahalakshmi and Khurana 1997). The first successful report of fertile transgenic wheat plant was reported by Cheng *et al.* (1997). Patnaik *et al.* (2006) reported efficient production of transgenic wheat plant using *Agrobacterium tumifaciens* with a frequency of nearly 1. 28-1. 77 in both bread wheat as well as drum wheat. Application of biotechnology for the production of transgenic plant is also essential to meet the demand for food as its application may increase yield, increase pest resistance, induce herbicide tolerance, increased salt tolerance and increase the nutritive value.

Like transgenic animal transgenic plants are also constructed. In transgenic animals, the expression systems are particularly cost intensive and requires removal of oncogenic DNA, pathogenic micro-organisms and endotoxins during downstream processing. Transgenic plants offer additional advantages for mass production of recombinant proteins and antibodies, with enormous levels of biomass, straightforward product storage, distribution with low production cost and are devoid of onchogenic DNA, pathogens and endotoxins present in downstream processing of microbial and animal cell culture used for the production of recombinant antibodies. Furthermore, plant suspension cultures can be easily be scaled up to large fermentation volumes and can be grown under full cGMP conditions. The use of crops for the production of preventive, diagnostics and therapeutics have unique advantages in terms of delivery and long term storage as seeds.

Recently, expression of hepatitis B surface, antigens (HBsAg) have been studied under two strong promoters i.e. CaMV and Patatin Class I promoters, in genetically engineered potato plants. The middle (M) and major or small (S) hepatitis B surface antigens were extracted from transgenic potato plants and characterized by sedimentation in

sucrose and cesium chloride gradient system and it was observed that both M and S HBV protein aggregated into particles. These may be a strong candidate in development of hepatitis vaccine and transgenic potato (Domonsky *et al.* 1998). A series of expression vectors for HbsAg have been evaluated in potato plants. From these line a potato has been selected which gave as high as 18 microgram of HbsAg per gram of fresh potato. Mice fed with peeled potato slice expressing HBs AG developed serum IgM and IgG response that were specific to HbsAg whereas control mice fed with non-transformed potato failed to make any antibody.

In an another study Thanavala *et al.* (1998) created transgenic tobacco plants which also express HbsAg. Mice immunized with a crude extract of the tobacco derived HbsAg elicited an antibody and T-cell response specific to HbsAg that was qualitative similar to commercially available yeast derived HbsAg.

The ICRISAT, Hyderabad developed the first transgenic ground nut in the world. The ground nut was genetically modified with gene for the resistance to Indian pea nut clump virus (IPCV) which is widespread in the country. IPCV is transmitted by a soil born fungus *Polymyxa graminis* and hence it is difficult to control. The technology behind the development of genetic engineered ground nut involved transferring the coat protein and polymerase gene of ICPV through the vector *Agrobacterium tumefaciens.*

Transgenic soyabean was genetically modified to make it tolerant to a herbicide namely glycophosphate. For that a gene that encode the enzyme 5-enolpyruvylshikimate-3-phosphate synthase was transferred from *Agrobacterium* sp. Strain CP_4 into commercial cultivators of soyabean.

Practice Assignment XVIII

1. The growth hormone used by Palmitter to produce first transgenic mouse was obtained from:
   (a) Rat (b) Cat
   (c) Horse (d) Sheep.
2. The first transgenic sheep produced was called:
   (a) Crazy (b) Tracy
   (c) Mercey (d) Farcey.
3. Previously the hybrid animals or plants are produced by:
   (a) Natural breeding (b) Selection procedure
   (c) Both of the above (d) None of the above.

4. Transgenic animals are:
   (a) Genetically modified animals
   (b) Harbour unrelated gene in its genome
   (c) Both of the above
   (d) None of the above.
5. Transgenesis is the process of:
   (a) Inserting foreign construct gene into DNA of living animals
   (b) Gene may be transferred from one species to same species
   (c) Gene may be transferred from one species to the another species
   (d) All of the above.
6. Transgenis animals :
   (a) Are susceptible to various diseases
   (b) Here large number of animals are required
   (c) Both of the above
   (d) None of the above.
7. Getting milk from transgenic animal is useful as :
   (a) Milk borne protein do not cross into blood
   (b) Less danger for the production of undesired side effects
   (c) Is cost effective
   (d) All of the above.
8. Mammary gland expression in transgenic animals can be exploited for the production of:
   (a) Pharmaceutical products (b) Various human proteins
   (c) Both of the above (d) None of the above.
9. Omega-3 fatty acids have been produced in mouse by transfer of gene called:
   (a) Fat-1 from Caenorhabdis elegans roundworm
   (b) Human beta-globulin locus
   (c) Cystein aminoacid gene from pigs
   (d) All of the above.
10. The first transgenic plant developed was of;
   (a) Tomato (b) Potato
   (c) Pea (d) Cabbage.

Answers:

1. - (a), 2. -(b), 3. -(c), 4. -(c), 5. -(c), 6. -(d), 7. -(c), 8. -(d), 9. -(c), 10. -(a).

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Chapter 19

Apoptosis, Necrosis, Senescence and Quiescence

Apoptosis:

Apoptosis was first described by Kerr *et al.* (1972). The term apoptosis in which the second "P" is not pronounced was identified by Andrew Wyllie in the same year. It is widely accepted as a normal physiological process during cell development and differentiation (Vaux and Korsmeyer 1999). Apoptosis is an energy dependent process by which individual cells undergo programmed cell death in response to various extrinsic and intrinsic factors (Gadaleta *et al.* 2002) without inducing inflammatory responses. Normal development of organ requires not only cell division and cell differentiation but also elimination of cell by apoptosis. Such as, in the central nervous system, the number of neuron is largest in the midway foetal life, but later extensive apoptosis causes the number of neurons to be just half. The same process occurs in ovaries of the female when the number of immature egg cells in the ovaries is gradually reduced from midway in pregnancy until birth. Panisup *et al.* (2005) observed apoptotic changes in the Bursa of Fabricius showing negligible inflammatory changes infected with infectious bursal disease virus in chicken. It consists of a predictable series of morphologically defined events such as thymic dendritic cells (TDC) play an important role in clonal deletion (apoptosis) of autoreactive immature thymocytes and also in defence against chemical insult and infection. Many of the RNA viruses (such as paramyxovirus, arbovirus etc.) and DNA viruses (like adenovirus and herpes viruses etc) have been shown to induce apoptosis in the infected cells, but their mechanism of apoptosis differ in different viruses. However, some of the transforming viruses can inhibit apoptosis and cause cell differentiation instead. The viruses take up different pathway to enhance or inhibit the apoptotic process.

The inhibition of apoptosis may lead to cancer. Although most cell death abnormalities have been associated with specific autoimmune disorders such as lupus erythematosus and lymphoproliferative syndromes, it is evident that regulation of cell death is also pertinent to diseases expression in many organ specific disease such as rheumatoid arthritis and glomerulonephritis. In apoptosis, the active cell death process (ACDP) is a gene controlling arsenal for maintaining homeostasis in the body for survival (Lam, 1997). It starts working during embryonic life in response to the internal clock through a set of caspases (Thronbemy *et al.* 1998). However, in postnatal life apoptosis can be induced by a number of stimuli such as infectious agents like HIV in human beings, infectious bursal disease in chickens (Cao *et al.* 1995) and non-infectious agents like ionising radiation, steroids, cytokines, hyperthermia including poisons and toxins and other agents causing damage to DNA (Hockenberry 1995). Besides this withdrawal of growth factors and antigen-receptor engagements in case of T and B cells may also lead to apoptosis. In apoptosis DNA is fragmented in which the genome DNA is cleaved into oligonucleosomal fragments by multiples of 180-200 bp, the cytoplasm shows blebbing, plasma membrane has increased granularity and there is a fracturing of the cell into small apoptotic bodies containing DNA. Though in apoptosis DNA fragmentation is an important feature, it may not be so in every case, e.g., in oligodendrocytes. Certain types and cell lines, such as K 562, Raji do not fragment their DNA in this manner during apoptosis.

Pathway of Apoptosis:

Current research on apoptosis suggests that proteins belonging to the caspase family are involved in cell death during apoptosis. Fas/Apo-1 receptor (Fas R) also called CD_{95}, which is a cell membrane receptor (mFas), expressed on the cell surface of normal and malignant cells is known to induce cell death by apoptosis. Fas (CD95)/FasL interaction also downregulates immune responses by mediating T-cell apoptosis during activation induced cell death (AICD). Apoptosis occurs by both Intrinsic and Extrinsic pathway:

Intrinsic Pathway:

The intrinsic pathway is triggered by chromosomal abnormalities involving mutational change in gene resulting into appearance of P^{53} (onchogene) which initiates apoptosis in response to DNA damage in some cell types (e.g., skin cells) by inhibiting BCL-2 (family of cell

death regulator and is homologous to *Caenorhabditis elegans* CED-9 proteins expressed in all surviving cells during development) and activating BAX gene resulting into formation of BAX protein causing apoptosis. (Vaux and Strasser 1996).

Extrinsic Pathway:

Extrinsic pathway involves appearance of death receptor TNF at cell surface during unfavourable environment. When TNF acts with membrane receptor trimerization of the receptor occurs. The membrane receptor has TRADD protein. This protein is associated with procaspase-8. After removal of some sequence of procaspase-8, this complex acts on the BID protein which cause its fragmentation into Carboxy (C) and amino (N) terminal end. When these fragments act on mitochondrial membrane causing mitochondrial permeability transition which allows the disruption of inner transmembrane potential (ÄØm) and the release of proapoptotic factors that cytochromC and AIF (apoptotic inducing factors) (Susin *et al.* 1999). The electrical and volume homeostatis in mitochondria which is regulated by Bcl-XI might have a role in transmitting apoptotic signals. Apoptotic protease activating factor-1(Apaf-1) which is composed of caspase recruitment domain (CARD), cell death abnormal-4 (CED-4) like domain and WD-40 repeats has been shown to activate caspase-9 in a cytochromeC/dATP-dependent manner followed by caspase-3 activation (Li *et al.* 1997). Activated caspase-3 is able to cleave many substrate including PARD[Poly (ADP-ribose) polymerase] and DNA-Pk (DNA dependent protein kinase) and PKCä. Finally caspase activated DNAase and other hydrolytic enzymes are produced that lead to the morphological and biochemical consequences of apoptosis (Liu *et al.* 1997).

Recently, Lim *et al.* (2005) investigated apoptosis in Vero cells (C-1586) infected with Akabane, Aino and Chuzan virus (viruses belonging to RNA arthropod-borne virus group). They observed that *de novo* viral protein synthesis is critical for viral apoptosis. In addition, the activity of caspase-3 was also detected in Vero cells by indirect fluorescent assay.

The process of apoptosis can be divided into **three stages**:

The First Stage is Induction Stage:- In this stage there is initial signal for apoptosis by variety of stimuli like various stresses including deprivation of serum, growth factors or cytokines, heat shock and various carcinogenic reagents (Schutz-Osthoff *et al.* 1988).

The Second Stage is Execution Stage:- This stage involves the classic morphological and biochemical changes. Morphological changes includes condensation and peripheralization of chr ›matin, vacuolisation and loss of cytoplasm, fragmentation of nucleus, compaction of organelles, the disestablishment of communication with neighbouring cells, fusion of the endoplasmic reticulum with the outer cell membrane. The biochemical changes involves the activation of a specific series of cytoplasmic cellular proteases and endonucleases, caspases (Cryns and Yuan 1998). The activation of these self- catalytic caspases in the cytoplasm has been identified.

The Third Stage is Degrdation Stage:- This occurs due to the various morphological and biochemical changes mentioned above. Finally there is fragmentation of the cell DNA and other macromolecules into size of nucleosome units due to the activation of an endogenous nuclear endonucleases. Water is extracted from the cell resulting into marked decrease in cell size and increase in density. The increase in density has been used to isolate apoptotic from non-apoptotic cell (Martin *et al.* 1990). The shrunken apoptotic cells subsequently fragmented into sealed vesicles and thus formation of numerous membrane- bound apoptotic bodies containing DNA which are engulfed by the surrounding cells (Hay and Kannourakis 2002).

Detection of Apoptosis:

The DNA fragmentation which occurs in apoptosis can be visualised by agar gel electrophoresis. The reduction in cell size and volume in apoptosis can be studied by microscopy of flow cytometry. A number of dyes such as ethidium bromide or propidium iodide can enter in the apoptotic cell due to altered permeability, and thus number of dead cells can be obtained in flow cytometry. The following methods have been developed to quantify DNA fragmentation:

(i) FACS (Fluorescent Activation Cell Sorter) analysis:- In this method the isolated nuclei are stained with various dyes. The DNA content of apoptotic nuclei is less than that contained in intact diploid nuclei,

(ii) Terminal deoxy nucleotide transferase (Tdt) mediated dUTP-digoxigenin nick and labelling (TUNEL) assay :- This method is based on *in situ* labelling of DNA fragmentation sites in the nuclei of intact fixed cells. Tissue sections can also be stained by this technique.

Suppression of Apoptosis:

Sometimes through an active programme suppression of apoptosis is needed to maintain the cells in quiescent state. The execution of the quiescence program appears to be essential for the long term survival of peripheral lymphocytes and is dependent upon signals transduced through the B and T cell antigen receptors. In addition, the transcription factor LKLF is required component of this programme in T-cells.

Role of Apoptosis:

(i) It has important role in various fundamental biological processes such as organ development, wound healing, immune response and oncogenesis.
(ii) Plays important role in regulating total cell number.
(iii) It helps in adaptation of an organism to environment and resolution of inflammation by safe elimination of unwanted cells
(iv) It helps in removing the damaged, infected and potentially neoplastic cells and thus protect the human beings and different livestock from various diseases including cancer.

Necrosis:

Necrosis means the death of cells or tissues in the living body. Here cell dying by this process has no control over its fate. In some cases death is rapid or sudden, but in others various degenerative changes takes places, such as cloudy swelling and fatty degeneration, before the cell or tissues dies, and the process of dying is gradual one. Here the cell death is due to leakage of the lysosomal enzymes into the cytoplasm, swelling of the cell and eventual rupture of the plasma membrane. When a cell die in the living body there follows short lapse of time during which comparatively little histological change takes place and the cell presents an appearance identical with that shown by the healthy cell which has been killed by fixation.

There are several causes, which initiates necrosis. These include;

(i) Micro-organism and their Products:- Invasion of cells by different micro-organisms, such as *Mycobacterium tuberculosis, Corynebacterium diphtheriae* and toxins of Clostridium sp. May cause necrosis.

(ii) Physical Agents:- Various physical agents like electricity, extreme heat and cold, X-rays and prolonged pressure (by ligature or tumours) may lead to necrosis of the cells.

(iii) **Chemical Agents:-** various chemicals such as carbolic acids, mineral acids and caustics act directly on cells resulting into necrosis of cells.

(iv) **Enzymes:-** Fat splitting enzymes from the pancreas which gives rise to fat necrosis.

(v) **Interference with Nutrition:-** Interference with nutrition by arrest of circulation in tissues where a collateral blood supply is absent or insufficient to ensure the life of the tissues. When such obstruction occurs, infarction results and one of the main features of infarction is necrosis.

(vi) **Pressure Necrosis :-** It occurs as a result of long continued pressure at a particular place, generally seen in orthopaedic patients, who have to lie down for a longer period of time. In them pressure necrosis may be seen in mild form like bed sores.

(vii) **Lack of Nerve Supply:-** It has been observed that limbs and other parts suffer atrophy and necrosis when deprived of normal innervations.

Difference between Apoptosis and Necrosis:

Table. -10 : Depicting Difference Between Apoptosis and Necrosis.

Apoptosis	Necrosis
1. Apoptosis occurs at single cell level.	1. Necrosis occurs in a group of cells or in tissues at a particular locus.
2. It is an active type of programmed cell death that is highly regulated and involves the activation of cascade of molecular events leading to cell death.	2. It is a passive degeneration of cells characterized by catastrophic toxic events.
3. It is a programmed cell death and is initiated as a response to external signals from other cells, or as a result of changes in the intracellular macromolecules.	3. Necrosis is caused by external injury, damage or microbial agents.
4. Here during cell death there is no leakage of the cell content, instead there is fragmentation of DNA, the cytoplasm shows blebbing and increased granularity and there is fracturing of the cell into small apoptotic bodies containing DNA.	4. In necrosis cell dearth is due to leakage of the lysosomal enzymes into the cytoplasm, swelling of the cell and eventual rupture of the plasma membrane.

Apoptosis	Necrosis
5. Here no inflammatory changes are observed.	5 Necrosis is always accompanied by inflammatory changes.
6. In apoptosis there is shrinkage of cell.	6. In necrosis there is swelling of cells.

Senescence and Quiescence:- Both the phenomena have been observed during growth of cell culture.

Senescence:

Eukaryotic cell cycle has been considered to be an indefinitely repetitious and variable process. Some cells in the adults, such as those of skin or blood proliferate throughout most of our adult life whereas those of the nervous system stop dividing soon after the birth and never divide again in the lifetime. Closer consideration of tissues such as the skin or the blood suggests that even within an actively dividing tissue cells cease to divide and eventually die or are removed and these two processes are normally in balance. Senescence is essentially an irreversible phenomenon

The eventual loss of ability to divide is in fact a fundamental property of most types of cells within the body. The process by which cells lose the ability to produce progeny cells is termed *senescence.* In a senescent population of cells the growth fraction has declined to zero and no further increase in population is possible. Even within a population of cells actively undergoing cell proliferation, there may be a fraction of senescent cells that never divide. Such as, if normal cells from skin are removed and grown in culture their behavior can be studied over many generations under relatively controlled conditions. In most cases, it is found that normal cells divide and multiply quite rapidly but as time and number of generations increase, the overall rate of population increase begins to slow and eventually reach a plateau. At that point the growth fraction has dropped to zero and all the cells remain indefinitely in G1 phase. Closer inspection of this phenomenon shows that senescence does not occur instantaneously after the same period of chronological time or number of cell division but rather it is a progressive process. As a population of cells approaches senescence cell cycle time increases and become progressively more heterogeneous and at the same time the growth factor progressively decrease.

The phenomenon of senescence also exhibits some unexpected properties when different types of cells are considered. In case of human fibroblasts, the average number of cell doublings that occurs before the plateau phase is relatively constant for individual to individual, being approximately 50 generations. Secondly, the number of generations required for population to reach the plateau phase is strongly influenced not by the species but by the exact type of cells in question. While skin fibroblasts reach a plateau phase after approximately 50 generations, some cell types from early embryo can plateau and senescence after only 6-7 generations in culture. This means that cellular senescence appears to be programmed phenomenon whose kinetics is ultimately controlled by the identity of the type of the cell.

Quiescence:

A population of cells may cease to proliferate but retain the ability to divide further, this phenomenon is called *Quiescence*. The state of quiescence is brought about most usually by the manipulation of environment of the cells. Quiescence is reversible. If cells are rendered quiescent by alteration or manipulation of their environment they can be induced to reenter the cell cycle and proliferate by restoration of original conditions. Closer inspection of cells whose multiplication has been brought to a halt by quiescence, reveals that they are halted or altered not randomly but specifically in the G1 phase of cell cycle and the process of departure from quiescence and reinitiation of cell multiplication must necessarily involve events occurring in G1.

Difference between Senescence and Quiescence:

Senescence and quiescence can be considered as analogous phenomena. Both involve cessation, multiplication and progress through the cell cycle. While senescence is programmed and essentially irreversible, quiescence is dependent upon environmental stimuli and is reversible. Both are initiated by failure to progress through G1 phase. Taken together with G1 as the major variable phase in cell cycle, it may be concluded that G1 is the phase in which prominent control points in the cell cycle are exerted.

Practice Assignment XIX

1. In apoptosis the following changes takes place, EXCEPT,
   (a) The nuclear DNA is fragmented
   (b) Plasma membrane has increased granularity

(c) All the stage of inflammatory changes takes place
(d) There is featuring of cell into small apoptotic bodies containing DNA.

2. Apoptosis can be induced by:
   (a) Steroids
   (b) Agents that damages DNA
   (c) Cytokines
   (d) All of the above.
3. DNA fragmentation in apoptosis :
   (a) Does not occur in oligodendrocytes
   (b) Does not occur in Razi cell line
   (c) Both of the above (d) None of the above.
4. Apoptosis occurs in various stages, which may be characterized by:
   (a) Signalling by a variety of stimuli
   (b) Classical morphological and biochemical changes
   (c) Above changes leads to fragmentation of DNA
   (d) All of the above.
5. All of the changes occurs in apoptosis, Except :
   (a) Inflammatory changes
   (b) Programmed cell death
   (c) Fragmentation of DNA
   (d) Increased granularity of plasma membrane.
6. In necrosis death of cell may occur due to:
   (a) Various degenerative changes, like cloudy swelling and fatty degeneration
   (b) Leakage of lysosomal enzymes in the cytoplasm
   (c) Swelling of cell and eventually rupture of plasma membrane
   (d) All of the above.
7. Necrosis may be initiated by :
   (a) Micro-organisms and their products
   (b) Various physical agents
   (c) Various chemical agents including enzymes
   (d) All of the above.
8. All the events occurs in Senescence, Except :
   (a) Cells loose the ability to produce progeny
   (b) No further increase in population
   (c) It does not occur instantaneously
   (d) Is a reversible process.

9. In quiescence:
   (a) Cells may cease to proliferate but retain the ability to divide
   (b) Occurs mainly due to manipulation of the environment
   (c) Is a reversible process
   (d) All of the above.
10. The execution of senescence programme appears to be essential:
   (a) For the long term survival of peripheral lymphocytes
   (b) Dependent upon signals transducer through T and B cell
   (c) Both of the above
   (d) None of the above.

Answers:

1. -(c), 2. -(d), 3- (c), 4. -(d), 5. - (a), 6. -(d), 7. -(d), 8. -(d), 9. - (d), 10. -(c)

Chapter 20

Embryo Transfer Technology

Eversince the creation of mankind, the major thrust has been in the search of food for existence. Thus, human population had to create and sustain the ability to manipulate the nature to provide a continuous supply of food.Plants and hunted animals were utilized as food during the pre-historic period. Subsequent domestication of animals and their involvement in crop raising activities had made animal raising as a subsidiary to agriculture. However, livestock industry has shown the ability to sustain itself as a profitable industry in many spheres overpowering the traditional dominance exercised by agriculture and thus, bovine wealth plays an important role in India, where cattle (cows and buffaloes) is a prime dairy animal. Here cattle dairy farming have been recognized as socio-economic enterprise for rural development and thus various genetic improvement programme have been intensified. Traditionally, one of the most effective ways of rapidly bringing significant genetic improvement programme in dairy animals was based on evaluation of performance of a sire's progeny introduced through artificial insemination. This has resulted in substantial and steady progress in the selection of desired productive traits over many years.

The development of Embryo Transfer Technology (ETT) has emerged as a very powerful tool after artificial insemination for genetic improvement programme. Recent development in molecular biology and genetic engineering are providing new dimension to research and development for future application of farm animals and ETT now offers new opportunity to improve the dairy industry. So, "Embryo Transfer Technology refers to the technology by which fertilized ova are collected from the reproductive tract of genetically superior female (donor) and transferred to that of another female (recipient) which is genetically inferior".

Historical Perspective:

As early as in 1890 Walter Heape, a student at Cambridge University

performed the first embryo transfer experiment. He removed surgically two embryo (4-cell stage) from Angora rabbit and transferred it in a Belgian female hare. Afterwards the events occurring in different year are as follows:

1933 :- J. S. Nicholson successfully transferred embryo in rat.

1951:- El willett usefully transferred embryo in cattle.

1960:- Kvasnickni, a Russian scientist produced first successful embryo transplanted offspring in swine.

1964:- Mutter and co-workers transferred a bovine embryo non-surgically and getting a viable offspring.

1979:- Steptoe and Edwards got success in getting a human baby girl through ETT.

1983:- Drost got calf born by ETT.

1991:- Mishra and coworker successfully produced calf from frozen thawed buffalo embryo.

The chain of events in the entire process of embryo transfer technology includes selection of donor and recipient. The management includes, treatment of donor for production of optimum number of viable ova, breeding of the donor with quality semen, embryo collection from donor, evaluation of fertilized ova and then transfer it into recipient for growth. The steps of the ETT are as follows:

Superovulation:

Superovulation (multiple ovulation) is a key step for the production of large number of high quality embryo which is a well defined goal of successful embryo transfer programme. Various gonadotrophins are used for superovulation, but superovulating response to exogenous gonadotrophins remains " Achilles heal of embryo transfer" (Elsden 1986). Two types of gonadotrophins have been used to induce superovulation. These are pregnant mare serum gonadotrophins (PMSG) and Gonadotrophins from pituitary extracts of animals.

Pregnant Mare Serum Gonadotrophins (PMSG):- It is a glycoprotein having both FSH and LH activity, the ratio of FSH an LH may vary between batches. It's half life is approximately 40 hours and and it has been shown to persist upto 10 days. Inspite of long biological half life and undesirable side effects of production of post-superovulatory, anovulatory follicle(s), affecting endocrine profile and embryo quality PMSG has been extensively used in cow. Recently, by the introduction

of Neutro-PMSG, a monoclonal antibody, which is injected at the time of first insemination the above problems are overcome.

Gonadotrophins from Pituitary Extract of Animals:- these include follicular stimulating hormone (FSH), Folltropin-V, Ovagen and super-over.

Follicular stimulating hormone (FSH):- FSH-P is derived from pituitary gland of domestic animal and has been the most commonly used hormone to induce superovulation (Joshi *et al.* 1992).

Folltropin-V:- It is a highly purified procine pituitary extract containing very little amount of LH. Because of the low level of LH, the most of the detrimental effect like fertilization failure, poor embryo quality and embryo recovery are minimized and thus resulting in large number of transferable embryo. Recently, in order to reduce variability of superovulation response, bFSH produced by recombinant DNA technology has been used in cattle (Looney *et al.* 1988).

Gonadotrophin Releasing Hormone (GnRH):- A recent study to use GnRH agonist to improve synchronization of ovulation in superovulation has given encouraging result (Carvalho *et al.* 2000).

Other method of collection of oocyte is from ovaries collected from slaughter house. For this ovaries are transported to the laboratory in a thermos flask containing sterile physiological saline. Oviducts ipsilateral to the newly formed corpus luteum are removed from rest of the reproductive tract. Oocytes are searched under steriozoom microscope following aspiration of surface visible follicles (Das *et al.* 1996). So, finally the recovery of large number of oocytes with high developmental competence remains an ultimate goal for mass production of embryo. However, less number of oocytes recovered from ovary or high percentage of oocytes of poor quality are the main problem faced *in vitro* fertilization. Lonergan *et al.* (1994) observed that oocyte derived from large follicles are more competent than those derived from small follicles.

Recently, the aspiration of immature oocytes from live animals by ultrasonic guided non-surgical follicle aspiration has extended the application of this technology for genetic improvement of valuable stock (Gasparrini, 2002).

Steps of Embryo Production and Transfer :

Embryo production and transfer can be done both ***in vivo*** and ***in vitro***.

***In vivo* technique:-** The following steps are followed in vivo technique:

- An elite animal (say genetically superior high milking cow) is selected as donor and the animal of low productivity is selected as recipient.
- Ova is obtained by superovulation by using different suitable gonadotrophins hormones as mentioned above.
- Semen from genetically superior sire is collected or frozen semen of high quality is used for inseminating the donor.
- Six to seven days after insemination fertilized egg is collected from the uterus of donor by flushing method using 3-way Foley's catheter.
- The flushed fluid containing embryo is transferred to a searching petri dish and examined under sterio-microscope and good embryo is selected.
- The selected embryo is maintained in special medium at 37 ^{0}C in an incubator.
- One good quality of ovum is transferred to the surrogate mother having low genetic potential and whose oestrus cycles have been synchronized with that of donor.
- On an average 50% success is achieved so that 18 superior cows can be produced from one donor cow in a year.
- In case fresh transfers are not required embryo can be frozen for long time preservation.

Technique of Freezing Embryo:

Cryopreservation of embryo has become a routine procedure for success of embryo transfer in livestock species. For this the embryo is dehydrated with the help of a cryoprotectant like glycerol. After the equilibration is achieved embryos are cooled very slowly from 25^{0}C to - 38^{0}C. The embryo at -38^{0}C are plunged into liquid nitrogen (-196^{0}C) and stored for posterity and used whenever needed. Cryopresevation has the following advantages:

- It relieves for having simultaneous synchronization of oestrus in donor and recipient animals.
- Easy and safe transport of valuable germplasm throughout the globe.
- Conservation of superior genetic material.

- Protection of valuable strains of experimental animals against possible loss through disease, accident or genetic drift.
- Possibility of shortening the generation interval for progeny testing programme.

***In vitro* technique :-** The following steps are followed *in vitro*:

- The harvested immature oocyte (either retrieved from slaughter hose or collected from live animal-ovum pick up) is taken in culture medium using all aseptic technique.
- In order to mimic, *in vitro* the condition to which the oocyte is exposed *in vivo*, the first step is to reversely inhibit the resumption of meioses, which under normal circumstances occurs in fully grown oocytes on removal from the inhibitory environment of the follicle (Pincus and Enzmann 1935). However, Sirard and Bilodean (1990) demonstrated that the granulosa cells inhibit the resumption of meiosis in bovine oocyte *in vitro*.
- The oocytes are allowed to fertilize with high quality sperm.
- The ferlized ovum are allowed to develop upto transferable stage in suitable culture media or in **co-culture system** (*vide infra*). The culture condition influencing oocytes maturation include maturation medium, source of serum, supplementation with hormones, growth factors and follicular fluids etc. Large number of maturation media has been used to mature immature oocyte in cattle and buffalo but the selection of maturation media is very critical for the success of this process (Bavister *et al.* 1992). Choi *et al.* (1998) observed that follicular fluid from large follicles enhances *in vitro* oocyte maturation as compared to the small follicles. This finding was also substantiated by Jelodar *et al.* (2003).
- After the growth the good quality blastocysts are taken in straw and mounted in transfer gun. The recipient animal is examined rectally for the presence of corpus luteum and the embryo is pushed in the horn. The recipient animal is administered with antibiotics immediately after embryo transfer in order to avoid bacterial contamination.
- Care of recipient animal is done till the completion of pregnancy and steps should be taken for early detection of pregnancy.

Co-culture System:- A co-culture system has become an integral component of *in vitro* fertilization and has replaced the earlier *in vivo* system in which fertilized zygotes are transferred to the oviduct of

live animals till recovery of blastocysts. Different co-culture systems using oviduct cells are in vogue to-day (Bavister *et al.* 1992). Oviduct cells from homologous species (buffalo) and heterologous species (goat) were used as co-culture systems for buffalo by Yadav *et al.* (1998).

Difference between *in vitro* and *in vivo* technique has been depicted in Table- 11.

Table:- 11. Difference between *in vitro* and *in vivo* Technique.

S. N.	IN VITRO	IN VIVO
1.	Here environment is based largely on empirism rather than on precise knowledge of embryo needs. These inevitably provides a sub-optimal environment resulting in a discordant repertoire of biochemical signals that confound the genetic blueprint of at least some embryos (McEvoy *et al.* 2000).	Here the reproductive tract provides the appropriate developmental and trophic signals orchestrated by to ensure normal development.
2.	It is less successful than that of in vivo mature oocyte, possibly as a consequence of incomplete cytoplasmic maturation (Leibfried *et al.* 1980).	Here development of oocyte to pre-implantation stage embryo is more successful.
3.	In vitro produced embryo produces more IFN-tau.	Less IFN-tau is produced.
4.	Here embryo has darker cytoplasm of lower density (Pollard and Leibo 1994).	No such embryo is observed.
5.	Embryo contains more triglyceride and less lipid (Abd El Razek *et al.* 2000).	No such composition is observed.
6.	Has swollen blastomere (vanSoom et al., 1992) and more fragile zona pellucida(Dubey *et al.* 1997).	Less swollen blastomere and less fragile zona pellucida).

Embryo Sexing:

Embryo sexing enable us to have desired sex according to our requirement. If we want animals for meat purposes with faster growth and lean carcasses then certainly we would like to have more males,

but if we want animal for milk purposes than we will like to have more female for dairy purpose. Now these desired traits can be obtained by sexing embryo. There are two approaches for the control of sex of offspring: (i) regulation of sex of embryo and (ii) identification of sex of embryo.

(i) Regulation of Sex of Embryo are Based on:

- Sperm separation,
- Nuclear transplantation,
- Parthenogenesis and
- Removal of few cell from an embryo at an early stage of development and to make a preparation from these cells in which the chromosomes can be examined. Such removal of cells do not restrict its further developmental capacity.

(ii) Identification of Sex of Embryo:- this can be done by:

- Karyotyping-can be done by using quinacirine mustard.
- Identification of sex chromatin.
- Assay of sex linked enzymes, like glucose-6-phosphate dehydrogenase.
- Identification of DNA probe or Y-chromosome (refer page 230of chapter-18) and
- Serological determination of H-Y antigen which is a male specific antigen in mammals. These include attempts to develop monoclonal antibodies against H-Y antigen and to detect sex by means of ELISA.

Micromanipulation of Embryo:

The technique is based on the developmental and regulatory capacity of individual cells or groups of cells from embryo at early cleavage stage. By micromanipulation of embryo we can have

(i) Monozygotic twins in sheep, cattle, pigs and horses. This have been derived by separating the blastomeres from either two, four or eight-celled embryo into the equal groups.

(ii) A large number of identical animals from a single embryo by the simple technique of separating blastomeres. (Single blastomere from 8-celled embryo or group of two blastomere from 16-celled embryo seldom produce a viable foetus).

(iii) A set of identical quadruplet lambs derived from single 8-celled embryo.

(iv) Chimeric animals by this technique. Chimera is a composite animal living cells from more than one cell line. The main objective is to combine the best genetic traits of each donor in the chimeric animal. Sheep-goat chimera has been successfully produced.

(v) Many exciting possibilities for manipulation of animals of selected genotype.

Advantages of Embryo Transfer Technology:

1. It permits exploitation of superior female genotype giving more offspring from the same genetic donor than would arise under normal breeding condition.
2. Twin production, introduction of new gene into cloned herds, manipulation of embryo and transgenic animals have been possible by ETT.
3. Infertility, especially of the older animals is a major handicap in animal breeding programme. Such animals of superior genetic makeup could still be tapped through ETT to provide additional progenies.
4. ETT is being used at an ever-increasing rate in basic and applied research especially investigation of spermatozoal differentiation, viral infection of embryo and detection of carries of heritable disease.
6. It increases the reproductive capacity of a superior heifer or a cow, since semen from one male can be used for a large number of females. A single superior female can be made to give a large number of ovules through superovulation.
7. Old superior cow that are unable to maintain pregnancy can still donate ovules for embryo transfer.
8. We may not need to import a superior quality cow, instead we may have imported frozen embryo which can grow in surrogate mother. So, it is much cheaper to import frozen embryo than to import cows.
9. In an animal (cow and buffalo) *in vitro* production of embryo has considerable potential value in for disseminating genetic improvement and shortening the generation interval as compared to progeny testing.

10. ETT can be used to resolve several reproductive enigmas like embryo-utero relationship, endocrine requirement for maintenance of pregnancy, the relative influence of genetic and environment on foetal growth and the biology of zona pellucida and blastomere.

Practice Assignment XX

1. Identification of sex of embryo can be done by:
   (a) Karyotyping-can be done by using quinacirine mustard
   (b) Identification of sex chromatin
   (c) Assay of sex linked enzymes, like glucose-6-phosphate dehydrogenase
   (d) All of the above.
2. Traditional method of gene improvement was:
   (a) Artificial insemination
   (b) Embryo transfer technology
   (c) Both of the above
   (d) None of the above.
3. ETT refers to:
   (a) Collection of ova from elite donor
   (b) Fertilization of ova by sperm
   (c) Transfer of fertilised ova in a genetically inferior recipient
   (d) All of the above.
4. The first embryo experiment was done by:
   (a) Walter Heape (b) Dr. Dorset
   (c) Mutter (d) Nicholson.
5. Hormones used for superovulation are:
   (a) Pregnant mare serum gonadotrophin
   (b) FES-V
   (c) Folltropin-V
   (d) All of the above.
6. Embryo production and transfer can be done :
   (a) *In vivo* only (b) *In vitro* only
   (c) Both of the above (d) None of the above.
7. Freezing of embryo may help in:
   (a) Easy and safe transport of valuable germ plasm
   (b) In simultaneous synchronization of oestrus in donor and recipient
   (c) Conservation of superior genetic material
   (d) All of the above.

8. Micromanipulation of Embryo helps in getting:
   (a) Monozygotic twins
   (b) A large number of identical animals from a single embryo.
   (c) Chimeric animals can be produced by this technique
   (d) All of the above.
9. Regulation of sex of embryo are based on:
   (a) Sperm separation (b) Nuclear transplantation
   (c) Parthenogenesis (d) All of the above.
10. All the statement regarding *in vitro* fertilization is true, Except,
   (a) Here embryo has darker cytoplasm of lower density
   (b) Less IFN-tau is produced
   (c) Produces swollen blastomere and more fragile zona pellucida
   (d) Embryo contain more triglyceride and less lipid.

Answer:

1. -(d) , 2. -(a), 3. -(d), 4. -(a), 5. -(d), 6. -(c), 7. -(d) , 8. -(d) , 9. -(d), 10-(b) .

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