Post-Graduate Degree Programme (CBCS)
in
ZOOLOGY

SEMESTER-III

SOFT CORE THEORY PAPER

REPRODUCTIVE BIOTECHNOLOGY

ZDSE(MN)T 306

SELF LEARNING MATERIAL

DIRECTORATE OFOPEN AND DISTANCE LEARNING
UNIVERSITY OF KALYANI
KALYANI, NADIA,
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Director's Message

Satisfying the varied needs of distance learners, overcoming the obstacle of distance and reaching the unreached students are the threefold functions catered by Open and Distance Learning (ODL) systems. The onus lies on writers, editors, production professionals and other personnel involved in the process to overcome the challenges inherent to curriculum design and production of relevant Self Learning Materials (SLMs). At the University of Kalyani a dedicated team under the able guidance of the Hon'ble Vice-Chancellor has invested its best efforts, professionally and in keeping with the demands of Post Graduate CBCS Programmes in Distance Mode to devise a self-sufficient curriculum for each course offered by the Directorate of Open and Distance Learning (DODL), University of Kalyani.

Development of printed SLMs for students admitted to the DODL within a limited time to cater to the academic requirements of the Course as per standards set by Distance Education Bureau of the University Grants Commission, New Delhi, India under Open and Distance Mode UGC Regulations, 2017 had been our endeavour. We are happy to have achieved our goal.

Utmost care and precision have been ensured in the development of the SLMs, making them useful to the learners, besides avoiding errors as far as practicable. Further suggestions from the stakeholders in this would be welcome.

During the production-process of the SLMs, the team continuously received positive stimulations and feedback from Professor (Dr.) Manas Kumar Sanyal, Hon'ble Vice- Chancellor, University of Kalyani, who kindly accorded directions, encouragements and suggestions, offered constructive criticism to develop it within proper requirements. We gracefully, acknowledge his inspiration and guidance.

Sincere gratitude is due to the respective chairpersons as well as each and every member of PGBOS (DODL), University of Kalyani. Heartfelt thanks is also due to the Course Writers-faculty members at the DODL, subject-experts serving at University Post Graduate departments and also to the authors and academicians whose academic contributions have enriched the SLMs. We humbly acknowledge their valuable academic contributions. I would especially like to convey gratitude to all other University dignitaries and personnel involved either at the conceptual or operational level of the DODL of University of Kalyani.

Their persistent and co-ordinated efforts have resulted in the compilation of comprehensive, learner-friendly, flexible texts that meet the curriculum requirements of the Post Graduate Programme through Distance Mode.

All the Self Learning Materials are self writing and collected from e-book, journals and websites.

Director
Directorate of Open and Distance Learning
University of Kalyani

ZDSE(MN)T306:ReproductiveBiotechnology

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UNIT-I

Cell culture laboratory design and equipment, media and reagents

Objective: In this unit you will learn about Cell culture laboratory design and equipment, and also about media and reagents.

Introduction:

Animal cell culture basically involves the in vitro (in the laboratory) maintenance and propagation of animal cells in a suitable nutrient media. Thus, culturing is a process of growing cells artificially. Cell culture has become an indispensable technology in various branches of life sciences.

The general parameters need to be considered are: (i) Cell Quantitation (ii) Equipment and Medium (iii) pH and Buffer Systems (iv)Oxygen (v) Growth Kinetics (vi) Types of Culture Processes (vii) Other Practical Considerations. In the laboratories, small scale cultures of cells in flasks (usually 1-5 litre volume) are done for establishing the cell lines. Such cell cultures are useful for studying the morphology, growth, metabolism etc. Large-scale cultures are required for semi-industrial (100-1,000 I capacity) and large-scale industrial (5,000-20,000 I capacity) use of cells for production of wide range of biologically important compounds (e.g. enzymes, antibodies, hormones, interferon's, plasminogen activator, interleukins).

The terms fermenter and bioreactor are in common use while dealing with the industrial use of cells. A fermenter usually refers to the containment system for the cultivation of prokaryotic cells (bacteria, fungi), while a bioreactor grows the eukaryotic cells (mammalian, insect). Scale-up refers to the process of developing the culture systems in stages from a laboratory to the industry. Scale-up although tedious, labour intensive and expensive, is required for the production of commercially important products. For a better understanding of scale-up, certain basic and fundamental concepts of cell culture should be clear.

Cell Culture — General Considerations:
There are several parameters that need to be considered for appropriate growth, proliferation and maintenance of cells in culture.

A good understanding of these parameters, listed below is also necessary for scale-up:

i. Cell quantitation.

ii. Equipment and medium.

iii. pH and buffer systems.

iv. Oxygen.

v. Growth kinetics.

vi. Types of culture processes.

vii. Other practical considerations.

i. Cell Quantization:

The total number of cells in a culture can be measured by counting in a haemocytometer. It is however, not possible to identify the viable and non-viable cells by this method.

Cell viability:

The viability of cells can be detected by use of dyes e.g.tryphan blue. The principle is based on the fact that the dye is permeable to dead cells while the viable cells do not take up dye.

Indirect measurements for cell viability:

The viability of cells can be measured by their metabolic activity. Some of the most commonly used parameters are listed:

i. Glucose utilization.

ii. Oxygen consumption.

iii. Pyruvate production.

iv. Carbon dioxide formation.

In recent years, many laboratories have started measuring the activity of lactate dehydrogenase (LDH) to detect cell viability. Dead cells release LDH and therefore, this enzyme can be used to quantitatively measure the loss of cell viability.

ii. Equipment and Medium:

The various aspects of equipment and medium used in culture laboratory.

Culture vessels:
The materials made up of glass or stainless steel are commonly used for cell cultures. Borosilicate glass (e.g. Pyrex) is preferred as it can better withstand autoclaving for suspension cultures, wherein cell attachment to the surface has to be discouraged; the culture vessels are usually treated with silicone (siliconization).

Medium and nutrients:
Appropriate selection of the medium is done based on the nutritional requirements, and the purpose for which the cultured cells are required. Eagle's basal medium and minimal essential medium are the most commonly used. The media may be supplemented with serum.

Additional feeding of certain nutrients is often required as they are quickly utilized and get exhausted. These include glucose, glutamine and cystine. For suspension cultures, media lacking calcium and magnesium are used, since their absence minimizes the surface attachment.

Non-nutrient medium supplements:
Certain non-nutrient compounds are often added to the medium for improvement of cell cultures. Sodium carboxymethyl cellulose addition to medium helps to minimize mechanical damage that may occur due to forced aeration or the forces generated by stirred impeller. Polyglycol (trade name Pluronic F-68) in the medium reduces foaming in stirred and aerated cultures.

iii. pH and Buffer Systems:
The ideal pH for animal cell cultures is around 7.4. A pH below 6.8 inhibits cell growth. The factors that can alter pH include the stability of the medium, type of buffer and its buffering capacity, concentration of glucose and headspace.

The commonly used buffer of the in vitro culture carbon dioxide-bicarbonate system (2-5% CO_2 with 10-25 mM $NaHCO_3$) is comparable to the blood buffer. The presence of phosphates in the medium improves the buffering capacity. Some laboratories use HEPES instead of bicarbonate for more efficient buffering.
As glucose is utilized by the cells, pyruvic acid and lactic acid are produced which can alter the pH. If fructose and galactose (instead of glucose) are used, the acid formation is less, but the cell growth is reduced.

iv. Oxygen:
Oxygen has to be continuously supplied to the medium throughout the life of the culture. This has to be done without causing damage to the cells. Oxygen can be supplied to the cultures in one of the following ways.

Surface aeration:
In closed system static cultures, the headspace is used for the supply of oxygen. For instance, in a 1 litre flask with 100 ml medium 900 ml of the space containing air has about 0.27 g of O_2. This O_2 is capable of supporting 10^8 cells for about 450 hours.

Sparging:
The process of bubbling gas through the culture is referred to as sparging. This is an efficient means of O_2 supply, but may often damage the cells due to effects of the bubble on the cell membrane surfaces. Use of higher air bubbles minimizes the damaging effect.

Membrane diffusion:
Adequate diffusion of oxygen into the culture can be obtained through silicone tubing which is highly permeable to gases. This approach however, is inconvenient, besides the high cost of silicone tubing.

Medium perfusion:
The medium is perfused through an oxygenation chamber before it enters the culture system. This method ensures good O_2 saturation. Medium perfusion is in fact used in glass bead system and micro carrier systems.

v. Growth Kinetics:
The standard pattern of growth of cultured cells follows a lag phase, an exponential (log) phase and a Stationary phase. Growth of cells usually means an increase in cell numbers. However, increase in cell mass may occur without replication. The following terms are in common use to represent growth of cultured cells.

Specific growth rate:
The rate of cell growth per unit amount of biomass.

Doubling time:
The time required for a population of cells to double in number or mass.

Degree of multiplication (number of doublings):
The number of times a given inoculum has replicated.

vi. Types of Culture Processes:
The different culture processes and the growth patterns of cells (represented by cell density) are depicted in Fig 37.1. They are briefly described.

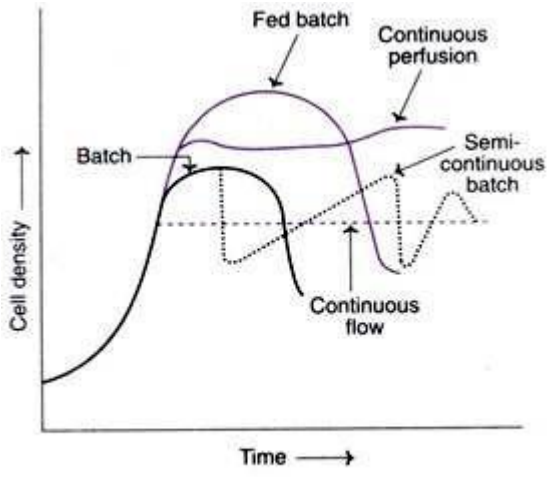


Fig. 37.1 : Comparison of different culture processes.

Batch culture:
In this technique, when the cells are inoculated into a fixed volume of the medium, they utilize the nutrients and grow, and simultaneously accumulate metabolites. As the nutrients get exhausted, toxic waste products accumulate and the cell multiplication ceases. Further, the cell density drops due to death of the cells.

Batch culture is a standard technique. Several modifications have been made to increase proliferation of cells, besides prolonging their life. The other culture processes described below are the modified batch cultures.

Fed batch culture:
There is a gradual addition of fresh medium so that the cell proliferation is much higher than the batch culture. Thus, in the fed batch culture there is an increase in the volume of culture.

Semi-continuous batch culture:
A portion of the culture medium is intermittently replaced with an equal volume of fresh medium. The growth pattern of the cells is fluctuating, with a rapid increase in cell density after each replacement of the medium.

Continuous perfusion culture:
There is a continuous addition of the medium to the culture and a withdrawal of an equal volume of used cell-free medium. The continuous perfusion process may close or open for circulation of the medium.

Continuous-flow culture:

In the continuous-flow culture, a homeostatic condition with no change in the cell numbers, nutrients and metabolites is attained. This is made possible by a balance

between the addition of the medium and withdrawal of medium along with cells. This is mostly suitable for suspension cultures.

vii. Other Practical Considerations:
Besides the parameters described above, there are several other practical considerations for in vitro culture and scale-up. Some important ones are given below.

Culture surface area:
The available surface is important for the cells to grow. In general, the culture processes are planned in such a way that the surface area is not a limiting factor.

Inoculation density of cells:
As such, there is no set rule for the density of inoculation. However, inoculation with high density is preferred for better growth.

Growth phase of cells:
Cells in the late exponential (log) phase are most suitable for inoculation. The cells at the stationary phase should be avoided since they have either prolonged lag phase or no growth at all.

Stirring rate of culture:
The stirring rates of different culture lines are developed in the laboratories. It is usually in the range of 100-500 rpm for most of the cultures.

Temperature of the medium:
It is advisable to warm the medium to 37°C before adding to the culture.

Culture Media for Animal Cells:
The selection of an appropriate growth medium for the in vitro cultivation of cells is an important and essential step. The mammalian cells of an organ in the body receive nutrients from blood circulation.

For culturing these cells in vitro, it is expected that they should be provided with the components similar to those present in blood. In general, the choice of the medium mostly depends on the type of the cells to be cultured, and the purpose of the culture (growth, differentiation, and production of desired products). The culture media may be natural or artificial.

a. Natural Media:
In the early years, the natural media obtained from various biological sources were used.

Body fluids:
Plasma, serum, lymph, amniotic fluid, ascitic and pleural fluids, aqueous humour from eyes and insect hemolymph were in common use. These fluids were tested for sterility and toxicity before their utility.

Tissue extracts:
Among the tissue extracts, chick embryo extract was the most commonly employed. The extracts of liver, spleen, bone marrow and leucocytes were also used as culture media. Some workers still prefer natural media for organ culture.

b. Artificial Media:
The artificial media (containing partly defined components) have been in use for cell culture since 1950.

The minimal criteria needed for choosing a medium for animal cell cultures are listed below:

i. The medium should provide all the nutrients to the cells.

ii. Maintain the physiological pH around 7.0 with adequate buffering.

iii. The medium must be sterile, and isotonic to the cells.

The basis for the cell culture media was the balanced salt solution which was originally used to create a physiological pH and osmolarity required to maintain cells in vitro. For promoting growth and proliferation of cells, various constituents (glucose, amino acids, vitamins, growth factors, antibiotics etc.) were added, and several media developed.

Addition of serum to the various media is a common practice. However, some workers in recent years have started using serum-free media. The physicochemical properties of media required for tissue cultures are briefly described. This is followed by a brief account on balanced salt solutions, commonly used culture media and the serum-free media.

Physicochemical Properties of Culture Media:

The culture media is expected to possess certain physicochemical properties (pH, O_2, CO_2, buffering, osmolarity, viscosity, temperature etc.) to support good growth and proliferation of the cultured cells.

pH:
Most of the cells can grow at a pH in the range of 7.0-7.4, although there are slight variations depending on the type of cells (i.e. cell lines). The indicator phenol red is most commonly used for visible detection of pH of the media.

Its colouration at the different pH is shown below:
At pH 7.4 — Red

At pH 7.0 — Orange

At pH 6.5 — Yellow

At pH 7.8 — Purple

CO_2, bicarbonate and buffering:

Carbon dioxide in the medium is in a dissolved state, the concentration of which depends on the atmospheric CO_2 tension and temperature. CO_2 in the medium exists as carbonic acid (H_2CO_3), and bicarbonate (HCO^-_3) and H^+ ions as shown below.

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO^-_3$$

As is evident from the above equation, the concentrations of CO_2, HCO^-_3 and pH are interrelated. By increasing the atmospheric CO_2, the pH will be reduced making the medium acidic.
Addition of sodium bicarbonate (as a component of bicarbonate buffer) neutralizes bicarbonate ions.

$NaHCO_3 \leftrightarrow Na^+ + HCO^-_3$
In fact, the commercially available media contain a recommended concentration of bicarbonate, and CO_2 tension for the required pH. In recent years HEPES (hydroxyethyl piperazine 2-sulfonic acid) buffer which is more efficient than bicarbonate buffer is being used in the culture media.
However, bicarbonate buffer is preferred by most workers because of the low cost, less toxicity and nutritional benefit to the medium. This is in contrast to HEPES which is expensive, besides being toxic to the cells. The presence of pyruvate in the medium results in the increased endogenous production of CO_2 by the cells. This is advantageous since the dependence on the exogenous supply of CO_2 and HCO^-_3 will be less. In such a case, the buffering can be achieved by high concentration of amino acids.

Oxygen:

A great majority of cells in vivo are dependent on the O_2 supply for aerobic respiration. This is in fact made possible by a continuous supply of O_2 to the tissues by hemoglobin. The cultured cells mostly rely on the dissolved O_2 in the medium which may be toxic at high concentration due to the generation of free radicals. Therefore, it is absolutely necessary to supply adequate quantities of O_2 so that the cellular requirements are met, avoiding toxic effects. Some workers add free-radical scavengers (glutathione, mercaptoethanol) to nullify the toxicity. Addition of selenium to the medium is also advocated to reduce O_2 toxicity. This is because selenium is a cofactor for the synthesis of glutathione.In general, the glycolysis occurring in cultured cells is more anaerobic

when compared to in vivo cells. Since the depth of the culture medium influences the rate of O_2 diffusion, it is advisable to keep the depth of the medium in the range 2-5 mm.

Temperature:

In general, the optimal temperature for a given cell culture is dependent on the body temperature of the organism, serving as the source of the cells. Accordingly, for cells obtained from humans and warm blooded animals, the optimal temperature is 37°C.In vitro cells cannot tolerate higher temperature and most of them die if the temperature goes beyond 40°C. It is therefore absolutely necessary to maintain a constant temperature (± 0.5°C) for reproducible results.

If the cells are obtained from birds, the optimal temperature is slightly higher (38.5°C) for culturing. For cold blooded animals (poikilotherms) that do not regulate their body heat (e.g. cold-water fish), the culture temperature may be in the range of 15-25°C. Besides directly influencing growth of cells, temperature also affects the solubility of CO_2 i.e. higher temperature enhances solubility.

Osmolality:

In general, the osmolality for most of the cultured cells (from different organisms) is in the range of 260-320 mosm/kg. This is comparable to the osmolality of human plasma (290 mosm/kg). Once an osmolality is selected for a culture medium, it should be maintained at that level (with an allowance of ± 10 mosm/kg). Whenever there is an addition of acids, bases, drugs etc. to the medium, the osmolality gets affected. The instrument osmometer is employed for measuring osmolalities in the laboratory.

Balanced Salt Solutions:

The balanced salt solutions (BSS) are primarily composed of inorganic salts. Sometimes, sodium bicarbonate, glucose and HEPES buffer may also be added to BSS. Phenol red serves as a pH indicator.

The important functions of balanced salt solutions are listed hereunder:

i. Supply essential inorganic ions.

ii. Provide the requisite pH.

iii. Maintain the desired osmolality.

iv. Supply energy from glucose.

In fact, balanced salt solutions form the basis for the preparation of complete media with the requisite additions. Further, BSS is also useful for a short period (up to 4 hours) incubation of cells.

The composition of two most widely used BSS namely Earle's BSS and Hank's BSS is given in Table 34.1.

Table 34.1 Composition (g/l) of balanced salt solutions (BSS)

Ingradient	*Earle's BSS*	*Hank's BSS*
NaCl	6.68	8.0
KCl	0.4	0.4
$CaCl_2$ (anhydrous)	0.02	0.14
$MgSO_4.7H_2O$	0.2	0.1
$NaHCO_3$	2.2	0.35
$NaH_3PO_4.H_2O$	0.14	—
$Na_2HPO_4.7H_2O$	—	0.09
KH_2PO_4	—	0.06
D-Glucose	1.0	1.0
Phenol red	0.01	0.01
HEPES, Na salt (buffer)	13.02	2.08

Complete Culture Media:

In the early years, balanced salt solutions were supplemented with various nutrients (amino acids, vitamins, serum etc.) to promote proliferation of cells in culture. Eagle was a pioneer in media formulation. He determined (during 1950-60) the nutrient requirements for mammalian cell cultures. Many developments in media preparation have occurred since then. There are more than a dozen media now available for different types of cultures.

Some of them are stated below:

EMEM—Eagle's minimal essential medium

DMEM—Dulbecco's modification of Eagle's medium

CMEM—Glasgow's modification of Eagle's medium

RPMI 1630 and RPMI 1640—Media from Rosewell Park Memorial Institute.

The other important culture media are Ham's F10, and F12, TC 199 and CMRL 1060. The detailed composition of three commonly used media namely Eagle's MEM, RPMI 1640 and Ham's F12 is given in Table 34.2. The complete media, in general, contains a large number of components amino acids, vitamins, salts, glucose, other organic supplements, growth factors and hormones, and antibiotics, besides serum. Depending on the medium, the quality and quantity of the ingredients vary. Some important aspects of the media ingredients are briefly described.

Table 34.2 Composition of three commonly used culture media

Component	*Eagle's MEM*	*RPMI 1640*	*Ham's F 12*
Amino acids			
L-Alanine			8.91
L-Arginine HCl	105	200	211
L-Asparagine H_2O		50	15.0
L-Aspartic acid		20	13.3
L-Cystine	24	50	24.0
L-Glutamic acid		20	14.7
L-Glutamine	292	300	146.2
Glycine		10	7.51
L-Histidine HCl H_2O	31	15	21.0
L-Isoleucine	52	50	3.94
L-Leucine	52	50	13.12
L-Lysine	58	40	36.54
L-Methionine	15	15	4.48
L-Phenylalanine	32	15	4.96
L-Proline		20	34.5
L-Serine		30	10.51
L-Threonine	48	20	11.91
L-Tryptophan	10	5	2.042
L-Tyrosine	36	20	5.43
L-Valine	46	20	11.7
Glutathione (red)		1	
L-Hydroxyproline		20	
Vitamins			
D-Biotin		0.2	0.007
Ca D-pantothenate	1	0.25	0.26
Choline chloride	1	3.0	13.96
Folic acid	1	1.0	1.32
i-Inositol	2		18.02
Nicotinamide	1	35	0.037
p-Aminobenzoic acid		1.0	
Pyridoxine HCl		1	0.062
Pyridoxal HCl	1		
Riboflavin	0.1	0.2	0.038
Thiamine HCl	1	1.0	0.34
Vitamin B_{12}		0.005	1.36

Table 34.2 contd. next column

Component	Eagle's MEM	RPMI 1640	Ham's F 12
Inorganic salts			
$CaCl_2.2H_2O$	200		44.1
$CaNO_3.4H_2O$		100	
$CuSO_4.5H_2O$			0.0025
$FeSO_4.7H_2O$			0.83
KCl	400	400	223
$MgSO_4.7H_2O$	220	100	133
NaCl	6800	6000	7599
$NaHCO_3$	2000	2000	1176
$Na_2HPO_4.7H_2O$		1512	268
$NaH_2PO_4.2H_2O$	150		
Other components			
D-Glucose	1000	2000	1801
Phenol red		5.0	1.2
Sodium pyruvate			110
Lipoic acid			0.21
Linoleic acid			0.084
Hypoxanthine			4.08
Putrescine 2HCl			0.16

Amino acids:

All the essential amino acids (which cannot be synthesized by the cells) have to be added to the medium. In addition, even the non-essential amino acids (that can be synthesized by the cells) are also usually added to avoid any limitation of their cellular synthesis. Among the non-essential amino acids, glutamine and/or glutamate are frequently added in good quantities to the media since these amino acids serve as good sources of energy and carbon.

Vitamins:

The quality and quantity of vitamins depends on the medium. For instance, Eagle's MEM contains only water soluble vitamins (e.g. B-complex, choline, inositol). The other vitamins are obtained from the serum added. The medium M 199 contains all the fat soluble vitamins (A, D, E and K) also. In general, for the media without serum, more vitamins in higher concentrations are required.

Salts:

The salts present in the various media are basically those found in balanced salt solutions (Eagle's BSS and Hank's BSS). The salts contribute to cations (Na^+, K^+, Mg^{2+}, Ca^{2+} etc.) and anions (CI^-, HCO^-_3, SO^{2-}_4, PO^{3-}_4), and are mainly responsible for the

maintenance of osmolality. There are some other important functions of certain ions contributed by the salts.

i. Ca^{2+} ions are required for cell adhesion, in signal transduction, besides their involvement in cell proliferation and differentiation.

ii. Na^{+}, K^{+} and CI^{-} ions regulate membrane potential.
iii. PO^{3-}_{4}, SO^{2-}_{4} and HCO^{-}_{3} ions are involved in the maintenance of intracellular charge; besides serving as precursors for the production of certain important compounds e.g. PO^{3-}_{4} is required for ATP synthesis.

Glucose:

Majority of culture media contain glucose which serves as an important source of energy. Glucose is degraded in glycolysis to form pyruvate/lactate. These compounds on their further metabolism enter citric acid cycle and get oxidized to CO_2. However, experimental evidence indicates that the contribution of glucose for the operation of citric acid cycle is very low in vitro (in culture cells) compared to in vivo situation. Glutamine rather than glucose supplies carbon for the operation of citric acid cycle. And for this reason, the cultured cells require very high content of glutamine.

Hormones and growth factors:

For the media with serum, addition of hormones and growth factors is usually not required. They are frequently added to serum-free media.

Other organic supplements:

Several additional organic compounds are usually added to the media to support cultures. These include certain proteins, peptides, lipids, nucleosides and citric acid cycle intermediates. For serum-free media, supplementation with these compounds is very useful.

Antibiotics:

In the early years, culture media invariably contained antibiotics. The most commonly used antibiotics were ampicillin, penicillin, gentamycin, erythromycin, kanamycin, neomycin and tetracycline. Antibiotics were added to reduce contamination. However, with improved aseptic conditions in the present day tissue culture laboratories, the addition of antibiotics is not required. In fact, the use of antibiotics is associated with several disadvantages.

i. Possibility of developing antibiotic-resistant cells in culture.

ii. May cause anti-metabolic effects and hamper proliferation.

iii. Possibility of hiding several infections temporarily.

iv. May encourage poor aseptic conditions.

The present recommendation is that for the routine culture of cells, antibiotics should not be added. However, they may be used for the development of primary cultures.

Serum:

Serum is a natural biological fluid, and is rich in various components to support cell proliferation. The major constituents found in different types of sera are listed in Table 34.3. The most commonly used sera are calf serum (CS), fetal bovine serum (FBS), horse serum and human serum. While using human serum, it must be screened for viral diseases (hepatitis B, HIV).

Table 34.3 Major constituents of serum

Proteins	
	Albumin
	Globulins
	Fetuin
	Fibronectin
	Transferrin
	Protease inhibitors (α_1-antitrypsin)
Amino acids	
	Almost all the 20
Lipids	
	Cholesterol
	Phospholipids
	Fatty acids
Carbohydrates	
	Glucose
	Hexosamine
Other organic compounds	
	Lactic acid
	Pyruvic acid
	Polyamines
	Urea
Vitamins	
	Vitamin A
	Folic acid
Growth factors	
	Epidermal growth factor
	Platelet-derived growth factor
	Fibroblast growth factor
Hormones	
	Hydrocortisone
	Thyroxine
	Triiodothyronine
	Insulin
Inorganics	
	Calcium
	Sodium
	Potassium
	Chlorides
	Iron
	Phosphates
	Zinc
	Selenium

Approximately 5-20% (v/v) of serum is mostly used for supplementing several media. Some of the important features of the serum constituents are briefly described.

Proteins:

The in vitro functions of serum protein are not very clear. Some of them are involved in promoting cell attachment and growth e.g. fetuin, fibronectin. Proteins increase the viscosity of the culture medium, besides contributing to buffering action.

Nutrients and metabolites:

Serum contains several amino acids, glucose, phospholipids, fatty acids, nucleosides and metabolic intermediates (pyruvic acid, lactic acid etc.). These constituents do contribute to some extent for the nutritional requirements of cells. This may however, be insignificant in complex media with well supplemented nutrients.

Growth factors:

There are certain growth factors in the serum that stimulate the proliferation of cells in the culture:

i. Platelet-derived growth factor (PDGF).

ii. Fibroblast growth factor (FGF).

iii. Epidermal growth factor (EGF).

iv. Vascular endothelial growth factor (VEGF).

v. Insulin-like growth factors (IGF-1, IGF-2).

In fact, almost all these growth factors are commercially available for use in tissue culture.

Hormones:

Hydrocortisone promotes cell attachment, while insulin facilitates glucose uptake by cells. Growth hormone, in association with somatomedins (IGFs), promotes cell proliferation.

Inhibitors:

Serum may also contain cellular growth inhibiting factors. Majority of them are artefacts e.g. bacterial toxins, antibodies. The natural serum also contains a physiological growth inhibitor namely transforming growth factor β (TGF-β). Most of these growth inhibitory factors may be removed by heat inactivation (at 56°C for 30 minutes).

Selection of Medium and Serum:

As already stated, there are around a dozen media for the cell cultures. The selection of a particular medium is based on the cell line and the purpose of culturing. For instance, for chick embryo fibroblasts and HeLa cells, EMEM is used.The medium DMEM can be used for the cultivation of neurons. A selected list of cells and cell lines along with the media and sera used is given in Table 34.4. In fact, information on the selection of appropriate medium for a particular cell line is available from literature.

TABLE 34.4 A selected list of the cells or cell lines along with the media and serum used for their culture

Cells or cell line	*Medium*	*Serum*
Chick embryo fibroblasts	EMEM	CS
Chinese hamster ovary (CHO)	EMEM, Ham's F12	CS
HeLa cells	EMEM	CS
Human leukemia	RPMI 1640	FB
Mouse leukemia	Fischer's medium, RPMI 1640	FB, HoS
Neurons	DMEM	FB
Mammary epithelium	RPMI 1640, DMEM	FB
Hematopoietic cells	RPMI 1640, Fischer's medium	FB
Skeletal muscle	DMEM, F 12	FB, HoS
Glial cells	MEM, F 12, DMEM	FB
3T3 eells	MEM, DMEM	CS

The selection of serum is also based on the type of cells being cultured.

The following criteria are taken into consideration while choosing serum:

i. Batch to batch variations.

ii. Quality control.

iii. Efficiency to promote growth and preservation of cells.

iv. Sterility.

v. Heat inactivation.

In recent years, there is a tendency to discontinue the use of serum, and switch over to more clearly defined media.

Supplementation of the Medium with Tissue Extracts:

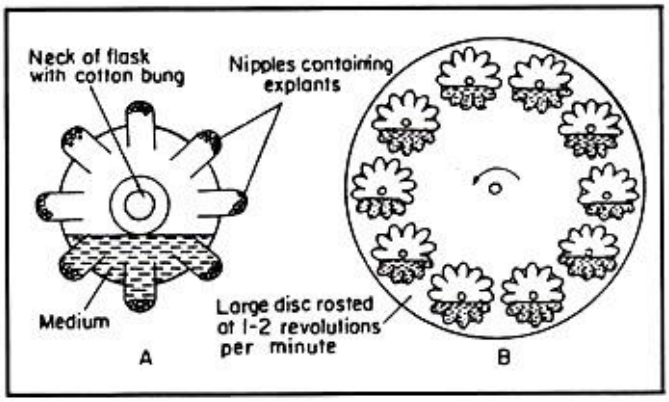
Besides serum, the culture media can also be supplemented with certain tissue extracts and microbial culture extracts. The examples are—chick embryo extract, proteolytic digests of beef heart, bactopeptone, lactalbumin hydrolysate, tryptose. The chick embryo extract was found to contain both high molecular weight and low molecular weight compounds that support growth and proliferation of cells.

A. Batch Culture:

Batch culture is a type of suspension culture where the cell material grows in a finite volume of agitated liquid medium. For instance, cell material in 20 ml or 40 ml or 60 ml liquid medium in each passage constitute a batch culture. Batch suspension cultures are most commonly maintained in conical flasks incubated on orbital platform shakers at the speed of 80-120 rpm.

Slowly Rotating Cultures:

Single cells and cell aggregates are grown in a specially designed flask, the nipple flask. Each nipple flask possesses eight nipple-like projections. The capacity of each flask is 250 ml. Ten flasks are loaded in a circular manner on the large flat disc of a vertical shaker (Fig 4.3). When the flat disc rotates at the speed of 1-2 rpm, the cell within each nipple of the flask are alternately bathed in culture medium and exposed to air.

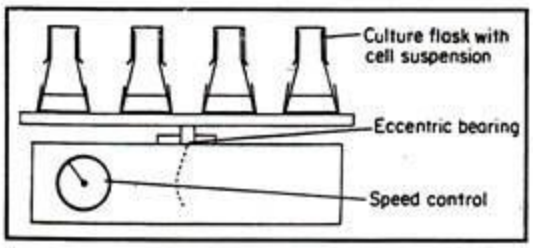


❒ Fig 4.3

A. Detail of a nipple flask. B. Large disc loaded with 10 nipple flasks used for growing cell suspension cultures

Shake Cultures:

It is a very simple and effective system of suspension culture. In this method, single cells and cell aggregates in fixed volume of liquid medium are placed in conical flasks. Conical flasks are mounted with the help of clips on a horizontal large square plate of an orbital platform shaker. The square plate moves by a circular motion at the speed of 60-180 rpm (Fig4.4).

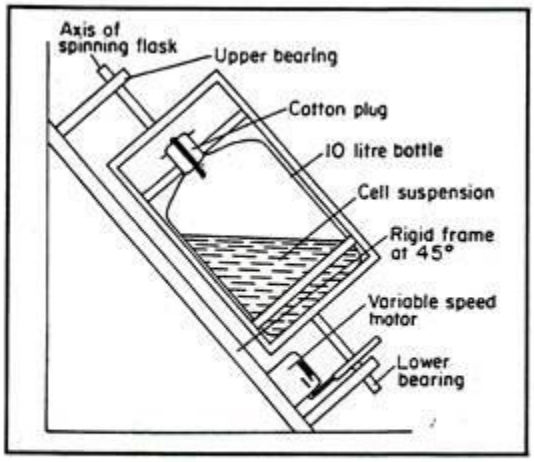


❒ Fig 4.4

Side view of a platform shaker loaded with suspension cultures contained in conical flasks

Spinning Cultures:

Large volumes of cell suspension may be cultured in 10L bottles which are rotated in a culture spinner at 120 rpm at an angle of 45° (Fig4.5).

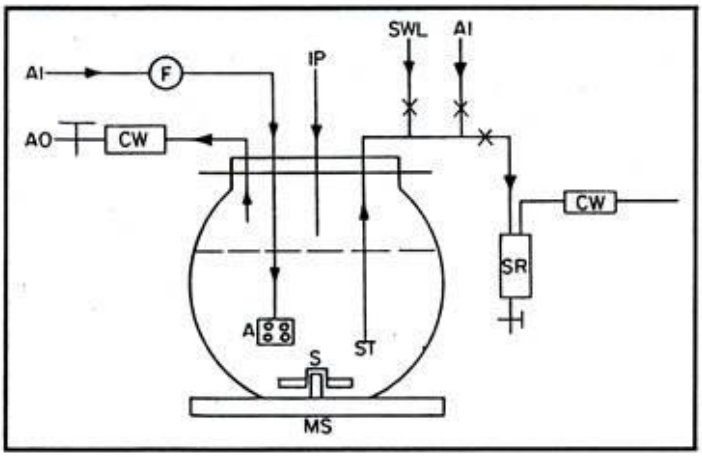


❒ Fig 4.5

Diagram of a 10 litre spinning culture apparatus

Stirred Culture:

This system is also used for large-scale batch culture (1.5 to 10 litres). In this method, the large culture vessel is not rotated but the cell suspension inside the vessel is kept dispersed continuously by bubbling sterile air through culture medium. The use of an internal magnetic stirrer is the most convenient way to agitate the culture medium safely. The magnetic stirrer revolves at 200-600 rpm. The culture vessel is a 5 to 10 litres round-bottom flask (Fig 4.6).



❒ Fig 4.6

Stirred batch culture unit. Arrow indicate direction of flow of air; AI = air input; F = sterilizing glass-fibre air filter; AO = air outlet; CW = cotton wool; IP = inoculation port; A = aerator; S = stirrer magnet; ST = sample tube; MS = magnetic stirrer; SWL = sterile water line; SR = sample receiver; (Diagram after Dr. P. King)

B. Continuous Culture System:

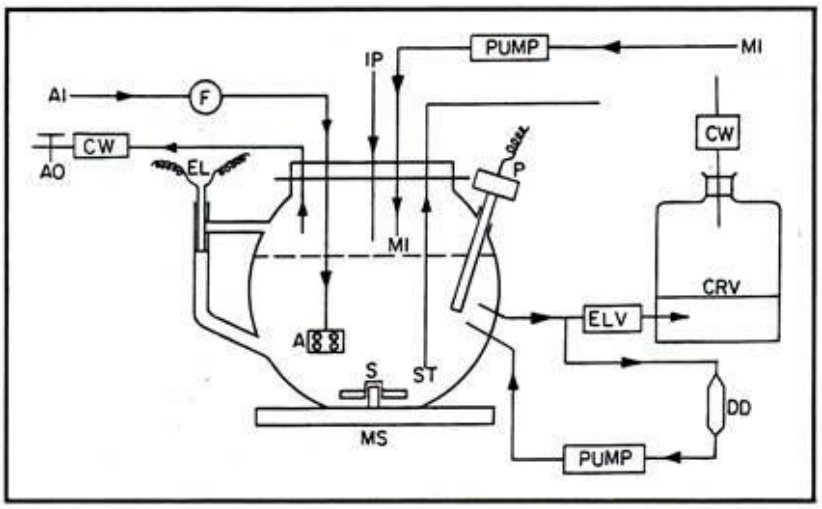
This system is very much similar to stirred culture. But in this system, the old liquid medium is continuously replaced by the fresh liquid medium to stabilize the physiological states of the growing cells. Normally, the liquid medium is not changed until the depletion of some nutrients in the medium and the cells are kept in the same medium for a certain period.

As a result active growth phase of the cell declines the depletion of nutrient. In continuous culture system, nutrient depletion does not occur due to continuous flow of nutrient medium and the cells always remain in the steady state of active growth phase.

Chemostats:

In this system, culture vessels are generally cylindrical or circular in shape and possess inlet and outlet pores for aeration and the introduction of and removal of cells and medium. The liquid medium containing the cells is stirred by a magnetic stirrer. The introduction of fresh sterile medium, which is pumped in at a constant rate into the vessel is balanced by the displacement of an equal volume of spent or old medium and cells.

Such a system can be maintained in a steady state so that new cells are produced by division at a rate which compensates the number lost in the outflow of spent medium. Thus in a steady state condition the density, growth rate, chemical composition and metabolic activity of the cells all remain constant. Such continuous cultures are ideal for studying growth kinetics and the regulation of metabolic activity in higher plants (Fig4.7).



❒ Fig 4.7

Chemostat culture. Arrows indicate direction of flow of liquid; AI = air input; F = sterilizing glass-fibre; AO = air output; CW = cottol wool; EL = volume-sensing electrodes; ELV = volume controlling outlet valve; MI = medium input; S = stirrer magnet; ST = sample tube; P = probe for oxygen tension; DD = density detector, CRV = culture receiving vessel; MS = magnetic stirrer; IP = inoculation port (Diagram after Dr. P. King)

Turbidostats:

The turbidity of a suspension culture medium changes rapidly when the cells increase in number due to their steady state growth. The changes of turbidity of the culture medium can be measured by the changes of optical density of the medium.Again, the pH of the medium changes due to increase of cell density. In turbidostats, an automatic monitoring unit is connected with the culture vessel and such unit adjusts the medium flow in such a way as to maintain the optical density or pH at a chosen, preset level.

Probable Questions:

1 What is batch culture? What is suspension culture?

2. Discuss various artificial and natural media used in animal cell culture.

3. Discuss in brief the physiochemical properties of culture media.

4. What is complete culture media. Give examples.

5. Describe Chemostats.

6. Discuss turbidostats.

7. Briefly discuss about equipment used in animal cell culture.

Suggested readings:

1. Biotechnology by P.K. Gupta

2. Gene Cloning by T. Brown.

3. Biotechnology by N. Kumarsen.

4. Biotechnology by B.D. Singh

UNIT-II

Animal cell culture techniques, concept of organoid culture

Objective: In this unit we will discuss about different types of techniques used in animal cell culture. We will also discuss about organoid culture.

Introduction:

Animal cell culture basically involves the in vitro (in the laboratory) maintenance and propagation of animal cells in a suitable nutrient media. Thus, culturing is a process of growing cells artificially. Cell culture has become an indispensible technology in various branches of life sciences.

Types of Animal Cell Culture:

i. Primary Culture:

When cells are surgically removed from an organism and placed into a suitable culture environment, they will attach, divide and grow. This is called a Primary Culture. There are two basic methods for doing this.

First, for Explant Cultures, small pieces of tissue are attached to a glass or treated in a plastic culture vessel and bathed in culture medium. After a few days, individual cells will move from the tissue explant onto the culture vessel surface or substrate where they will begin to divide and grow.

The second, more widely used method, speeds up this process by add-ing digesting (proteolytic) enzymes, such as trypsin or collagenase, to the tissue fragments to dissolve the cement holding the cells to-gether. This creates a suspension of single cells that are then placed into culture vessels con-taining culture medium and allowed to grow and divide. This method is called Enzymatic Dissociation.

The Basic Steps of Primary Cell Culture

The major advantages of primary cultures are the retention of:

1. The Capacity for Biotransformation:

In many cases, the metabolism of a primary cell culture has greater similarity to in vivo than that seen with sub-cellular fractions used as an exogenous source for biotrans-formation.

2. The Tissue-Specific Functions:

The sec-ond advantage of primary cultures is the retention of tissue specific functions. For example, primary cultures of rat myocar-dial cells consisting of synchronously beat-ing cells can be prepared. When these cul-tures were exposed to tricyclic antidepres-sants that are cardio toxic, beating were observed.

Limitations of Primary Cell Cultures:

One limitation of primary cultures is the ne-cessity to isolate cells for each experiment. Procedures to isolate cells require the disrup-tion of the tissue, often with proteolytic enzymes. This may result in the loss or damage of specific membrane receptors, damage to the integrity of the membrane, and loss of cellular products.

Sub-Culturing:

When the cells in the primary culture vessel have grown and filled up all of the available culture substrate, they must be Sub-cultured to give them room for continued growth. This is usually done by removing them as gently as possible from the substrate with enzymes. These are similar to the enzymes used in ob-taining the primary culture and are used to break the protein bonds attaching the cells to the substrate.

Some cell lines can be harvested by gently scraping the cells off the bottom of the culture vessel. Once released, the cell sus-pension can then be subdivided and placed into new culture vessels.

Once a surplus of cells is available, they can be treated with suitable cryoprotective agents, such as dimethylsulfoxide (DMSO) or glycerol, carefully frozen and then stored at cryogenic temperatures (below -130°C) until they are needed. The theory and techniques for cryopreserving cells are covered in the Corning Technical Bulletin: General Guide for Cryogenically Storing Animal Cell Cultures.

Characteristics of Cultured Animal Cells:

Cell Culture Systems:

Two basic culture systems are used for growing cells. These are based primarily upon the ability of the cells to either grow attached to a glass or treated plastic substrate, called as mono-layer culture systems, or floating free in the culture medium called as Suspension Culture Systems.

Monolayer cultures are usually grown in tissue culture treated dishes, T-flasks, roller bottles, Cell-STACK® Culture Chambers, or multiple well plates, the choice being based on the number of cells needed, the nature of the culture en-vironment, cost and personal preference.

Suspension Cultures are usually Grown Either:

1. In magnetically rotated spinner flasks or shaken Erlenmeyer flasks where the cells are kept actively suspended in the medium;

2. In stationary culture vessels such as T-flasks and bottles where, although the cells are not kept agitated, they are unable to attach firmly to the sub-strate. Many cell lines, especially those derived from normal tissues, are considered to be Anchorage-Dependent, that is, they can only grow when attached to a suitable sub-strate.

Some cell lines that are no longer considered normal (frequently designated as Transformed Cells) are frequently able to grow either attached to a substrate or floating free in suspension; they are Anchorage-Independent. In addition, some normal cells, such as those found in the blood, do not normally attach to sub-strates and always grow in suspension.

Types of Cells:

Cultured cells are usu-ally described based on their morphology (shape and appearance) or their functional characteristics.

There are three basic mor-phologies:

1. Epithelial:

Like: cells that are attached to a substrate and appear flattened and polygonal in shape.

2. Lymphoblast-Like:

Cells that do not at-tach normally to a substrate but re-main in suspension with a spherical shape.

3. Fibroblast-Like:

Cells that are attached to a substrate and appear elongated and bipolar, frequently forming swirls in heavy cultures. It is important to remember that the cul-ture conditions play an important role in determining shape and that many cell cul-tures are capable of exhibiting multiple morphologies.

Using cell fusion tech-niques, it is also possible to obtain hybrid cells by fusing cells from two different par-ents. These may exhibit characteristics of either parent or both parents. This tech-nique was used in 1975 to create cells ca-pable of producing custom tailored mono-clonal antibodies.

These hybrid cells (called Hybridomas) are formed by fusing two different but related cells. The first is a spleen-derived lymphocyte that is capable of producing the desired antibody. The sec-ond is a rapidly dividing myeloma cell (a type of cancer cell) that has the machin-ery for making antibodies but is not pro-grammed to produce any antibody.

The resulting hybridomas can produce large quantities of the desired antibody. These antibodies, called Monoclonal Antibod-ies due to their purity, have many impor-tant clinical, diagnostic, and industrial applications with a yearly value of well over a billion dollars.

Functional Characteristics:

The char-acteristics of cultured cells result from both their origin (liver, heart, etc.) and how well they adapt to the culture conditions. Bio-chemical markers can be used to determine if cells are still carrying on specialized functions that they performed in vivo (e.g., liver cells secreting albumin).

Morphologi-cal or ultra-structural markers can also be examined (e.g., beating heart cells). Fre-quently, these characteristics are either lost or changed as a result of being placed in an artificial environment. Some cell lines will eventually stop dividing and show signs of aging.

These lines are called Finite. Other lines which become immor-tal can continue to divide indefinitely and are called Continuous cell lines. When a “normal” finite cell line becomes immor-tal, it undergoes a fundamental irrevers-ible change or “transformation”. This can occur spontaneously or be brought about intentionally using drugs, radiation or vi-ruses.

Transformed Cells are usually easier and faster growing, may often have extra or abnormal chromosomes and frequently can be grown in suspension.Cells that have the normal number of chromo-somes are called Diploid cells; those that have other than the normal number are Aneuploid. If the cells form tumours when they are injected into animals, they are considered to be Neo-plastically Transformed

Advantages of Animal Cell Culture:

a. Controlled physiochemical environment (pH, temperature, osmotic pressure, O2, etc.)

b. Controlled and defined physiological con-ditions

c. Homogeneity of cell types (achieved through serial passages)

d. Economical, since smaller quantities of reagents are needed than in vivo.

e. Legal, moral and ethical questions of ani-mal experimentation are avoided.

Disadvantages of Animal Cell Culture:

a. Expertise is needed, so that behaviour of cells in culture can be interpreted and regulated.

b. Ten times more expensive for same quan-tity of animal tissue; therefore, reasons for its use should be compelling.

c. Unstable aneuploid chromosome constitu-tion.

Applications of Animal Cell Culture:

A. Model Systems:

Cell cultures provide a good model system for studying;

a. Basic cell biology and biochemistry.

b. The interactions between disease-causing agents and cells.

c. The effects of drugs on cells.

d. The process and triggers for aging.

f. Nutritional studies.

B. Toxicity Testing:

Cultured cells are widely used alone or in conjunction with animal tests to study the effects of new drugs, cosmetics and chemicals on survival and growth in a wide variety of cell types. Especially important are liver and kidney derived cell cultures.

C. Cancer Research:

Since both normal cells and cancer cells can be grown in cul-ture, the basic differences between them can be closely studied. In addition, it is possible, by the use of chemicals, viruses and radiation, to convert normal cultured cells to cancer causing cells.

Thus, the mechanisms that cause the change can be studied. Cultured cancer cells also serve as a test system to determine suitable drugs and methods for selectively destroy-ing types of cancer.

D. Virology:

One of the earliest and major uses of cell culture is the replication of vi-ruses in cell cultures (in place of animals) for use in vaccine production. Cell cultures are also widely used in the clinical detec-tion and isolation of viruses, as well as basic research into how they grow and in-fect organisms.

E. Cell-Based Manufacturing:

While cul-tured cells can be used to produce many important products, three areas are gen-erating the most interest.

The first is the large-scale production of viruses for use in vaccine production. These include vaccines for polio, rabies, chicken pox, hepatitis B and measles.

The second is the large-scale production of cells that have been genetically engi-neered to produce proteins that have me-dicinal or commercial value. These include monoclonal antibodies, insulin, hormones, etc. The third is the use of cells as replace-ment tissues and organs. Artificial skin for use in treating burns and ulcers is the first commercially available product.

However, testing is underway on artificial organs such as pancreas, liver and kidney. A po-tential supply of replacement cells and tis-sues may come out of work currently be-ing done with both embryonic and adult stem cells.

These are cells that have the potential to differentiate into a variety of different cell types. It is hoped that learn-ing how to control the development of these cells may offer new treatment approaches for a wide variety of medical conditions.

F. Genetic Counselling:

Amniocentesis, a diagnostic technique that enables doctors to remove and culture fetal cells from preg-nant women, has given doctors an impor-tant tool for the early diagnosis of fetal disorders. These cells can then be exam-ined for abnormalities in their chromosomes and genes using karyotyping, chromosome painting and other molecular techniques.

G. Genetic Engineering:

The ability to transfect or reprogram cultured cells with new genetic material (DNA and genes) has provided a major tool to molecular biolo-gists wishing to study the cellular effects of the expression of these genes (new pro-teins).

These techniques can also be used to produce these new proteins in large quantity in cultured cells for further study. Insect cells are widely used as miniature cells factories to express substantial quan-tities of proteins that they manufacture after being infected with genetically engi-neered baculoviruses.

H. Drug Screening and Development:

Cell-based assays have become increas-ingly important for the pharmaceutical in-dustry, not just for cytotoxicity testing but also for high throughput screening of com-pounds that may have potential use as drugs. Originally, these cell culture tests were done in 96 well plates, but increas-ing use is now being made of 384 and 1536 well plates.

I. Gene Therapy:

In modern molecular bio-logy, Gene Therapy is an experimental technique that involves insertion of cloned/altered genes into cells using r-DNA tech-nology to replace defective genes causing genetic abnormalities or to prevent poten-tial disorders.

Followings are the purpose of Gene Therapy:

a. Swapping harmful mutant alleles with functional ones by selective reverse mutation.

b. Deactivating improperly functioning mutated gene.

c. Inserting a new gene into the body to help battle a disease.

d. Interchanging non-functional gene with normal gene through homologous recombination.

Primary culture:

The culture produced by the freshly isolated cells or tissues taken from an organism is the primary culture. These cell are heterogenous and slow growing, and represent the tissue of their origin with regard to their properties.

The three types of technique are: (1) Mechanical Disaggregation (2) Enzymatic Disaggregation and (3) Primary Explant Technique.

Primary culture broadly involves the culturing techniques carried following the isolation of the cells, but before the first subculture. Primary cultures are usually prepared from large tissue masses. Thus, these cultures may contain a variety of differentiated cells e.g. fibroblasts, lymphocytes, macrophages, epithelial cells.

With the experiences of the personnel working in tissue culture laboratories, the following criteria/ characteristics are considered for efficient development of primary cultures:

a. Embryonic tissues rather than adult tissues are preferred for primary cultures. This is due to the fact that the embryonic cells can be disaggregated easily and yield more viable cells, besides rapidly proliferating in vitro.

b. The quantity of cells used in the primary culture should be higher since their survival rate is substantially lower (when compared to subcultures).

c. The tissues should be processed with minimum damage to cells for use in primary culture. Further, the dead cells should be removed.

d. Selection of an appropriate medium (preferably a nutrient rich one) is advisable. For the addition of serum, fetal bovine source is preferred rather than calf or horse serum.

e. It is necessary to remove the enzymes used for disaggregation of cells by centrifugation.

Techniques for Primary Culture:

Among the various techniques devised for the primary culture of isolated tissues, three techniques are most commonly used:

1. Mechanical disaggregation.

2. Enzymatic disaggregation.

3. Primary explant technique.

An outline of these techniques is depicted in Fig. 36.1, and the procedures are briefly described:

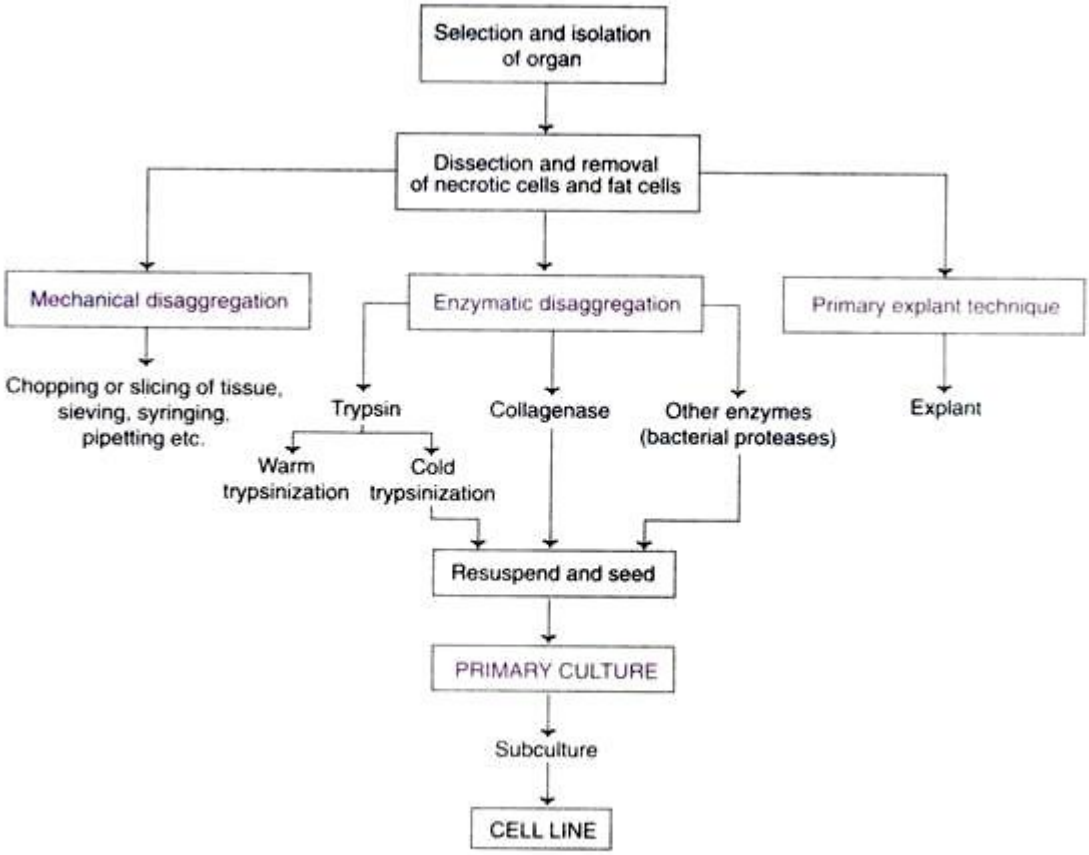


Fig. 36.1 : Different techniques used for primary culture.

Technique # 1. Mechanical Disaggregation:

For the disaggregation of soft tissues (e.g. spleen, brain, embryonic liver, soft tumors), mechanical technique is usually employed. This technique basically involves careful chopping or slicing of tissue into pieces and collection of spill out cells.

The cells can be collected by two ways:

i. Pressing the tissue pieces through a series of sieves with a gradual reduction in the mesh size.

ii. Forcing the tissue fragments through a syringe and needle.

Although mechanical disaggregation involves the risk of cell damage, the procedure is less expensive, quick and simple. This technique is particularly useful when the availability of the tissue is in plenty, and the efficiency of the yield is not very crucial. It must however, be noted that the viability of cells obtained from mechanical techniques is much lower than the enzymatic technique.

Technique 2. Enzymatic Disaggregation:

Enzymatic disaggregation is mostly used when high recovery of cells is required from a tissue. Disaggregation of embryonic tissues is more efficient with higher yield of cells by use of enzymes. This is due to the presence of less fibrous connective tissue and

extracellular matrix. Enzymatic disaggregation can be carried out by using trypsin, collagenase or some other enzymes.

Disaggregation by trypsin:

The term trypsinization is commonly used for disaggregation of tissues by the enzyme, trypsin.

Many workers prefer to use crude trypsin rather than pure trypsin for the following reasons:

i. The crude trypsin is more effective due to the presence of other proteases

ii. Cells can tolerate crude trypsin better.

iii. The residual activity of crude trypsin can be easily neutralized by the serum of the culture media (when serum-free media are used, a trypsin inhibitor can be used for neutralization).

Disaggregation of cells can also be carried out by using pure trypsin which is less toxic and more specific in its action. The desired tissue is chopped to 2-3 mm pieces and then subjected to disaggregation by trypsin. There are two techniques of trypsinization-warm trypsinization and cold trypsinization (Fig. 36.2).

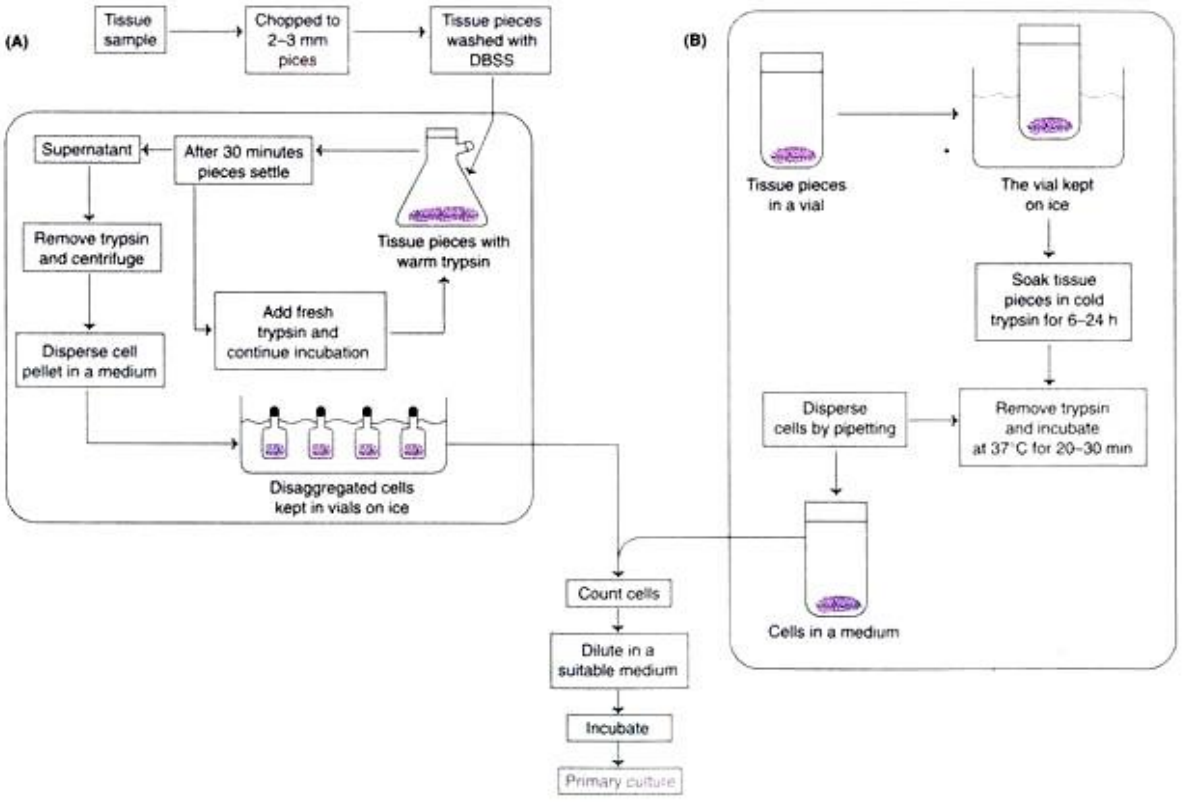


Fig. 36.2 : Preparation of primary culture by trypsin disaggregation (A) Warm trypsinization (B) Cold trypsinization (DBSS–Dissection basal salt solution).

Warm trypsinization (Fig. 36.2A):

This method is widely used for disaggregation of cells. The chopped tissue is washed with dissection basal salt solution (DBSS), and then transferred to a flask containing warm trypsin (37° C). The contents are stirred, and at an interval of every thirty minutes, the supernatant containing the dissociated cells can be collected. After removal of trypsin, the cells are dispersed in a suitable medium and preserved (by keeping the vial on ice).

The process of addition of fresh trypsin (to the tissue pieces), incubation and collection of dissociated cells (at 30 minutes intervals) is carried out for about 4 hours. The disaggregated cells are pooled, counted, appropriately diluted and then incubated.

Cold trypsinization (Fig. 36.2B):

This technique is more appropriately referred to as trypsinization with cold pre-exposure. The risk of damage to the cells by prolonged exposure to trypsin at 37°C (in warm trypsinization) can be minimized in this technique.

After chopping and washing, the tissue pieces are kept in a vial (on ice) and soaked with cold trypsin for about 6-24 hours. The trypsin is removed and discarded. However, the tissue pieces contain residual trypsin. These tissue pieces in a medium are incubated at 37°C for 20-30 minutes. The cells get dispersed by repeated pi-pettings. The dissociated cells can be counted, appropriately diluted and then used.

The cold trypsinization method usually results in a higher yield of viable cells with an improved survival of cells after 24 hours of incubation. This method does not involve stirring or centrifugation, and can be conveniently adopted in a laboratory. The major limitation of cold trypsinization is that it is not suitable for disaggregation of cells from large quantities of tissues.

Limitations of trypsin disaggregation:

Disaggregation by trypsin may damage some cells (e.g. epithelial cells) or it may be almost ineffective for certain tissues (e.g. fibrous connective tissue). Hence other enzymes are also in use for dissociation of cells.

Disaggregation by collagenase:

Collagen is the most abundant structural protein in higher animals. It is mainly present in the extracellular matrix of connective tissue and muscle. The enzyme collagenase (usually a crude one contaminated with non-specific proteases) can be effectively used for the disaggregation of several tissues (normal or malignant) that may be sensitive to trypsin.

Highly purified grades of collagenase have been tried, but they are less effective when compared to crude collagenase. The important stages in collagenase disaggregation, depicted in Fig. 36.3, are briefly described hereunder.

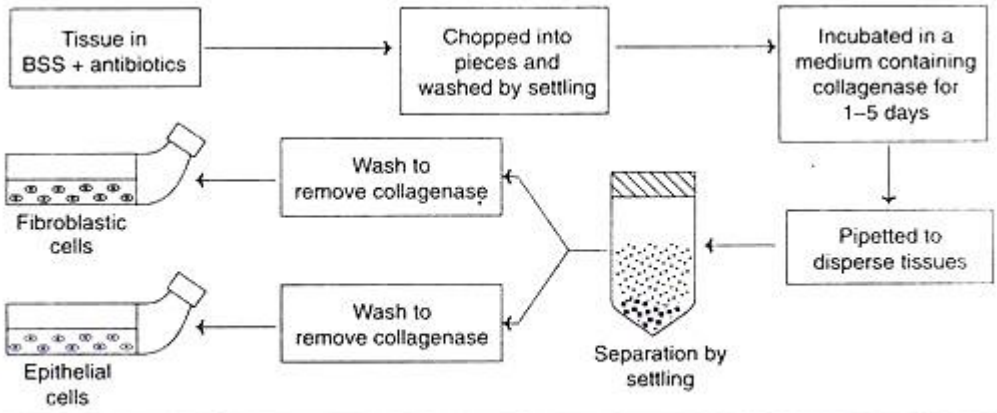


Fig. 36.3 : Important stages in collagenase disaggregation of tissue for primary culture (BSS–Basal salt solution).

The desired tissue suspended in basal salt solution, containing antibiotics is chopped into pieces. These pieces are washed by settling, and then suspended in a complete medium containing collagenase. After incubating for 1-5 days, the tissue pieces are dispersed by pipetting. The clusters of cells are separated by settling. The epithelial cells and fibroblastic cells can be separated.

Collagenase disaggregation has been successfully used for human brain, lung and several other epithelial tissues, besides various human tumors, and other animal tissues. Addition of another enzyme hyaluronidase (acts on carbohydrate residues on cell surfaces) promotes disaggregation. Collagenase in combination with hyaluronidase is found to be very effective for dissociating rat or rabbit liver. This can be done by per-fusing the whole organ in situ. Some workers use collagenase in conjunction with trypsin, a formulation developed in chick serum, for disaggregation of certain tissues.

Use of other enzymes in disaggregation:

Trypsin and collagenase are the most widely used enzymes for disaggregation. Certain bacterial proteases (e.g.pronase, dispase) have been used with limited success. Besides hyaluronidase, neuraminidase is also used in conjunction with collagenase for effective degradation of cell surface carbohydrates.

Technique 3. Primary Explant Technique:

The primary explant technique was, in fact the original method, developed by Harrison in 1907. This technique has undergone several modifications, and is still in use. The simplified procedure adopted for primary explant culture is depicted in Fig. 36.4, and briefly described below.

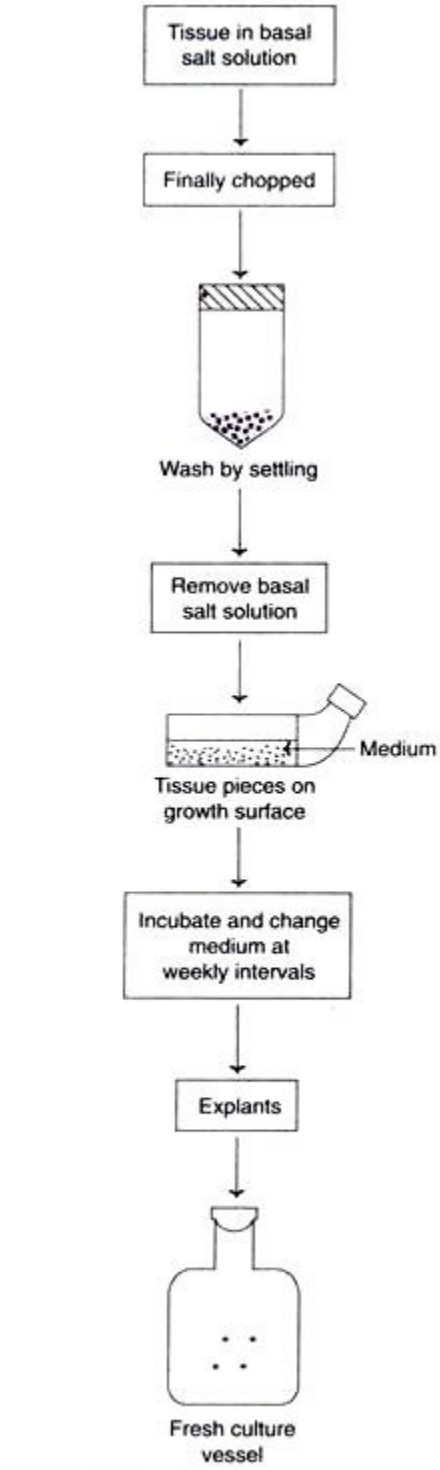


Fig. 36.4 : Primary explant technique for primary culture.

The tissue in basal salt solution is finely chopped, and washed by settlings. The basal salt solution is then removed. The tissue pieces are spread evenly over the growth surface. After addition of appropriate medium, incubation is carried out for 3-5 days. Then the medium is changed at weekly intervals until a substantial outgrowth of cells is observed. Now, the explants are removed and transferred to a fresh culture vessel.

The primary explant technique is particularly useful for disaggregation of small quantities of tissues (e.g. skin biopsies). The other two techniques mechanical or enzymatic disaggregation however, are not suitable for small amounts of tissues, as there is a risk of losing the cells.

The limitation of explant technique is the poor adhesiveness of certain tissues to the growth surface, and the selection of cells in the outgrowth. It is however, observed that the primary explant technique can be used for a majority of embryonic cells e.g. fibroblasts, myoblasts, epithelial cells, glial cells.

Separation of Viable and Non-Viable Cells:

It is a common practice to remove the nonviable cells while the primary culture is prepared from the disaggregated cells. This is usually done when the first change of the medium is carried out. The very few left over non-viable cells get diluted and gradually disappear as the proliferation of viable cells commences.

Sometimes, the non-viable cells from the primary cultures may be removed by centrifugation. The cells are mixed with ficoll and sodium metrizoate, and centrifuged. The dead cells form a pellet at the bottom of the tube.

Medical Ethics and Safety Measures in Culture Techniques:

Since the culture techniques involve the use of animal or human tissues, it is absolutely necessary to follow several safety measures and medical ethics. In fact, in some countries there are established legislation/norms for selection and use of tissues in cultures. For example, in United Kingdom, Animal Experiments (Scientific Procedures) Act of 1986 is followed.

The handling of human tissues poses several problems that are not usually encountered with animal tissues. While dealing with fetal materials and human biopsies, the consent of the patient and/his or her relatives, besides the consent of local ethical committee is required. Further, taking any tissue (even in minute quantities) from human donors requires the full consent of the donor in a prescribed format.

The following issues need to be fully considered while dealing with human tissues:

1. The consent of the patient and/or relatives for using tissues for research purposes.

2. Ownership of the cell lines developed and their derivatives.

3. Consent for genetic modification of the cell lines.

6. Patent rights for any commercial use of cell lines.

In the general practice of culture techniques using human tissues, the donor and/or relatives are asked to sign a disclaimer statement (in a prescribed pro-forma) before the tissue is taken. By this approach, the legal complications are minimized.

Safety measures:

Handling of human tissues is associated with a heavy risk of exposure for various infections. Therefore, it is absolutely necessary that the human materials are handled in a biohazard cabinet. The tissues should be screened for various infections such as hepatitis, tuberculosis, HIV, before their use. Further, the media and apparatus, after their use must be autoclaved or disinfected, so that the spread of infections is drastically reduced.

Subculture Methods:

Subculture (or passage) refers to the transfer of cells from one culture vessel to another culture vessel. Subculture usually (not always) involves the subdivision of proliferating cells that enables the propagation of a cell line.

The term passage number is used to indicate the number of times a culture has been sub-cultured.

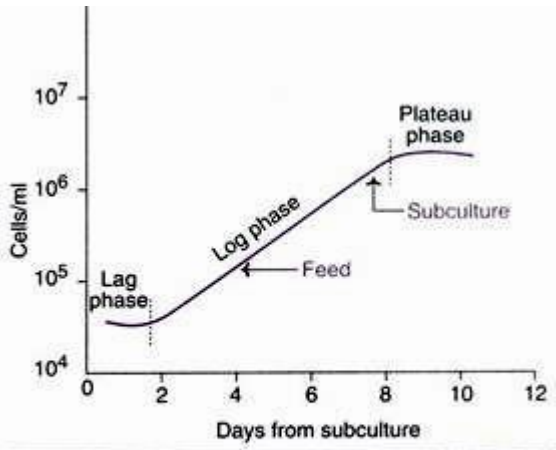


Fig. 36.5 : Depiction of a standard growth of cells in a culture (Note : the cell concentration is expressed in semilog plot).

There are physical and enzymatic methods for dissociation of monolayer cultures (Table. 36.3).

Table 36.3 Commonly used cell dissociation methods for monolayer cultures

Method	*Applicable to*
Physical	
Mechanical shaking	Loosely adherent cells
Scraping	Cells sensitive to proteases
Enzymatic	
Trypsin	Most of the continuous cell lines
Trypsin + collagenase	Dense and multilayer cultures
Dispase	Removal of epithelium in sheets
Pronase	Single–cell suspensions

Mechanical shaking and cell scraping are employed for cultures which are loosely adhered, and the use of proteases has to be avoided. Among the enzymes, trypsin is the most frequently used. For certain cell monolayers, which cannot be dissociated by trypsin, other enzymes such as pronase, dispase and collagenase are used. Prior to cell dissociation by enzymes, the monolayers are usually subjected to pretreatment of EDTA for the removal of Ca^{2+}.

Criteria for Subculture of Monolayers:

The sub-culturing is ideally carried out between the middle of the log phase and the time before they enter plateau phase (Fig. 36.6). Subculture of cells should not be done when they are in lag phase. The other important criteria for subculture of monolayers are briefly described.

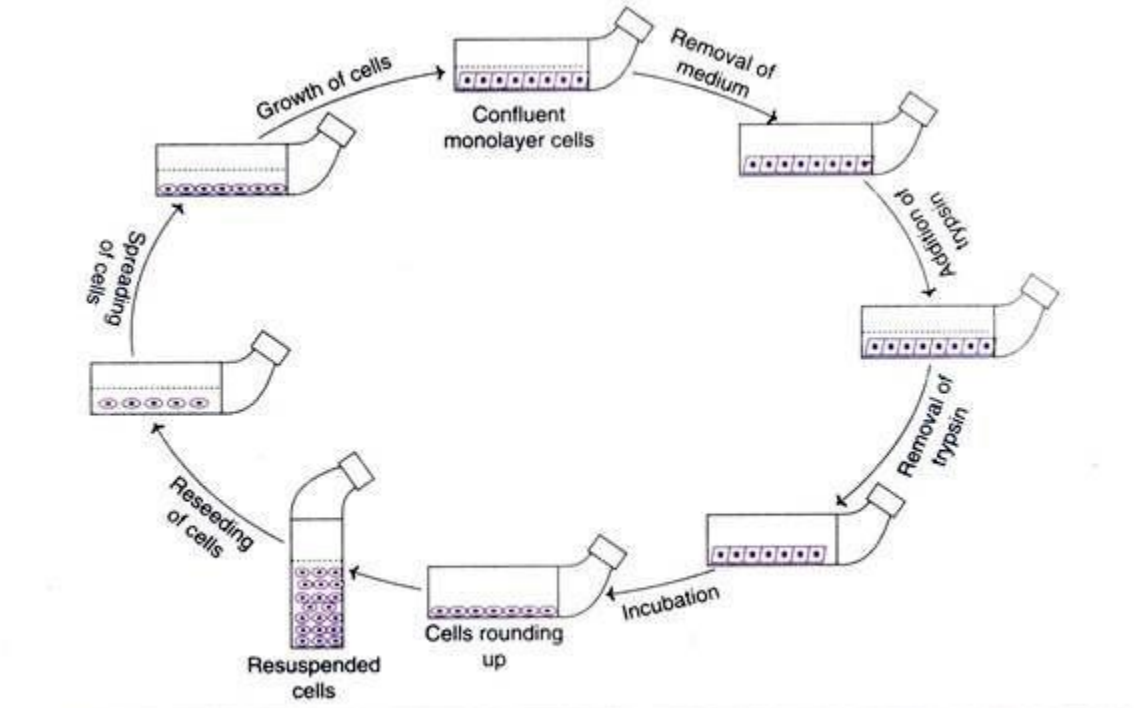


Fig. 36.6 : Technique of monolayer subculture.

Culture density:

It is advisable to subculture the normal or transformed monolayer cultures, as soon as they reach confluence. Confluence denotes the culture stage wherein all the available growth area is utilized and the cells make close contact with each other.

Medium exhaustion:

A drop in pH is usually accompanied by an increase in culture cell density. Thus, when the pH falls, the medium must be changed, followed by subculture.

Scheduled timings of subculture:

It is now possible to have specified schedule timings for subculture of each cell line. For a majority of cell cultures, the medium change is usually done after 3-4 days, and sub-culturing after 7 days.

Purpose of subculture:

The purpose for which the cells are required is another important criteria for consideration of sub-culturing. Generally, if the cells are to be used for any specialized purpose, they have to be sub-cultured more frequently.

Techniques of Monolayer Subculture:

The subculture of monolayer cells basically consists of the following steps (Fig. 36.6)

1. Removal of the medium

2. Brief exposure of the cells to trypsin.

3. Removal of trypsin and dispersion in a medium.

4. Incubation of cells to round up.

5. Re-suspension of the cells in a medium for counting and reseeding.

6. Cells reseeded and grown to monolayers.

Cell concentration at subculture:
Most of the continuous cell lines are sub-cultured at a seeding concentration between 1 × 10^4 and 5 × 10^4 cells/ ml. However for a new culture, subculture has to be started at a high concentration and gradually reduced.

Type # 2. Suspension Cultures:

Majority of continuous cell lines grow as monolayers. Some of the cells which are non-adhesive e. g. cells of leukemia or certain cells which can be mechanically kept in suspension can be propagated in suspension. The transformed cells are sub-cultured by this method. Subculture by suspension is comparable to culturing of bacteria or yeast.

Advantages of Cell Propagation by Suspension:

i. The process of propagation is faster.

ii. The lag period is usually shorter.

iii. Results in homogeneous suspension of cells.

iv. Treatment with trypsin is not required.

v. Scale-up is convenient.

vi. No need for frequent replacement of the medium.

vii. Maintenance is easy.

viii. Bulk production of cells can be conveniently achieved.

Criteria for Suspension Subculture:

The criteria adopted for suspension subculture are the same as that already described for monolayer subcultures.

The following aspects have to be considered:

i. Culture density.

ii. pH change representing medium exhaustion.

iii. Schedule timings of subculture.

iv. Purpose of subculture.

Technique of Suspension Culture:

The cells can be suspended in a culture flask (a stirrer flask) containing the desired medium (Fig. 36.7). The medium is continuously stirred with a magnetic pendulum rotating at the base of the flask. The cells have to periodically examined for contamination or signs of deterioration.

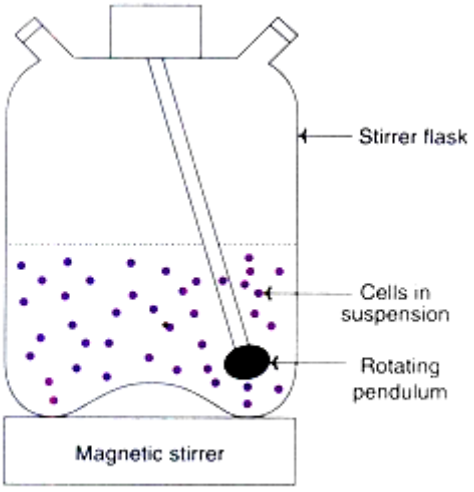


Fig. 36.7 : Suspension culture in a stirrer flask.

Cell Lines:

The development and various other aspects of primary culture are described above. The term cell line refers to the propagation of culture after the first subculture.In other words, once the primary culture is sub-cultured, it becomes a cell line. A given cell line contains several cell lineages of either similar or distinct phenotypes.

It is possible to select a particular cell lineage by cloning or physical cell separation or some other selection method. Such a cell line derived by selection or cloning is referred to as cell strain. Cell strains do not have infinite life, as they die after some divisions.

Types of Cell Lines:

Finite Cell Lines :

The cells in culture divide only a limited number of times, before their growth rate declines and they eventually die. The cell lines with limited culture life spans are referred to as finite cell lines. The cells normally divide 20 to 100 times (i.e. is 20-100 population doublings) before extinction. The actual number of doublings depends on the species, cell lineage differences, culture conditions etc. The human cells generally divide 50-100 times, while murine cells divide 30-50 times before dying.

Continuous Cell Lines :

A few cells in culture may acquire a different morphology and get altered. Such cells are capable of growing faster resulting in an independent culture. The progeny derived from these altered cells has unlimited life (unlike the cell strains from which they originated). They are designated as continuous cell lines.

The continuous cell lines are transformed, immortal and tumorigenic. The transformed cells for continuous cell lines may be obtained from normal primary cell cultures (or cells strains) by treating them with chemical carcinogens or by infecting with oncogenic viruses. In the Table. 36.1, the different properties of finite cell lines and continuous cell lines are compared.

Table 36.1 Comparison of properties of finite and continuous cell lines

Property	*Finite cell line*	*Continuous cell line*
Growth rate	Slow	Fast
Mode of growth	Monolayer	Suspension or monolayer
Yield	Low	High
Transformation	Normal	Immortal, tumorigenic
Ploidy	Euploid (multiple of haploid chromosomes)	Aneuploid (not an exact multiple of haploid chromosomes)
Anchorage dependence	Yes	No
Contact inhibition	Yes	No
Cloning efficiency	Low	High
Serum requirement	High	Low
Markers	Tissue specific	Chromosomal, antigenic or enzymatic

The most commonly used terms while dealing with cell lines are explained below.

Split ratio:

The divisor of the dilution ratio of a cell culture at subculture. For instance, when each subculture divided the culture to half, the split ratio is 1: 2.

Passage number:

It is the number of times that the culture has been sub-cultured.

Generation number:

It refers to the number of doublings that a cell population has undergone. It must be noted that the passage number and generation number are not the same, and they are totally different.

Nomenclature of Cell Lines:

It is a common practice to give codes or designations to cell lines for their identification. For instance, the code NHB 2-1 represents the cell line from normal human brain, followed by cell strain (or cell line number) 2 and clone number 1. The usual practice in a culture laboratory is to maintain a log book or computer database file for each of the cell lines.

While naming the cell lines, it is absolutely necessary to ensure that each cell line designation is unique so that there occurs no confusion when reports are given in literature. Further, at the time of publication, the-cell line should be prefixed with a code designating the laboratory from which it was obtained e.g. NCI for National Cancer Institute, Wl for Wistar Institute.

Commonly used cell lines:

There are thousands of cell lines developed from different laboratories world over. A selected list of some commonly used cell lines along with their origin, morphology and other characters are given in Table. 36.2.

Table 36.2 A selected list of commonly used cell lines

Cell line	*Species of origin*	*Tissue of origin*	*Morphology*	*Ploidy*	*Characteristics*
IMR-90	Human	Lung	Fibroblast	Diploid	Susceptible to human viral infections.
3T3-A31	Mouse	Connective tissue	Fibroblast	Aneuploid	Contact inhibited, readily transformed
BHK21-C13	Hamster (Syrian)	Kidney	Fibroblast	Aneuploid	Readily transformable
CHO-k1	Chinese hamster	Ovary	Fibroblast	Diploid	Simple karyotype
NRK49F	Rat	Kidney	Fibroblast	Aneuploid	Induction of suspension growth by TGF-α, β.
BRL 3A	Rat	Liver	Epithelial	Diploid	Produces IGF-2
Vero	Monkey	Kidney	Fibroblast	Aneuploid	Viral substrate and assay
HeLa-S_3	Human	Cervical carcinoma	Epithelial	Aneuploid	Rapid growth, high plating efficiency.
Sk/HEP-I	Human	Hepatoma	Endothelial	Aneuploid	Factor VIII
Caco-2	Human	Colo-rectal carcinoma	Epithelial	Aneuploid with polarised support.	Forms tight monolayer
MCF-7	Human	Breast tumor (effusion)	Epithelial	Aneuploid	Estrogen receptor positive.
Friend	Mouse	Spleen	Suspension	Aneuploid	Hemoglobin, growth hormone.

Selection of Cell Lines:

Several factors need to be considered while selecting a cell line.

Some of them are briefly described:

1. Species:

In general, non-human cell lines have less risk of biohazards, hence preferred. However, species differences need to be taken into account while extrapolating the data to humans.

2. Finite or continuous cell lines:

Cultures with continuous cell lines are preferred as they grow faster, easy to clone and maintain, and produce higher yield. But it is doubtful whether the continuous cell lines express the right and appropriate functions of the cells. Therefore, some workers suggest the use of finite cell lines, although it is difficult.

3. Normal or transformed cells:

The transformed cells are preferred as they are immortalized and grow rapidly.

4. Availability:

The ready availability of cell lines is also important. Sometimes, it may be necessary to develop a particular cell line in a laboratory.

5. Growth characteristics:

The following growth parameters need to be considered:

i. Population doubling time

ii. Ability to grow in suspension

iii. Saturation density (yield per flask)

iv. Cloning efficiency.

6. Stability:

The stability of cell line with particular reference to cloning, generation of adequate stock and storage are important.

7. Phenotypic expression:

It is important that the cell lines possess cells with the right phenotypic expression.

Maintenance of Cell Cultures:

For the routine and good maintenance of cell lines in culture (primary culture or subculture) the examination of cell morphology and the periodic change of medium are very important.

Cell Morphology:

The cells in the culture must be examined regularly to check the health status of the cells, the absence of contamination, and any other serious complications (toxins in medium, inadequate nutrients etc.).

Replacement of Medium:

Periodic change of the medium is required for the maintenance of cell lines in culture, whether the cells are proliferating or non-proliferating. For the proliferating cells, the medium need to be changed more frequently when compared to non-proliferating cells. The time interval between medium changes depends on the rate of cell growth and metabolism.

For instance, for rapidly growing transformed cells (e.g. HeLa), the medium needs to be changed twice a week, while for slowly growing non-transformed cells (e.g. IMR-90) the medium may be changed once a week. Further, for rapidly proliferating cells, the sub-culturing has to be done more frequently than for the slowly growing cells.

The following factors need to be considered for the replacement of the medium:

1. Cell concentration:

The cultures with high cell concentration utilize the nutrients in the medium faster than those with low concentration; hence the medium is required to be changed more frequently for the former.

2. A decrease in pH:

A fall in the pH of the medium is an indication for change of medium. Most of the cells can grow optimally at pH 7.0, and they almost stop growing when the pH falls to 6.5. A further drop in pH (between 6.5 and 6.0), the cells may lose their viability.

The rate of fall in pH is generally estimated for each cell line with a chosen medium. If the fall is less than 0.1 pH units per day, there is no harm even if the medium is not immediately changed. But when the fall is 0.4 pH units per day, medium should be changed immediately.

3. Cell type:

Embryonic cells, transformed cells and continuous cell lines grow rapidly and require more frequent sub-culturing and change of medium. This is in contrast to normal cells, which grow slowly.

4. Morphological changes:

Frequent examination of cell morphology is very important in culture techniques. Any deterioration in cell morphology may lead to an irreversible damage to cells. Change of the medium has to be done to completely avoid the risk of cell damage.

Probable Questions:

1. What is primary culture?
2. Discuss limitations of primary cell culture.
3. Define sub culturing.
4. State advantages of animal cell culture.
5. State disadvantages of animal cell culture.
6. State applications of animal cell culture.
7. What are the purposes of gene therapy?
8. Discuss different techniques of primary cell culture.
9. How monolayer of subculture is performed?
10. How suspension culture is done?
11. Differentiate finite and continuous cell lines.

Suggested readings:

1. Biotechnology by P.K. Gupta

2. Gene Cloning by T. Brown.

3. Biotechnology by N. Kumarsen.

4. Biotechnology by B.D. Singh

UNIT-III

Assisted Reproductive Technology and IVF

Objective: In this unit we will discuss assisted reproductive technology and In vitro fertilization procedures.

In vitro fertilization:

In a normal pregnancy, a male sperm penetrates a woman's egg and fertilizes it inside her body after ovulation, when a mature egg has been released from the ovaries. The fertilized egg then attaches itself to the wall of the uterus, or womb, and begins developing into a baby. This is known as natural conception. However, if natural or unassisted conception is not possible, fertility treatment is an option. IVF has been used since the late 1970s. On 25 July 1978, the first "test-tube baby," Louise Brown, was born. Robert Edwards and Patrick Steptoe, who collaborated on the procedure, are considered to be the pioneers of IVF. In 2010, Robert Edwards received the 2010 Nobel Prize in Physiology or Medicine "for the development of in-vitro fertilization."

In July 2013, an American couple had the first baby to be born through IVF as a result of next-generation DNA sequencing, a new way of screening embryos that improves IVF success rates and significantly reduces the cost of treatment. DNA sequencing technology helps doctors screen embryos created by IVF to identify those most likely to lead to successful pregnancies. In vitro fertilisation (IVF) is a process by which an egg is fertilised by sperm outside the body: in vitro. IVF is a major treatment for infertility when other methods of assisted reproductive technology have failed. The process involves monitoring and stimulating a woman's ovulatory process, removing ovum or ova (egg or eggs) from the woman's ovaries and letting sperm fertilise them in a fluid medium in a laboratory.

The fertilised egg (zygote) cultured for 2-6 days in a growth medium and is then transferred to the mother's uterus with the intention of establishing a successful pregnancy. The first successful birth of a "test tube baby," Louise Brown, occurred in 1978. Louise Brown was born as a result of natural cycle IVF where no stimulation was made. Robert G. Edwards, the physiologist who developed the treatment, was awarded the Nobel Prize in Physiology or Medicine in 2010.

The term in vitro, from the Latin meaning in glass, is used, because early biological experiments involving cultivation of tissues outside the living organism from which they came, were carried out in glass containers such as beakers, test tubes, or petri dishes. Today, the term in vitro is used to refer to any biological procedure that is

performed outside the organism it would normally be occurring in, to distinguish it from an in vivo procedure, where the tissue remains inside the living organism within which it is normally found. A colloquial term for babies conceived as the result of IVF, "test tube babies," refers to the tube-shaped containers of glass or plastic resin, called test tubes, that are commonly used in chemistry labs and biology labs.

However, in vitro fertilisation is usually performed in the shallower containers called Petri dishes. One IVF method, autologous endometrial coculture, is actually performed on organic material, but is still considered in vitro.

Procedure:

Techniques may differ depending on the clinic, but IVF usually involves the following steps:

1. Suppressing the natural menstrual cycle

The woman receives a drug, usually in the form of a daily injection for about 2 weeks, to suppress their natural menstrual cycle.

2 Super ovulation

Fertility drugs containing the fertility hormone follicle stimulating hormone (FSH) are given to the woman. FSH makes the ovaries produce more eggs than usual. Vaginal ultrasound scans can monitor the process in the ovaries.

3. Retrieving the eggs

The eggs are collected through a minor surgical procedure known as "follicular aspiration." A very thin needle is inserted through the vagina and into an ovary. The needle is which is connected to a suction device. This sucks the eggs out. This process is repeated for each ovary. In 2011, researchers suggested that collecting 15 eggs from the ovaries in one cycle gives the highest chance of a successful pregnancy. Frozen or donated eggs may also be used.

4. Insemination and fertilization

The eggs that have been collected are placed together with male sperm and kept in an environmentally controlled chamber. After a few hours, the sperm should enter the egg. Sometimes the sperm is directly injected into the egg. This is known as an intracytoplasmic sperm injection (ICSI).Frozen sperm, retrieved through testicular biopsy, may be used. This is believed to be as effective as fresh sperm in achieving a successful pregnancy. The fertilized egg divides and becomes an embryo. At this point, some centres offer pre-implantation genetic diagnosis (PGD) which can screen an embryo for genetic disorders. This is somewhat controversial and is not always used. One or two of the best embryos are selected for transfer. The woman is then given

progesterone or human chorionic gonadotrophin (hCG) to help the lining of the womb receive the embryo.

5. Embryo transfer

Sometimes, more than one embryo is placed in the womb. It is important that the doctor and the couple wishing to have a child discuss how many embryos should be transferred. Normally, a doctor will only transfer more than one embryo if no ideal embryos are available. The transfer of the embryo is done using a thin tube, or catheter. It enters the womb through the vagina. When the embryo sticks to the lining of the womb, healthy embryo growth can begin.

The important techniques employed in assisted reproductive technology are listed below:

i. Intrauterine insemination (IUI).

ii. In vitro fertilization and embryo transfer (IVF and ET).

iii. Gamete intra-fallopian transfer (GIFT).

iv. Zygote intra-fallopian transfer (ZIPT).

v. Intra-vaginal culture (IVC).

vi. Cytoplasmic transfer (CT).

vii. Micromanipulation (Intra-cytoplasmic sperm injection (ICSI), sub-zonal insertion (SUZI).

viii. Cryopreservation.

ix. Assisted hatching (AH).

Among these techniques, the most commonly used procedure is in vitro fertilization and embryo transfer. Important features of different types of ART are briefly described.

Intrauterine Insemination (IUI):

The infertile women (due to endometriosis, idiopathic infertility) without blockage or damage to fallopian tubes can be effectively treated by intrauterine insemination. The women with adequate ovulation and below the age of 40 years are considered for IUI.

The women are usually super-ovulated by administering gonadotrophins. This results in multiple egg development. The IUI is timed to coincide with ovulation. The semen is washed and the highly motile sperms are separated. By using a thin and soft catheter, the sperms are placed either in the cervix or in uterine cavity. The women subjects are advised to remain lying down for about 15-30 minutes following IUI. Insemination should be carefully timed for good success. If it is done, a little before the expected time of ovulation, the chances for fertilization are much higher. IUI is usually successful in the first 3-4 attempts. In any case, this approach is not recommended for more than a maximum of 6 ovulation cycles. The success rates of IUI vary considerably and are in the range of 15-30%.

In Vitro Fertilization and Embryo Transfer (IVF and ET):

In vitro fertilization broadly deals with the removal of eggs from a women, fertilizing them in the laboratory, and then transferring the fertilized eggs (zygotes) into the uterus a few days later.

Indications for IVF:

Infertility due to the following causes may be considered for IVF.

i. Failed ovulation induction

ii. Tubal diseases

iii. Cervical hostility

iv. Endometriosis

v. Idiopathic infertility (in men and women).

Ideal Subjects for IVF:

Although it is not always possible to have a choice in the selection of subjects, the following criteria are preferred.

i. Woman below 35 years.

ii. Presence of at least one functional ovary.

iii. Husband with normal motile sperm count (i.e. normal seminogram).

iv. The couple must be negative for HIV and hepatitis.

Methodology of IVF:

The in vitro fertilization broadly involves the following steps.

1. Induction of superovulation.

2. Monitoring of ovarian response.

3. Oocyte retrieval.

4. Fertilization in vitro.

5. Embryo transfer.

Induction of Superovulation:

It is well known that the success rate IVF is much higher when more embryos (3-5) are transferred. This is possible only with controlled ovarian hyper-stimulation (COH). The other advantages of COH include improvement in the quality of oocyte, control of ovulation timing, besides overcoming the ovulatory dysfunction. The following drug regimens are in use to induce superovulation.

i. Clomiphene citrate (CC).

ii. CC + human menopausal gonadotrophin (hMG).

iii. CC + follicle stimulating hormone (FSH).

iv. Human menopausal gonadotrophin.

v. Follicle stimulating hormone.

vi. Gonadotrophin releasing hormone agonists (GnRHa) + hMG (or FSH).

It is now common to use GnRH agonists to induce ovulation. These compounds act through a process called down regulation of the physiologic hypothalamic- pituitary-ovarian feedback mechanism to effectively suppress spontaneous ovulation.

Monitoring of Ovarian Response:

The follicular growth or ovarian response can be monitored by increase in serum estradiol level, increase in follicular diameter and thickening of endometrial bed.

Oocyte Retrieval:

The most common method for oocyte retrieval is carried out through vaginal route under ultrasound guidance. This method is simple and less invasive, and can be performed with analgesics only. It is easy to recognize the oocyte as a single cell surrounded by a mass of cumulus cells. The recovered oocytes are maintained in vitro culture for 4-6 hours.

Fertilization in Vitro:

The semen specimens are collected (just prior to oocyte retrival) via masturbation, processed, and incubated in protein-supplemented media for 3-4 hours prior to fertilization. The incubation results in sperm capacitation.

The retrieved oocytes are also cultured in protein-supplemented media for about 6-8 hours. For the purpose of IVF, 50,000-1, 00,000 capacitated sperms are placed in culture with a single oocyte. The signs of fertilization may be demonstrated 16-20 hours later by the presence of two pronuclei within the developing embryo. There is no need to change the regime for a single failure of IVF. Many a times, success occurs in the subsequent cycles. The two most important criteria for the success of IVF are sperm density and motility.

Embryo Transfer:

Embryo at a stage between pronuclei and blastocyst stage are transferred. Conventionally, 4- 8 cell stage embryos are transferred between 48-60 hours following insemination. The transfer procedure is carried out by use of a catheter.

Not more than three embryos are transferred (per cycle) to minimize multiple pregnancies. However, in the women above the age of 40 years, higher number of embryo may be transferred. (Note: Excess oocytes and embryos are cryopreserved for further use. This will reduce the cost, besides the risk of ovarian hyper stimulation). Luteal phase support is given by administration of progesterone for about two weeks. By this time, the diagnosis of pregnancy can be assessed by estimating human chorionic gonadotrophin (hCG).

Success Rates of IVF:

Success of IVF varies from programme to programme and within the same programme, the success rate is dependent on the correct diagnosis of the patient, and age. The overall pregnancy rate in IVF is in the range of 25-35% per oocyte retrieval. The take home baby rate is about 15-20% per procedure.

The success rate of IVF is rather low due to the following reasons:

i. Increased risk of abortion

ii. Multiple pregnancy

iii. Ectopic pregnancy

iv. Low birth weight baby

v. Premature delivery.

The World's Picture of Test Tube Babies:

By employing in vitro fertilization and embryo transfer, the world's first test tube baby (Louise Brown) was born in UK on 28th July 1978. The world's second test tube baby (Kanupriya alias Durga) was born in Kolkata on 3rd October 1978. A team led by Subhash Mukherjee carried IVF and ET in India. Scientists responsible for the "birth of test tube babies were severely criticized then.
In fact, IVF turned out to be one of the major achievements of medical sciences in the last century. It has become a novel way of treating infertility. Today, there are more than a million test tube babies born all over the world. In 2003, the world celebrated the silver jubilee of IVF with much fanfare.

Gamete Intra-Fallopian Transfer (GIFT):

Gamete intra-fallopian transfer involves the transfer of both sperm and unfertilized oocyte into the fallopian tube. This allows the fertilization to naturally occur in vivo. The prerequisite for GIFT procedure is that the woman should have at least one normal fallopian tube.

The induction of ovulation and the monitoring procedures for GIFT are almost the same as described for IVF. A couple of hours prior to oocyte retrieval, semen specimens are collected. Two oocytes along with 2-5 lakhs motile sperms for each fallopian tube are placed in a plastic tube container. It is then inserted (by laparoscopy) 4 cm into the distal end of the fallopian tube, and the oocyte sperm combination is injected. The overall pregnancy rate is as high as 30- 40%. The take home baby rate is about 25%. This is much higher when compared to IVF. But the major limitation is the requirement of laparoscopy (a major surgical procedure) to transfer oocytes and sperms into the fallopian tubes.

Zygote Intra-Fallopian Transfer (ZIFT):

ZIFT is suitable when the infertility lies in men, or in case of failure of GIFT.The wife's oocytes are exposed to her husband's sperms in the laboratory. The fertilized eggs (zygotes) within 24 hours are transferred to the fallopian tube by using laparoscopy. ZIPT has an advantage over GIFT with male factor infertility. Further, it can be known whether the wife's oocytes have been fertilized by her husbands' sperms.

Intra Vaginal Culture (IVC):

The body's own environment is appropriately utilized in intra-vaginal culture. The retrieved oocytes and sperms are placed in a culture medium inside a sealed container. This is inserted into the vagina. The container is held by a vaginal diaphragm. Thus, the oocytes and sperms are maintained at the normal body temperature (in contrast to any incubator in the laboratory). Two to 3 three days later, the container is opened, and the fertilized and dividing zygotes are transferred into the uterus. This procedure appears simple, but the success rate is very low. Only a few centers practice this.

Cytoplasmic Transfer (CT):

Cytoplasm includes many things, the most important being mitochondria which provide energy to the cell. It is possible that deficiency in the mitochondria may leave the oocyte without the necessary power for cell division, after fertilization. This may result in abnormal cell division and poor development of embryo.

It is therefore logical to think of the transfer of cytoplasm from a donor (with active mitochondria) into the oocyte of a woman. The advantage with cytoplasmic transfer is that the mother's own genetic material is passed on to the offspring.

Two methods of cytoplasmic transfer have been developed:

1. Transfer of a small amount of cytoplasm by a tiny needle from a donor to a recipient oocyte.

2. Transfer of a large amount of cytoplasm which is fused with the recipient's cytoplasm by applying electricity.

The procedure of cytoplasmic transfer is tedious and technically difficult, besides the cost factor. At least two viable pregnancies have been so far reported in literature by this approach.

Micromanipulation:

Micromanipulation involves in vitro micro-surgically assisted fertilization procedures. This is required when the sperms are unable to penetrate the zona pellucida of oocyte and fertilize. Micromanipulations are usually done in severe cases of male factor infertility.

A diagrammatic representation of micromanipulation is depicted in Fig 18.3.

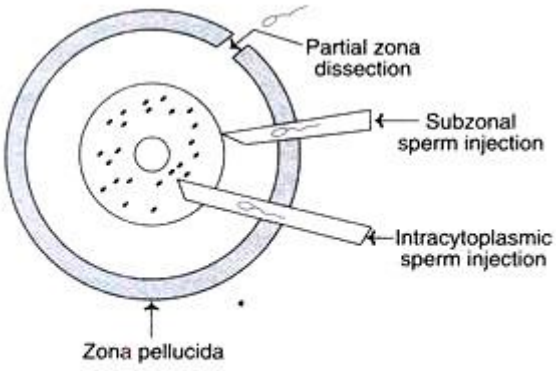


Fig. 18.3 : Micromanipulation for fertilization of an egg (microsurgically assisted fertilization).

Intra-Cytoplasmic Sperm Injection (ICSI):

Intra-cytoplasmic sperm injection is a new and novel infertility treatment utilizing the micromanipulation technology. Many of the previous treatment processes for male infertility have been abandoned in favour of ICSI. The male factor infertility could be due to low sperm counts, poor sperm motility, and poor quality of sperm to penetrate oocyte.

By partial zona dissection (PZD), the zona pellucida is opened using either chemical dissolution or a sharp instrument. A single spermatozoon can be directly injected into the cytoplasm of the oocyte through the micro-puncture of zona pellucida. A micropipette is used to hold the oocyte while the spermatozoon is deposited inside the ooplasm of the oocyte. Besides using normal sperms, round-headed sperms, sperms collected directly from the epididymis and previously cryopreserved sperms can be used in ICSI.

Among the micromanipulation techniques ICSI is the most successful one with a fertilization rate of about 65%. Attempts are on to improve this further. In fact, ICSI has revolutionized assistant reproductive technology by utilizing the sperms of husbands who were once considered to be unsuitable for fertilization process.

Sub-zonal Insertion (SUZI):

In sub-zonal insertion, the zona pellucida is punctured and sperms (1-30 in number) are injected into an area between the zona and the egg. It is expected that one of the sperms will fertilize the egg. The major limitation of SUZI is polyspermy since it is not possible to control the number of sperms that enter the egg.

Round Spermid nucleus Injection (ROSNI):

There are a few men who cannot manufacture sperms, and therefore they have a zero sperm count. For these men, it is possible to take out the round spermatids (immature cells) directly from the testicle, isolate the nucleus (containing the genetic material) and inject it into the partner's eggs. ROSNI is a recent exciting breakthrough to solve the problem of male infertility through micromanipulation.

Cryopreservation:

Preservation in a frozen state is regarded as cryopreservation. Cryopreservation is very useful in assisted reproductive technology.

i. Semen can be cryopreserved. This may be from the donors, cancer patients (before the commencement of treatment).

ii. Fertilized eggs after IVP or ICSI can be preserved.

iii. Embryos can also be preserved for transfer at a later stage.

Human embryos have been successfully preserved in the presence of cryoprotectants (1, 2-propanediol/dimethyl sulfoxide/glycerol) and stored at -196°C under liquid nitrogen. At appropriate time, the embryos are thawed, cryoprotectants removed and then transferred. Many test tube babies in fact have been born as a result of application of freezing technology.

Cryopreservation of semen:

Semen cryopreservation (commonly called **sperm banking or sperm freezing**) is a procedure to preserve sperm cells. Semen can be used successfully indefinitely after cryopreservation. For human sperm, the longest reported successful storage is 24 years. It can be used for sperm donation where the recipient wants the treatment in a different time or place, or as a means of preserving fertility for men undergoing vasectomy or treatments that may compromise their fertility, such as chemotherapy, radiation therapy or surgery.

Freezing

The most common cryoprotectant used for semen is glycerol (10% in culture medium). Often sucrose or other di-, trisaccharides are added to glycerol solution. Cryoprotectant media may be supplemented with either egg yolk or soy lecithin, with the two having no statistically significant differences compared to each other regarding motility, morphology, ability to bind to hyaluronate in vitro, or DNA integrity after thawing.

Additional cryoprotectants can be used to increase sperm viability and fertility rates post-freezing. Treatment of sperm with heparin binding proteins prior to cryopreservation showed decreased cryoinjury and generation of ROS. The addition of nerve growth factor as a cryoprotectant decreases sperm cell death rates and increased motility after thawing. Incorporation of cholesterol into sperm cell membranes with the use of cyclodextrins prior to freezing also increases sperm viability.

Semen is frozen using either a controlled-rate, slow-cooling method (slow programmable freezing or SPF) or a newer flash-freezing process known as vitrification. Vitrification gives superior post-thaw motility and cryosurvival than *slow programmable freezing*.

Thawing :

Thawing at 40 °C seems to result in optimal sperm motility. On the other hand, the exact thawing temperature seems to have only minor effect on sperm viability, acrosomal status, ATP content, and DNA. As with freezing, various techniques have been developed for the thawing process, both discussed by Di Santo et al. (2012)

Refreezing :

In terms of the level of sperm DNA fragmentation, up to three cycles of freezing and thawing can be performed without causing a level of risk significantly higher than following a single cycle of freezing and thawing. This is provided that samples are refrozen in their original cryoprotectant and are not going through sperm washing or other alteration in between, and provided that they are separated by density gradient centrifugation or swim-up before use in assisted reproduction technology.

Effect on quality :

Some evidence suggests an increase in single-strand breaks, condensation and fragmentation of DNA in sperm after cryopreservation. This can potentially increase the risk of mutations in offspring DNA. Antioxidants and the use of well-controlled cooling regimes could potentially improve outcomes. In long-term follow-up studies, no evidence has been found either of an increase in birth defects or chromosomal abnormalities in people conceived from cryopreserved sperm compared with the general population.

Cryopreservation of embryo:

Cryopreservation of embryos is the process of preserving an embryo at sub-zero temperatures, generally at an embryogenesis stage corresponding to pre-implantation, that is, from fertilisation to the blastocyst stage.

Indications:

Embryo cryopreservation is useful for leftover embryos after a cycle of in vitro fertilisation, as patients who fail to conceive may become pregnant using such embryos without having to go through a full IVF cycle. Or, if pregnancy occurred, they could return later for another pregnancy. Spare oocytes or embryos resulting from fertility treatments may be used for oocyte donation or embryo donation to another woman or couple, and embryos may be created, frozen and stored specifically for transfer and donation by using donor eggs and sperm.

Method:

Embryo cryopreservation is generally performed as a component of in vitro fertilization (which generally also includes ovarian hyperstimulation, egg retrieval and embryo transfer). The ovarian hyperstimulation is preferably done by using a GnRH agonist rather than human chorionic gonadotrophin (hCG) for final oocyte maturation, since it decreases the risk of ovarian hyperstimulation syndrome with no evidence of a difference in live birth rate (in contrast to fresh cycles where usage of GnRH agonist has a lower live birth rate).

The main techniques used for embryo cryopreservation are vitrification versus slow programmable freezing (SPF). Studies indicate that vitrification is superior or equal to SPF in terms of survival and implantation rates. Vitrification appears to result in decreased risk of DNA damage than slow freezing.

Direct Frozen Embryo Transfer: Embryos can be frozen by SPF in ethylene glycol freeze media and transfer directly to recipients immediately after water thawing without laboratory thawing process. The world's first crossbred bovine embryo transfer calf under tropical conditions was produced by such technique on 23 June 1996 by Dr. Binoy S Vettical of Kerala Livestock Development Board, Mattupatti

Prevalence

World usage data is hard to come by but it was reported in a study of 23 countries that almost 42,000 frozen human embryo transfers were performed during 2001 in Europe.

Pregnancy outcome and determinants

In current state of the art, early embryos having undergone cryopreservation implant at the same rate as equivalent fresh counterparts. The outcome from using cryopreserved embryos has uniformly been positive with no increase in birth defects or development abnormalities, also between fresh versus frozen eggs used for intracytoplasmic sperm injection (ICSI). In fact, pregnancy rates are increased following frozen embryo transfer, and perinatal outcomes are less affected, compared to embryo transfer in the same cycle as ovarian hyperstimulation was performed. The endometrium is believed to not be optimally prepared for implantation following ovarian hyperstimulation, and therefore frozen embryo transfer avails for a separate cycle to focus on optimizing the chances of successful implantation. Children born from vitrified blastocysts have significantly higher birth weight than those born from non-frozen blastocysts. For early cleavage embryos, frozen ones appear to have at least as good obstetric outcome, measured as preterm birth and low birth weight for children born after cryopreservation as compared with children born after fresh cycles. Oocyte age, survival proportion, and number of transferred embryos are predictors of pregnancy outcome.

Pregnancies have been reported from embryos stored for 16 years. A study of more than 11,000 cryopreserved human embryos showed no significant effect of storage time on post-thaw survival for IVF or oocyte donation cycles, or for embryos frozen at the pronuclear or cleavage stages.[11] In addition, the duration of storage had no significant effect on clinical pregnancy, miscarriage, implantation, or live birth rate, whether from IVF or oocyte donation cycles. A study in France between 1999 and 2011 came to the result that embryo freezing before administration of gonadotoxic chemotherapy agents to females caused a delay of treatment in 34% of cases, and a live birth in 27% of surviving cases who wanted to become pregnant, with the follow-up time varying between 1 and 13 years.

Assisted Hatching (AH):

Improper implantation of the embryo in the uterus is one of the limiting factors in the success of ART in humans. Assisted hatching is a novel approach for the proper implantation of the embryo in the endrometrium. The embryos in the uterus possess an outer coating namely zona pellucida (the shell). These embryos must be hatched to remove the shell, a step necessary for implantation. In certain women, particularly above 40 years age, natural hatching does not occur, and requires outside assistance.

Assisted hatching is carried out by using a Laser to make a small hole in the shell of the embryo. These embryos when transferred into the uterus hatch and get implanted. During the course of AH for 3-4 days, the women are kept on steroids (to suppress mother's immunity) and antibiotics (to counter infections). Better results are reported with this approach.

Pre-implantation Genetic Diagnosis (PGD):

The genetic defects in ovum before fertilization or in the embryo before implantation can be identified by a new medical tool namely pre-implantation genetic diagnosis. It is estimated that about 60% of the ART driven pregnancies are lost due to chromosomal abnormalities. This can be minimized or prevented by using PGD.

A direct determination of chromosomal abnormalities prior to implantation ensures a successful pregnancy and ultimate delivery of a healthy baby. One group of workers has reported an increase in the pregnancy rate from 15 to 30% by employing pre-implantation genetic diagnosis.DNA Amplification and Analysis:

The latest in PGD is the direct DNA analysis. This can be carried out by removing a single cell from 6-8-cell embryo. The DNA is removed and amplified by employing polymerase chain reaction. Direct DNA analysis is useful for the diagnosis of several genetic diseases e.g. cystic fibrosis, sickle-cell anaemia, haemophilia, Duchene's muscular dystrophy, Tay-Sachs disease.

Ethical Advantages of PGD:

PGD is highly advantageous from the ethical point of view, since the embryos with genetic disorders can be discarded in the very stages without the formation of offspring's with undesirable characteristics

The Negative Aspects of Art:

There are certain limitations/disadvantages associated with assisted reproductive technology in humans. Some highlights are given. It must however, be noted that the advantages of ART outweigh the disadvantages.

Ovarian Hyper-stimulation Syndrome (OHSS):

Due to administration of hormones and drugs, ovarian hyper-stimulation is frequently associated with complications, sometimes even life- threatening. OHSS is more severe in women who conceived in the same cycle, and received hCG as luteal support (following embryo transfer).

Risks Associated with Pregnancy:

ART is associated with multiple pregnancy, increased risk for anemia, gestational diabetes and premature labour. Low birth weight and prematurity are closed linked with mortality and morbidity.

Premature Menopause:

Controlled ovarian hyper-stimulation (COH) causes multiple follicular utility. There is a risk of premature menopause as COH may reduce the ovarian follicles, besides faster aging. Sometimes, a single COH may use ovarian follicles, which in the normal course are equivalent to two years of ovulation during the natural menstrual cycle.

Ovarian Cancer:

The use of fertility drugs and injuries to epithelium increase the risk of ovarian cancer at least by three times when compared to normal women.

Probable Questions:

1. Define in vitro fertilization. Why it is necessary?
2. Describe different steps of IVF?
3. What is cryopreservation? What is cryoprotectant? Give examples.
4. Describe intrauterine insemination.
5. When IVF is chosen?
6. Describe in brief Gamete Intra-Fallopian Transfer (GIFT).
7. Describe in brief Zygote Intra-Fallopian Transfer (ZIFT).
8. What are the two methods of Cytoplasmic transfer? Describe any one process.
9. Describe Intra-Cytoplasmic Sperm Injection (ICSI).
10. How cryopreservation of embryo is done?
11. What is the importance of Pre-implantation Genetic Diagnosis (PGD).

Suggested readings:

1. Biotechnology by P.K. Gupta
2. Gene Cloning by T. Brown.
3. Biotechnology by N. Kumarsen.
4. Biotechnology by B.D. Singh.

UNIT-IV

An overview of cloning techniques

Objective: In this unit we will discuss different techniques related to cloning

Introduction:

Gene cloning involves separation of specific gene or DNA fragments from a donor cell, attaching it to small carrier molecule called vector and then replicating this recombinant vector into a host cell.

The procedure consists of following steps:

a. Isolation of DNA to be Cloned:

The DNA of interest, i.e., target DNA may be genomic DNA or complementary DNA or synthetic DNA. The genomic DNA of interest if contained in a particular restriction fragment, that can be isolated from gel after electrophoresis.

Otherwise, a complementary DNA (cDNA) fragment is prepared directly by using mRNA as template. The polyadenylated mRNAs are separated from other types of RNAs through affinity column chromatography. These mRNAs are then copied to cDNAs with the help of reverse transcriptase. In these cases as the cDNA is obtained from mRNA, so it must contain the uninterrupted coding sequence of gene and the recombinant DNA molecule will synthesize the eukaryotic gene product in prokaryotic cell. One can also synthesize the desired DNA fragment by machine.

b. Insertion of Foreign DNA Fragment into a Vector:

The cDNA thus isolated above or obtained from gene bank is fragmented by using the specific restriction enzyme to develop specific cohesive ends. The cloning vector is also treated with the same restriction enzyme, so that the cohesive ends are generated (Fig. 18.5).

For insertion of double stranded cDNA into a cloning vector, it is necessary to add to both termini single stranded DNA sequence which should be complementary to a tract of DNA at the termini of linearized vector. In order to get efficient formation of recombinant DNA molecules, addition of sticky ends on both termini is necessary.

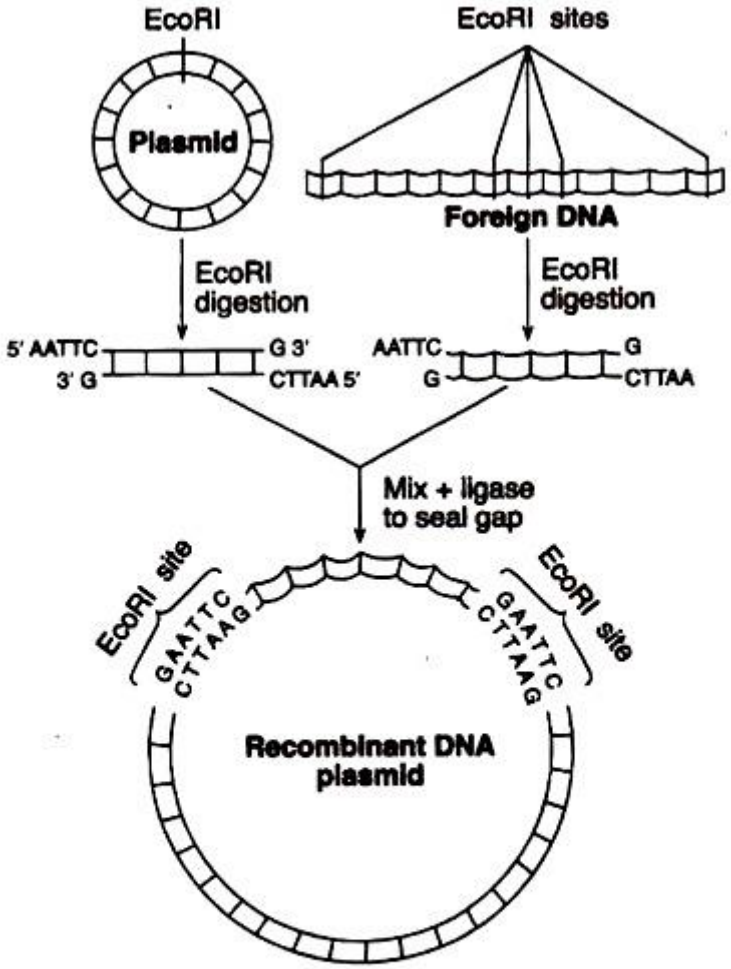


Fig. 18.5: Construction of a recombinant DNA plasmid through the use of the restriction enzyme Eco RI. The Eco RI cuts both the plasmid and the foreign DNA and by mixing, we get recombinant DNA plasmid (after Russel, 1987)

There are two methods for generation of cohesive ends on the double stranded cDNA:

(i) Use of linkers

(ii) Homopolymer tails.

Linkers are the chemically synthesized double stranded DNA oligonucleotides containing on it one or more restriction sites for cleavage by restriction enzymes (Fig. 18.6).

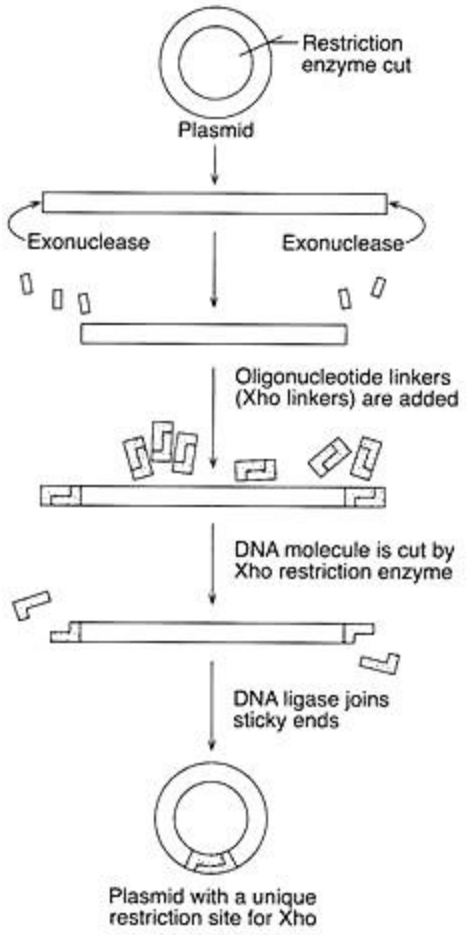


Fig. 18.6: Addition of a "linker" (carrying a restriction site) to a vector molecule

Linkers are ligated to blunt end by T4-DNA ligase. Using terminal transferase the synthesis of homopolymer tails of the defined length at both 3′ termini of double stranded DNA and vector is possible. If the poly- T tail is added at the termini of foreign DNA, then poly-A tail is added at the restriction site of the vector, so that the complementary sticky ends are formed and they get annealed by T4-DNA ligase (Fig. 18.7).

c. Transfer of Recombinant DNA into Bacterial Cell:

Before the recombinant DNA can be bulked up by cloning, it must be taken up by a suitable bacterial host cell, which is then said to be transformed, i.e., a host bacterial cell must accept the plasmid with the foreign gene, get it incorporated into its genome and start transcribing that gene.

The event of entering the plasmid with foreign DNA into the cell is known as **"transformation"**. A mild heat shock is given to the mixture which results in the uptake at higher frequency of the DNA. The selection of transformed cells is done by allowing the bacteria to grow in antibiotic selection medium.

Cloning in Eukaryotes:

In eukaryotes the nucleus is separated from the rest of cell through nuclear membrane, many of the genes are split genes with exons and introns. As such genetic engineering with eukaryotes needs special methods.

When eukaryotic genes are cloned in prokaryotes, the split genes cannot be correctly expressed, because prokaryotes do not have the machinery for splicing out the RNA transcribed from the introns of a gene. So the eukaryotic cells are needed for cloning and expression of cloned eukaryotic genes.

Among eukaryotes, DNA cloning has been done in yeast, mouse and in higher plant species. In yeast, a 2μ plasmid DNA is an appropriate cloning vehicle, which can be transferred through efficient transformation method. This involves protoplast production followed by PEG directed introduction of DNA into protoplasts.

d. Detection of Recombinant Clone:

From the large number of colonies produced by transformation to select or screen out the few colonies which contain the recombinant plasmid — the use of antibiotics is one of the most easy and useful methods for this purpose. The transformed cells can be plated on selection medium containing different antibiotics. The colonies which grow, can be said to have a plasmid, as the antibiotic resistance gene of plasmid enables the bacteria to grow. For example, the plasmid pBR 322 contains genes for ampicillin resistance (amp^r) and tetracycline resistance (tet^r). Thus the trans-formants can be detected by their plating potential on medium containing either (or both) of these antibiotics**.**

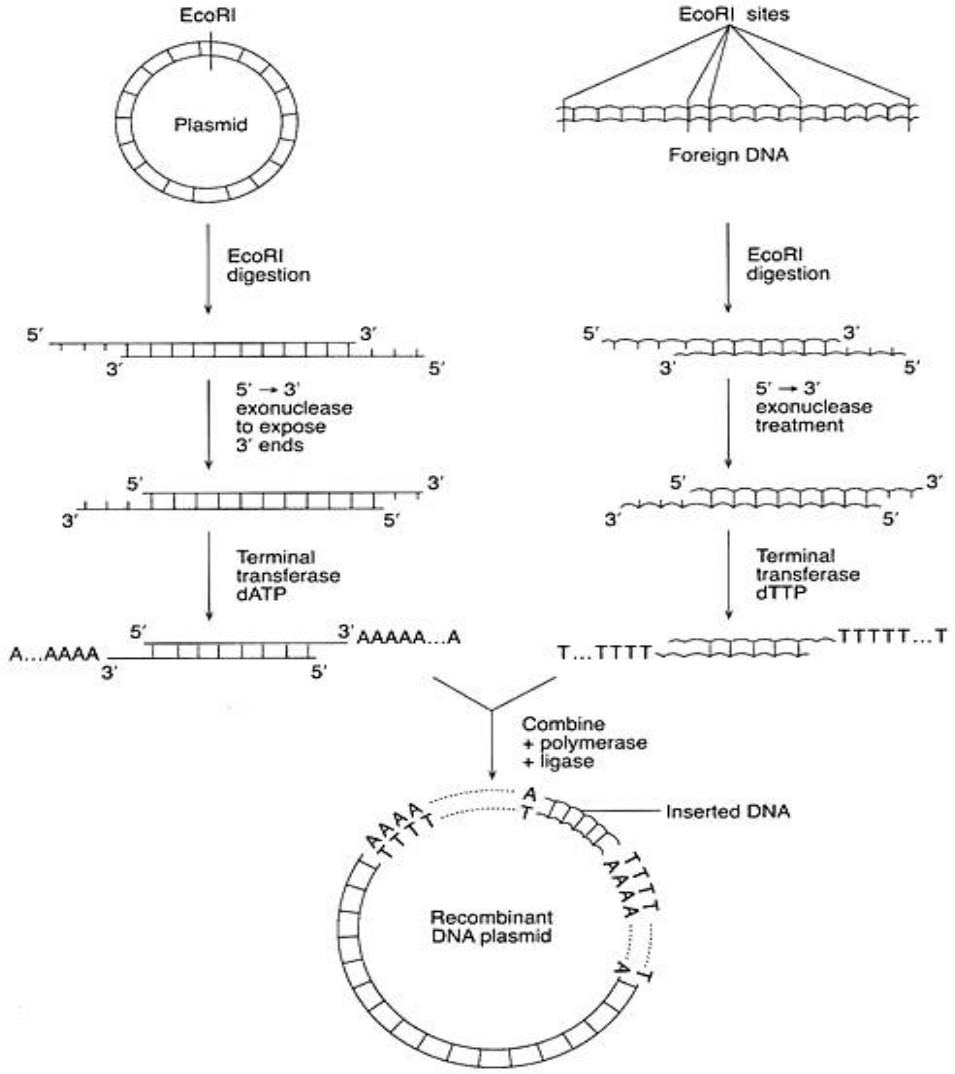


Fig. 18.7: Construction of a recombinant DNA plasmid using the enzyme terminal transferase to synthesize complementary ends on the linearized plasmid and a restriction enzyme generated fragment of foreign DNA (after Russel, 1987)

The presence of cloned DNA fragments can be detected by insertional inactivation of suitable genetic system. For example, DNA fragment of interest can be inserted into one of the antibiotic- resistance genes (tet^r) of pBR322, inactivating that gene (tet^s) and other remains active (amp^r).

To selectively kill cells with antibiotics, the original master plate with ampicillin in medium is subjected to replica plating method with both ampicillin and tetracycline. Bacteria with recombinant plasmid do not grow on replica plate, only with non-recombinant plasmid will grow. Recombinant colonies are thus identified and selected from master plate (Fig. 18.8).

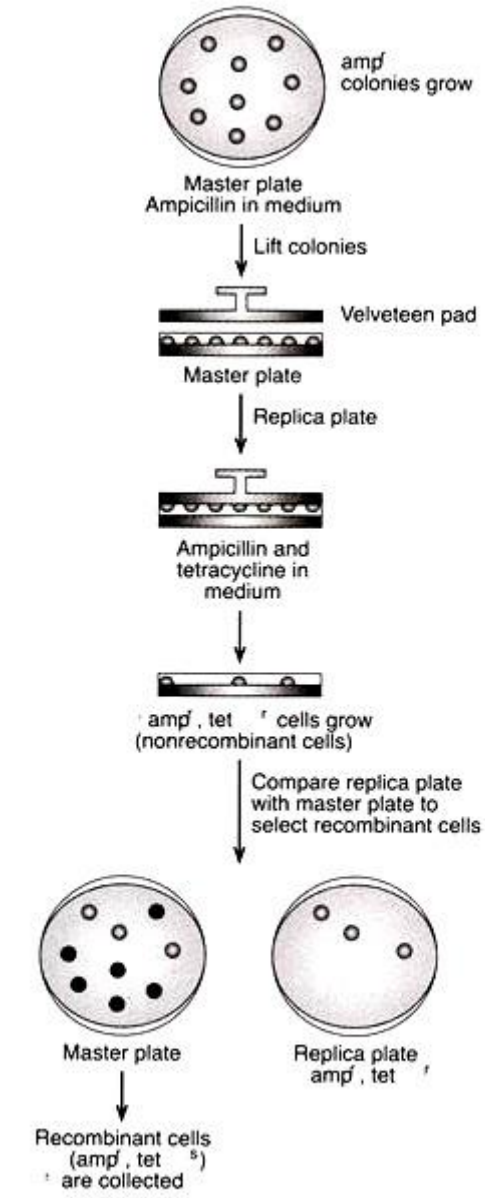


Fig. 18.8: Selecting for recombinant cells after transformation (after Barnum)

The detection of recombinant clones can also be done by using chromogenic substrates. The most popular system uses X-gal, a colourless substrate on cleavage by β-galactosidase, a blue coloured product is formed, then the expression of lac Z gene can be detected easily.

Host cells that are Lac^- are used, so that the Lac^+ phenotype will only arise when the vector is present. Furthermore, if a DNA fragment is cloned into lac Z gene (Eco RI site of Charon 16A), any recombinants will be lac Z and therefore will not produce β-galactosidase and plaques will remain colourless in presence of X-gal.

The Vectors which are used in Gene cloning are discussed below:

1. Plasmid Vectors:

Plasmids are the extra chromosomal genetic elements commonly found in bacteria and are mostly made of double-stranded circular DNA. They are used as vectors in gene cloning, because they have a replication origin in their DNA making them competent of autonomous replication, and also because they generally have one or two restriction sites for several restriction enzymes.

Many plasmids have been genetically engineered to add useful properties. The number of copies of plasmids may vary from one to several per host cell. Some plasmids under relaxed replication control can have larger number of copies which may be sometimes as high as 1,000. Such high copy-number plasmids are specially suitable for cloning.A commonly used small cloning vector is the plasmid pBR 322 which has a circular double- stranded DNA having 4,363 base pairs, compared to the E. coli chromosome having about 4,700 x 10^3 base pairs. It carries single restriction sites for the restriction enzymes EcoR1, Hind III, Bam HI and Sal I. Thus, each of these enzymes can make single cleavages at their respective sites of pBR 322 DNA, where the foreign DNA can be inserted.

Many plasmids also have genes for antibiotic resistance which can be profitably used as selectable markers. For example, pBR322 has two genes specifying resistance to ampicillin (amp^r) and tetracycline (tet^r). Presence of two such marker genes is more advantageous than a single marker, provided one of the marker genes has a restriction site within it. When a foreign DNA fragment is inserted within the resistance gene, it results in the inactivation of the resistance gene, just as a transposon causes inactivation of a gene.

Such inactivation of an antibiotic resistance gene makes the host ceil in which the plasmid is present susceptible to the particular antibiotic. This property can be utilized for identification and selection of the host cells in which the foreign gene has been cloned. For example, pBR 322 possesses a restriction site for Hind III in the tet^r gene.When a foreign gene is cloned using Hind III in pBR 322, tet^r gene is inactivated and the host cells containing the recombinant plasmid show resistance to ampicillin because the amp' gene is intact, but not to tetracycline. This makes possible the identification of the host cells containing the cloned foreign gene, because they cannot grow on a medium in which tetracycline has been incorporated at an inhibitory concentration. Other host cells which take up only the vector DNA without the cloned gene will grow in such a medium, because their tet^r gene is intact.

Naturally occurring plasmids do not usually possess all the desirable properties which are useful for their use as cloning vectors. So they must be suitably altered by molecular biological techniques. For example, the plasmid pBR 322 was developed by several alternations. Other plasmids which are used as cloning vectors include pSC 101, pUC 8, pHC 79 etc. (p stands for plasmid).A very useful plasmid used as a vector for introducing

foreign genes into plants is the Ti-plasmid of a plant pathogenic bacterium, Agrobacterium tumefaciens which infects many dicotyledonous plants causing a disease, known as crown-gall. When this organism infects a host plant, it transfers naturally a portion of the Ti-plasmid into the plant. This portion is known T-DNA. The T-DNA segment has been extensively altered by molecular biological methods to make the Ti-plasmid suitable as a vector for inserting foreign DNA into many plants to produce transgenic plants.

2. Bacteriophage Vectors:

The most commonly used bacteriophage vector is the λ-(lambda) phage of *E. coli*.The λ-DNA after it infects a host cell can either enter into a lysogenic state by integration into the host DNA, or can lead to a lytic cycle producing progeny phages. In the phage head, the DNA is present as a linear double-stranded helix.

After entering into the host cell, the molecule forms a covalently closed circular DNA with the help of single-stranded ends having complimentary base sequences (cos-sites). The λ-genome is 48.5 x 10^3 base-pair long and has a single restriction site for Eco R1. To be used as a cloning vector, the λ-DNA requires to be processed by genetical engineering techniques, so that it retains the genes necessary for carrying out the lytic cycle only and the Eco R1 recognition sequence.
The central portion of the genome which is not essential for the lytic cycle is removed to accommodate the inserted foreign DNA into λ-DNA, so that the recombinant DNA has the appropriate length to be packaged into the phage head.

One problem with the phage vectors is the length of the foreign DNA that is to be inserted. If the length of the insert is too long or too short, the recombinant DNA cannot be packaged into the phage head. The insert in case of λ-DNA should be about 10 to 20 kb, preferably 15 kb long. After the λ-DNA is processed, its length becomes too short to be packaged into the phage head.The insertion of a foreign DNA having an appropriate length (-15 kb) brings back the length to an appropriate size which can be packaged into a λ -head. The recombinant phage can then be used for propagation in E. coli yielding large number of phage particles which carry the recombinant DNA.

The insertion of a foreign DNA fragment of appropriate length which carries a gene of choice is carried out by treatment of both insert and processed λ-DNA with Eco R1. The foreign DNA, with its sticky-ends, forms base pairs with those of λ-DNA fragments to yield a recombinant DNA. The recombinant molecule is then packaged into a phage head. The phage on infection produces a circular DNA and multiplies producing a lytic cycle. From the progeny phage particles, recombinant DNA can be isolated (Fig. 9.131).For cloning larger DNA molecules, i.e. larger than 20 kb, the bacteriophage PI of E. coli can be used. This phage is also a temperate one like λ-phage, but it has a DNA genome of about 100 kb, i.e. double of λ-phage. Of its 100 kb genome, only about 15 kb is essential for replication in the host. So, a processed vector prepared from phage PI

can be used for cloning much larger DNA fragments of 80 to 85 kb. The phage PI DNA is a circular molecule. The general procedure of inserting a foreign DNA into P1 vector is similar to that of λ -DNA.

3. Cosmid Vectors:

Cosmids are hybrids of plasmids and λ -phage. The cos-sites of λ-DNA are joined to a plasmid, like Col El, to produce a typical cosmid. A cosmid is generally a circular DNA molecule containing its own replication origin sequence, a selectable marker gene — like one coding for antibiotic resistance — one or two restriction sites and the cos-site of X-DNA. The cos-site helps in circularization of the cosmid DNA. The cosmid derived from Col El has a rifampicin-resistance marker (rif^r), two restriction sites for Hind III and the cos-site of λ-phage (Fig. 9.132).

Cosmids are suitable for cloning larger molecules of DNA. On the average, DNA fragments having a size of 35 to 45 kb can be inserted into a cosmid, whereas, in plasmids, the insert size is between 5 kb to 10 kb, and, in λ-vectors, the insert size is usually about 15 kb.

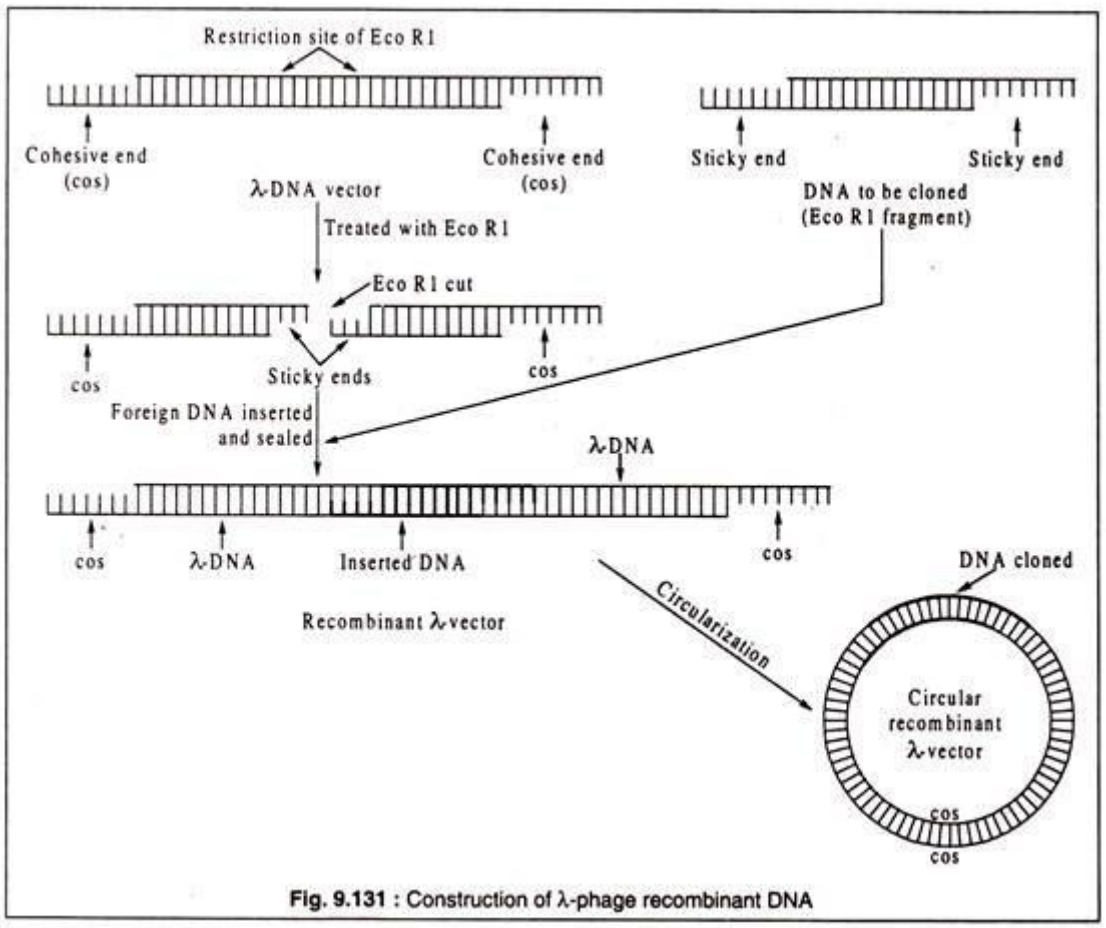


Fig. 9.131 : Construction of λ-phage recombinant DNA

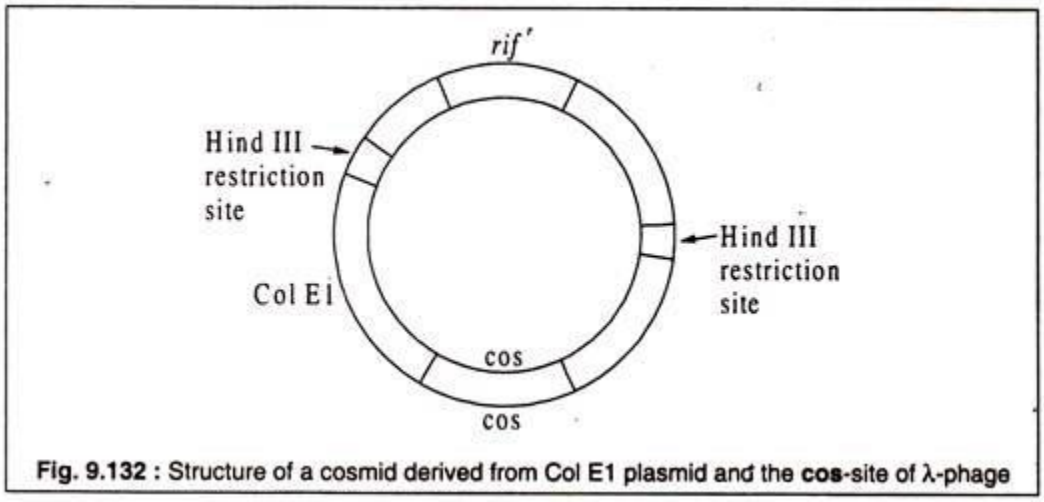


Fig. 9.132 : Structure of a cosmid derived from Col E1 plasmid and the **cos**-site of λ-phage

Procedure for Cloning Recombinant DNA

The first step in a gene cloning programme is to construct a recombinant DNA molecule containing a donor DNA segment in which a selected gene is located and a vector DNA. The donor may belong to any taxonomic group. The DNA of the donor is cleaved into fragments using any of the many restriction enzymes. The vector DNA has also to be cleaved by the same enzyme, so that both DNAs have similar sticky ends. When the donor fragments are mixed with vector fragments, the single-stranded sticky-ends form base-pairs. The free ends are then joined by DNA ligase to obtain a recombinant DNA molecule as shown in Fig. 9.130 and Fig. 9.131 using two different types of vectors.

Although joining of two DNA fragments obtained by digesting them with the same restriction enzyme is not difficult, identification of a recombinant DNA containing a specific donor gene is by no means so simple. Because, the donor DNA is often a very large molecule containing many restriction sites of a single restriction enzyme.

As a result, restriction digest consists of many fragments, only one or a few of these fragments include the gene or parts of the gene to be cloned. The insertion of these fragments into vector yields recombinant DNA, of many sorts, only a few of which contain the desired gene. In a cloning experiment it becomes essential to identify the particular recombinant molecules in which the gene is present. A straight-forward way of doing this is to identify the gene in a clone by its product. Other methods involve use of a purified DNA containing the gene of choice for insertion into the vector to prepare the recombinant molecule. The next step is the introduction of the recombinant DNA into a suitable host. This depends on the nature of vector chosen. For insertion of comparatively small DNA fragments, the vector of choice is one of the plasmids which has been suitably tailored for the purpose.

The recombinant circular plasmid vector is introduced into its bacterial host, mostly E. coli, by forced transformation, because E. coli is not normally transformable. The

bacteria which have taken up the recombinant plasmid can be identified with suitable markers. For example, if the plasmid contains a gene conferring resistance to say ampicillin, only those transformed bacteria containing the plasmid can grow in an ampicillin-containing agar and the non-transformed bacteria are eliminated. This is only a preliminary selection. Because of reasons, most of the recombinant plasmids do not have the desired donor gene. For further screening, the technique of colony hybridization is often adopted to identify the bacterial clones with the desired gene.

The cloning procedure using a plasmid vector is shown in a simplified diagrammatic manner in Fig. 9.133:

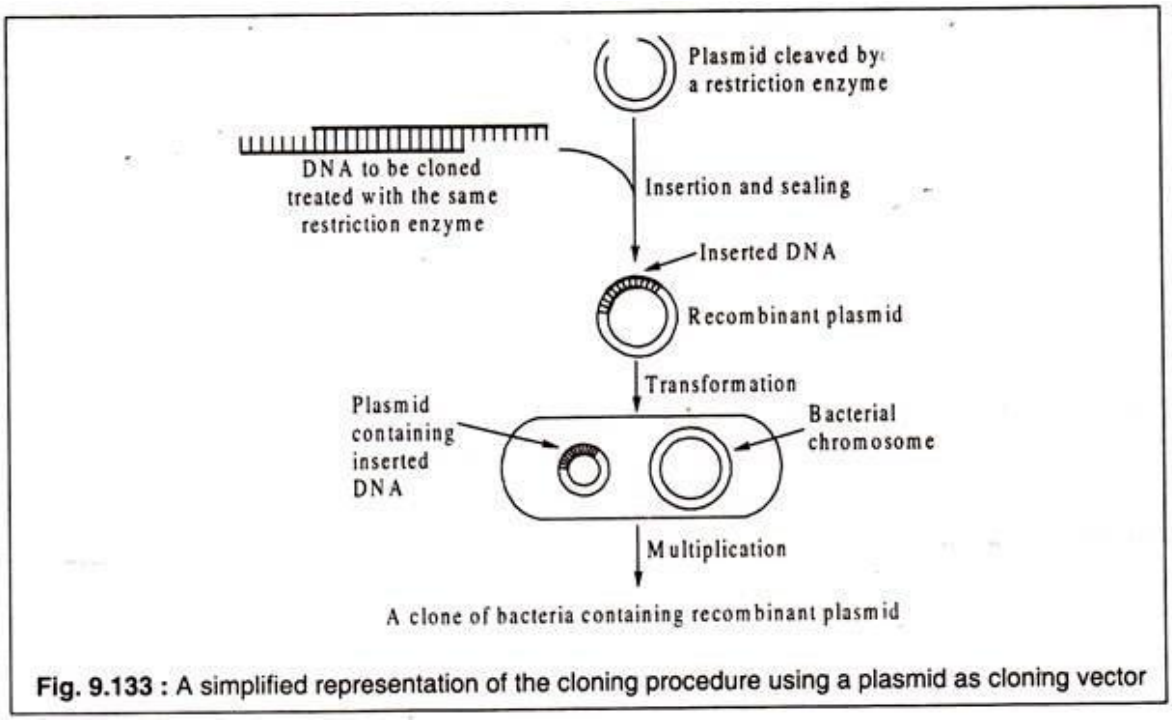


Fig. 9.133 : A simplified representation of the cloning procedure using a plasmid as cloning vector

When bacteriophage vectors are used for cloning, the recombinant DNA obtained by joining the DNA segment of the donor and the processed phage DNA is packaged into the phage head. The recombinant phage particles are allowed to infect appropriate host bacteria by the natural infection process.

The recombinant DNA of the phage multiplies producing large number of progeny phage particles which are released by lysis of the host cells. Recombinant DNA molecules can be isolated from the progeny phage particles for experimental purpose.

The procedure is shown in Fig. 9.134:

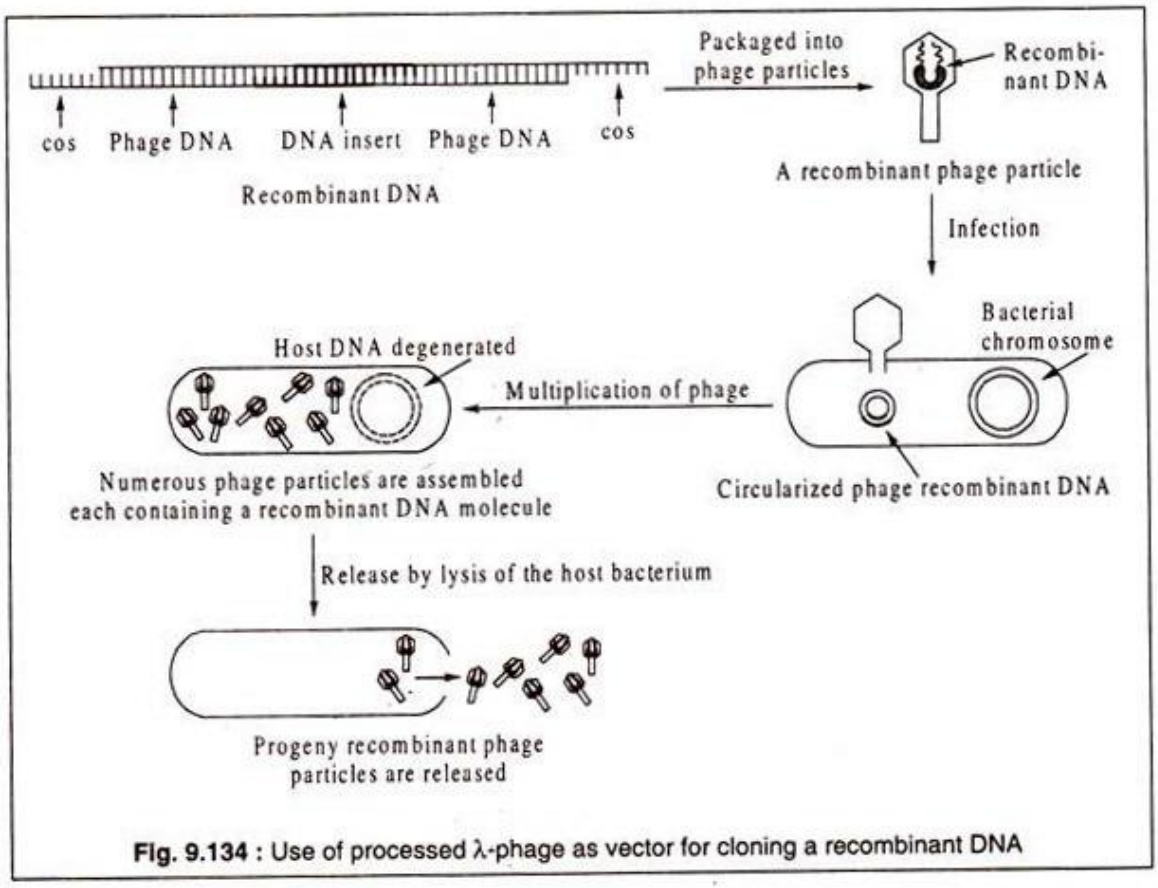


Fig. 9.134 : Use of processed λ-phage as vector for cloning a recombinant DNA

The cloning procedure using cosmid vectors is more or less similar to that of phage vectors, except that they are capable of inserting larger DNA fragments of about 40 kb size. Another important distinguishing feature of cosmid vectors is that they multiply in infected hosts as plasmids and do not form phage progeny. Therefore, the question of lysis does not arise.

All the three types of vectors — plasmids, phage and cosmids — are used for cloning recombinant DNA in bacterial hosts, mainly E. coli.

Some- vectors have been developed by genetic engineering techniques which can exist in two different hosts. These are called shuttle vectors. A vector of this type is YEp 24 which can replicate in both yeast and E. coli. These vectors contain sequences of an E. coli plasmid and a part of the yeast genome. Each part has its own replication origin and some other genes as well as restriction sites. A structure of a shuttle vector of yeast and E. coli is shown in Fig. 9.135.

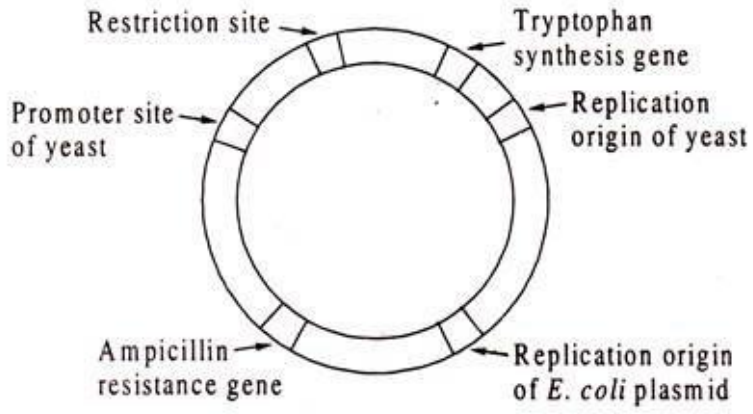


Fig. 9.135 : The structure of a circular yeast *E. coli* YE_p shuttle vector. The vector contains a part of an *E. coli* plasmid including its replication origin and an antibiotic resistance marker (ampicillin resistance), and a part of yeast genome including a promoter sequence, replication origin, a restriction enzyme recognition sequence and a marker gene, like one coding for tryptophan synthesis

Such shuttle vectors have proved useful in cloning eukaryotic genes like mammalian genes. One problem of cloning eukaryotic genes in prokaryotes is that the bacterial RNA polymerase may fail to initiate transcription of the eukaryotic gene transferred into a bacterial host, because of its inability to interact with the eukaryotic promoter.

Another problem is due to the presence of introns in the eukaryotic primary transcripts (hn-RNA) which the bacteria are unable to remove for they do not have the biochemical machinery. The first problem can be effectively solved by coupling the eukaryotic gene next to a bacterial promoter. As E. coli is selected generally as the host, its lac-promoter is often used for this purpose. The second problem of removal of introns can be solved, if the eukaryotic gene to be cloned is made free of the intron segments before its joining to the vector DNA. This can be achieved indirectly by preparing an eukaryotic gene from its m-RNA using reverse transcriptase. The processed form of m-RNA which is translated to yield the gene product is devoid of introns. A complimentary copy of m-RNA produced by reverse transcription gives a DNA without introns. Such a DNA, known as complementary DNA (c-DNA) can then be used for obtaining a recombinant DNA by joining with a suitable vector.

Probable Questions:

1. Describe different steps of gene cloning with suitable diagram.
2. What is the difference between adaptor and linker?
3. What is homopolymer tailing?
4. How recombinant cells are detected?
5. What are the characteristics of a ideal vector?
6. Describe the role of plasmid as vector.
7. What is the difference between cloning vector and expression vector?
8. What is shuttle vector?
9. Describe the role of cosmids as vector.
10. Describe the role of bacteriophage as vector.

Suggested Readings:

1. Biotechnology by P.K. Gupta
2. Gene Cloning by T. Brown.
3. Biotechnology by N. Kumarsen.
4. Biotechnology by B.D. Singh

UNIT-V

Nuclear transfer technology

Objective: In this unit we will discuss about nuclear transfer technology

Introduction: The development of a single celled fertilized zygote to an animal capable of reproduction involves not only cell division but the differentiation or specialization to numerous cell types forming each tissue and organ of the adult animal. The technique of nuclear transfer allows the reconstruction of an embryo by the transfer of genetic material from a single donor cell, to an unfertilized egg from which the genetic material has been removed. Successful development of live offspring from such embryos demonstrates that the differentiated state of the donor nucleus is not fixed and can be reprogrammed by the egg cytoplasm to control embryo and fetal development. Nuclear transfer has many applications in agriculture and human medicine. This article will review some of the factors associated with the success of embryo development following nuclear transfer and outline the potential uses of the technology.

In animals reproduction occurs by sexual means; fertilization of the female egg by the male-derived sperm results in the production of a single cell or zygote, which begins development and results in the production of offspring. The genome or genetic information present in the majority of the cells of the body consists of two sets of genes, one contributed by the sperm (the paternal genome) and the other by the egg (the maternal genome). During the development of egg and sperm cells the genetic information is rearranged by the process of meiosis, and thus each fertilized zygote contains a unique genome and results in the formation of a unique individual. During development the zygote has to grow and divide; with each division the genome is copied and each cell inherits a copy of this novel genome which is located within the nucleus (also referred to as nuclear or chromosomal DNA). In contrast, a clone is defined as a population of cells or organisms derived from a single cell or organism through the process of asexual reproduction. Under natural circumstances, the occurrence of 'clones' in vertebrates is restricted to the production of identical twins as a result of the division of a single embryo to form two identical but separate individuals (also termed monozygotic, derived from a single zygote). In some species division of the embryo may result in the formation of more than two clones (e.g. in the Echinida). Such 'splitting' of embryos may also be carried out experimentally in the laboratory to produce identical offspring.

In contrast to the 'splitting' of embryos, Dolly was produced by a technique known as nuclear transfer. In this process the genetic material is removed from an unfertilized

egg and replaced with the nuclear genetic material taken from a cell at a later developmental stage or, as in the case of Dolly, a cell derived from an adult animal. No fertilization occurs and therefore there is neither maternal nor paternal DNA present in the resulting zygote. In normal sexual reproduction the egg and the sperm are not equal contributors; although both contribute genetic information, the egg contains numerous other factors essential for development. In particular, the egg contains extrachromosomal DNA located in the mitochondria; these intracellular organelles are inherited primarily through the maternal line. In the true sense of the word the animals produced by nuclear transfer are not 'clones' as eggs used for the process are obtained from different females and therefore maternally inherited factors (i.e. mitochondria) will differ between the resultant offspring. Although the offspring are generated by asexual means, cell duplication or splitting did not occur and therefore may be more aptly described as 'genomic copies'.

Background to the development of 'cloning':

As described, the product of sexual reproduction the zygote inherits a single maternal and a single paternal copy of the genome. From this combined genetic material and the maternally inherited organelles, proteins, mRNAs, etc., found in the unfertilized egg, an embryo, fetus and finally an adult animal develop. The single-celled zygote must grow and multiply to produce the vast number of cells which make up an individual, and in addition groups of cells must develop along specific pathways in order to produce all of the cell types which make up the tissues and organs of the mature adult. The controls of the processes which determine this specialization, termed differentiation, form one of the most fundamental questions in developmental biology. Today we understand that genetic information is encoded by DNA, which is assembled into chromatin and makes up the chromosomes. However, although early developmental biologists realized that the zygote must contain all of the information required to produce a complete individual, the nature and control of this information was unknown.

Two theories arose to explain the control of differentiation; August Weismann (1892) proposed that during development the genetic material was 'shared out', i.e. individual cells received only that information which was required to fulfil their particular specialized function. Credence to this theory was lent by experiments using sea-urchin embryos. Wilhelm Roux (1888) killed a single blastomere at the two-cell stage by puncturing it with a hot needle; following further development a half embryo was produced. However, in subsequent experiments, Driesch (1892) and others separated the blastomeres at the two-cell stage and found that anatomically whole, but dwarfed embryos were produced (for a comprehensive history see Di Berardino, 1997). These experiments demonstrated that at least during early embryonic development, each cell retains all of the genetic material. In an extension of these experiments, Hans Spemann constricted salamander zygotes, using human baby hair, restricting the nuclei

to one half of the cytoplasm. The half zygote containing the genetic material continued to divide. After several divisions the constriction was relaxed sufficiently for a nucleus to traverse the cytoplasmic bridge. The previously enucleated portion of cytoplasm then resumed cleavage and development. This technique resulted in the production of dwarfed but twinned offspring. After considering the results of these experiments, Spemann proposed the transfer of nuclei from more advanced developmental stages back to zygotes from which the genetic material had been removed (Spemann, 1938).

This suggestion by Spemann, which is perceived as the birth of nuclear transfer technology, was originally proposed as a method to study the role of the genetic material in cellular differentiation or, more specifically, whether information contained within the genes was lost or inactivated during development and differentiation. We now know, from many lines of research, that development and cellular differentiation is the result of highly specific temporal and spatial control of gene expression. During these processes DNA is not lost and the majority of cells in an adult animal retain two copies of the genome as originally inherited in the zygote.

A brief history of cloning:

Although Spemann provided a route for the investigation of differentiation by nuclear transfer, further experiments were not reported until the 1950s when Briggs & King (1952) reported the development of swimming tadpoles after the transfer of nuclei from early-stage frog embryos into enucleated eggs. Subsequently, John Gurdon reported the production of adult *Xenopus* (South African Clawed Toads) after transferring nuclei from tadpole intestinal epithelial cells (Gurdon & Uehlinger, 1966); however, in subsequent experiments no adults were produced when cells taken from adult animals were used as nuclear donors (Gurdon et al. 1975). In mammals, the techniques for embryo reconstruction were not developed until much more recently, one reason being the small size of mammalian eggs, 120–150 μm, in comparison with those of various amphibians, which are > 1 mm. Therefore, significantly different equipment is required; however, there were early reports of nuclear transfer in the rabbit during the late 1970s and then McGrath & Solter (1983a) demonstrated that by using microsurgery, pronuclei could be exchanged between mouse zygotes and development still be obtained. Subsequently, Willadsen (1986) produced live lambs after transferring nuclei from 8- to 16-cell sheep embryos into enucleated MII oocytes. Following these reports, successful nuclear transfer was reported in cattle, sheep and mice by a number of laboratories, but development to term was restricted to the use of early cleavage-stage embryos as nuclear donors.

Methodology of nuclear transfer:

In mammalian species, enucleated MII oocytes (unfertilized eggs) have now become the recipient cell of choice owing to the lack of development obtained when using enucleated zygotes (i.e. in cattle: Robl et al. 1987; pig: Prather et al. 1989). This restriction may be due to the removal of zygotic factors, which are essential for early development that may be associated with the pronuclei. MII oocytes to be used as nuclear recipients may be obtained from a variety of sources dependent upon species.

In ruminants, particularly cattle, oocytes may be aspirated from ovarian follicles following slaughter and matured *in vitro*. Additionally, matured oocytes may be flushed from the oviducts of donor animals following superovulation regimes and immature oocytes may be aspirated from follicles *in vitro* or following ovariectomy (for a review see Campbell & Wilmut, 1994). Having obtained a suitable recipient oocyte, the genetic material located on the meiotic spindle is removed by microsurgery. Briefly, a small amount of cytoplasm is aspirated from directly beneath the first polar body using a fine glass pipette. Fluidity of the cell membranes allows both the oocyte and the aspirated karyoplast to reseal following manipulation. The enucleation procedure can be monitored by staining the aspirated karyoplast with a DNA-specific fluorochrome, e.g. Hoechst 3332 (for methodology see Campbell et al. 1998). Following enucleation, the genetic material from the donor cell (karyoplast) must be introduced into the enucleated recipient cell (cytoplast). In the mouse, Sendai virus was originally used to cause fusion of the two cells (McGrath & Solter, 1983b); however, more recently, the development of piezo-aided injection has proved of considerable use (Wakayama et al. 1998). In farm animal species, the use of a DC electrical pulse (electrofusion) has become the method of choice; although viral and chemical methods have been used, these have proved less reproducible and in the case of chemical fusion may be toxic. At this point the reconstructed embryo is able to begin development, but many factors are involved in the successful development of such reconstructed embryos. These include other techniques associated with the methods of activation (induction of fertilization responses), culture, and biological factors relating to both the cytoplast and the karyoplast (for reviews see Campbell & Wilmut, 1994, 1998). Of great importance for successful development is co-ordination of the nuclear and cytoplasmic cell cycle phases of both donor and recipient cells (Campbell et al. 1996a).

Animal Cloning:

Animal cloning is more difficult than plant cloning because animal cells lose their totipotency on reaching the gastrula stage of animal development. However, animal tissue cultures from tumours and embryonic tissue cells have been successful. Standard techniques are available for isolating animal cells and tissues from different systems.

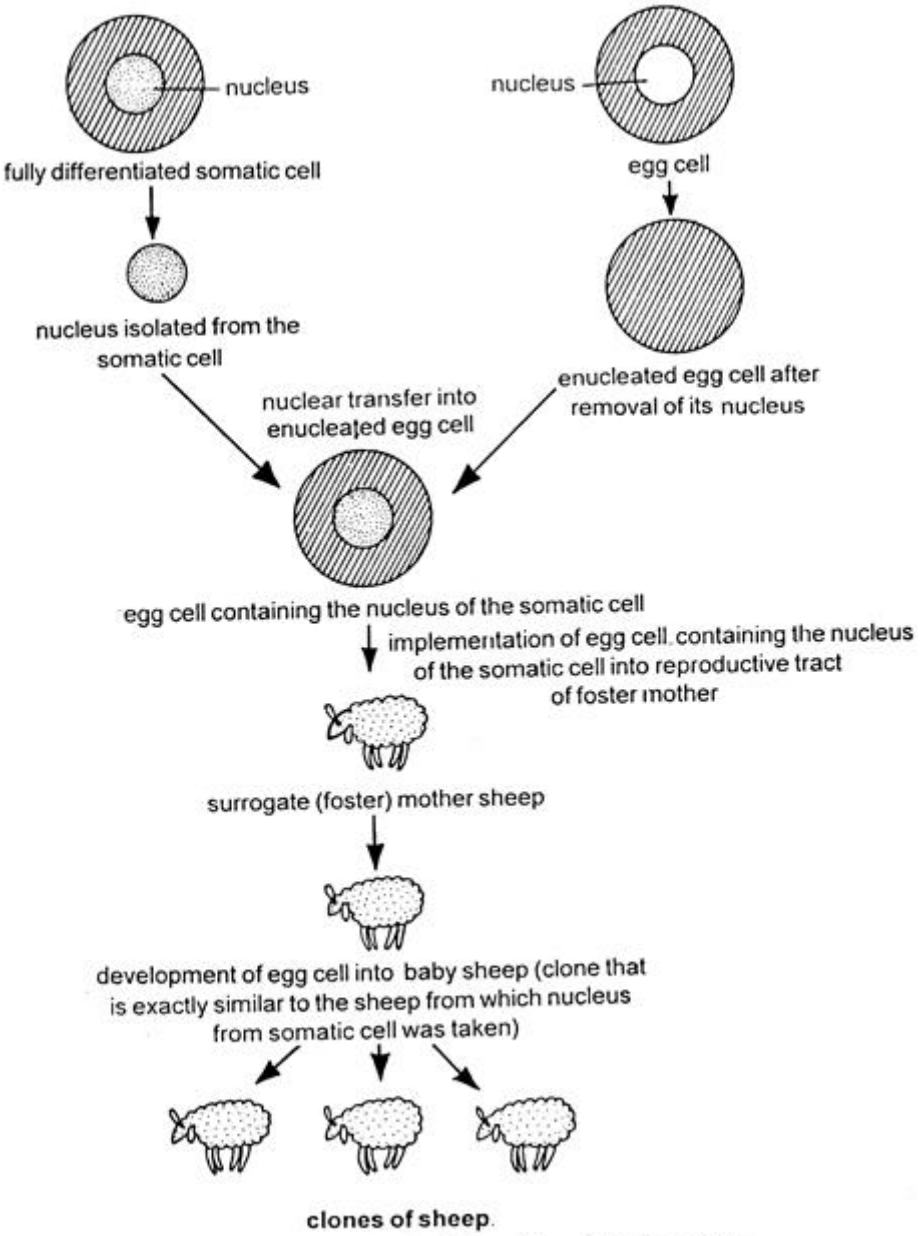


Fig. 8.15. Steps involved in the cloning of the sheep **Dolly.**

Some more important examples of animal cloning are tissue culture, somatic cell fusion, cell cloning and creating transgenics.

Gene Transfer in Animals:

Gene transfer in animals is mostly through direct methods such as electroporation or microinjection or using particle gun. In creating 'Dolly' the cloned sheep, fertilised egg of its mother was removed by micro-needle and nucleus from an udder cell of a donor sheep was microinjected in the egg after removing egg nucleus. The egg developed into 'Dolly' with genes identical to its mother.

How Sheep 'Dolly' Cloned?

Ian Wilmut and his associates at the Roslin Research Institute, Scotland, took cells from ewe (mother sheep's udder). An udder cell is different from a skin cell or a muscle cell or a nerve cell. They managed to store these udder cells in nutrient deprived culture. This checked the starved cells from dividing, and switched off their active genes.

Now, one udder cell complete with its nucleus was selected, as this nucleus carries the mother's genetic information.

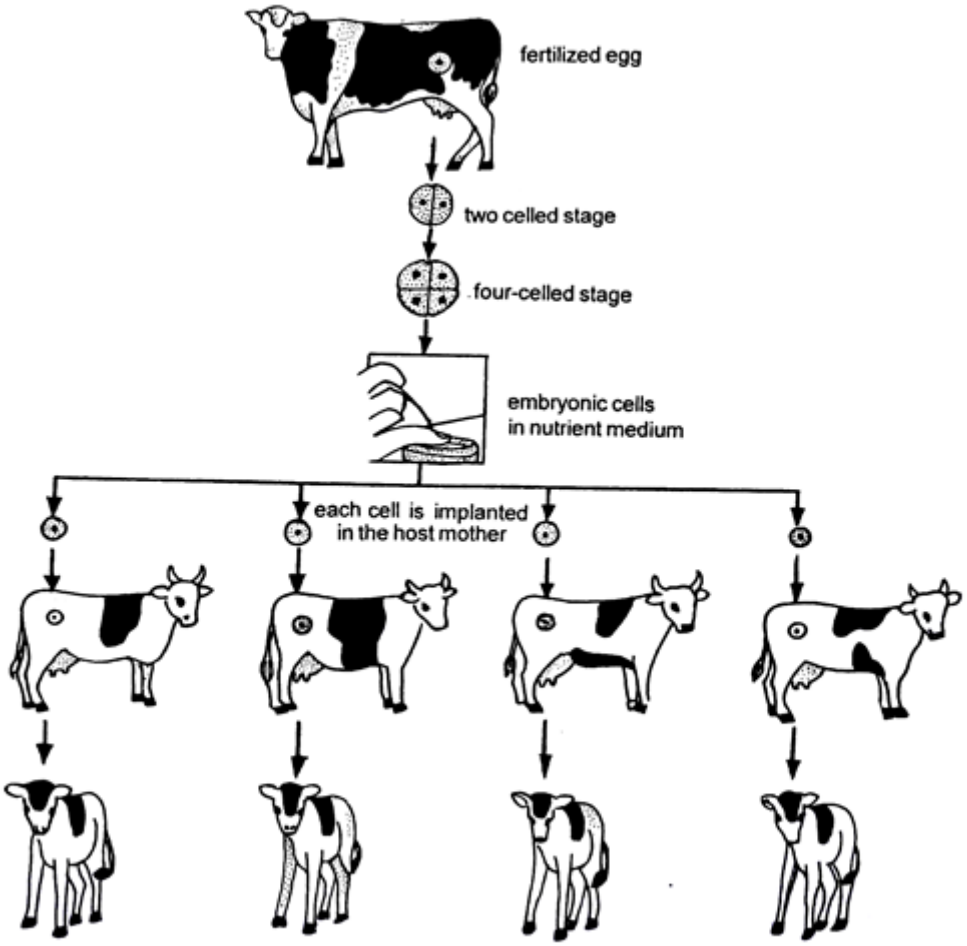


Fig. 8.16. Japanese method of cloning cattle.

Meanwhile, unfertilized egg cell was taken from a different ewe (host mother sheep). Its nucleus was sucked out leaving an empty cell containing all the necessary components to produce an embryo. This cell was now ready to receive udder cell nucleus. They now fused udder cell nucleus with the empty egg cell by electrical stimulation. Then this egg cell had the mother's nucleus.

When a normal or altered egg is implanted in a different female is termed 'surrogate mother'. This means, substitute mother. Then the altered egg was cultured for six days.

Out of many resulting embryos, one was implanted in the uterus of the surrogate mother, where it grew into a lamb. Thus, Dolly was born genetically identical to mother sheep as her first cell nucleus came from mother's cell.

How Calves are Cloned in Japan?

Scientists from Japan have cloned cattle in a different way.

They have got success in growing as many as eight identical calves from one fertilized cell of their mother.

The process is as follows:

When the mother cow has mated with the bull, she has a fertilized egg in her womb. Now this zygote divides in two and then in four and then in eight. This embryo is carefully removed from the womb, and the embryonic cells are separated using as enzyme.

Each isolated cell is kept in a nutrient medium and later implanted in the womb of a different 'host mother' cow.

The host mother's womb must accept the cell and make it grow. Each cell may grow into a normal baby calf, if all goes well.

First Human Clone:

The birth of the first human clone (December 26, 2002), a baby girl called Eve by scientists, was announced by Brigitte Boisselier, head of a company named Clonaid. According to Boisselier, the child is an exact genetic duplicate of her mother.

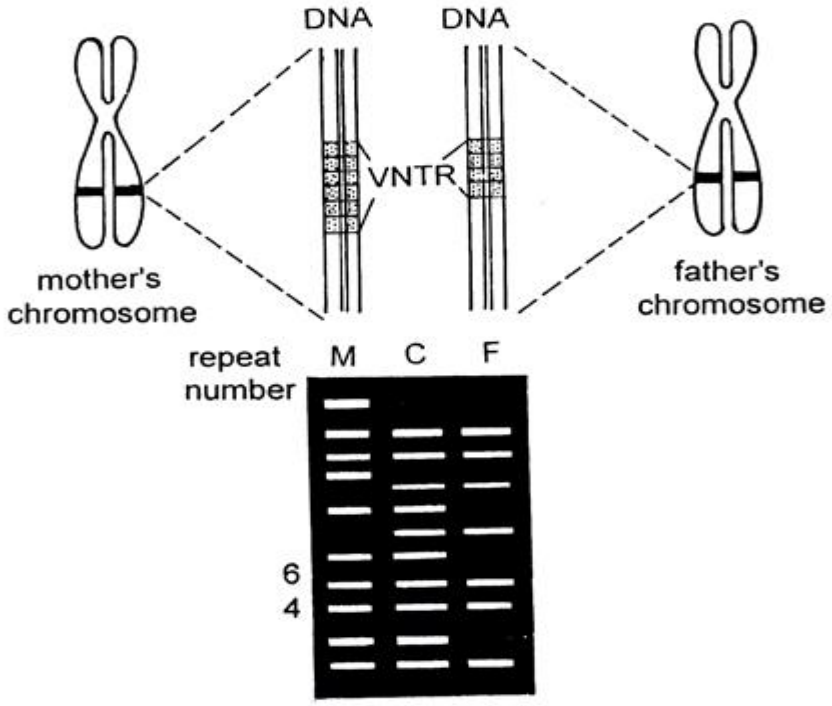


Fig. 8.17. Variable Number Tandem Repeats (VNTRs). M = mother C = child, F = father.

To clone, scientists slip the nucleus of an adult cell, like a skin cell, into an unfertilized egg from which its own genetic material has been removed.

Then, if stimulated to act like a fertilized egg, the newly altered genetic material can then direct the egg to divide and grow into an embryo, then a foetus, then a newborn, if all goes well. Yet, as scientists have discovered, only rarely does all go well.

In animal work so far only about 1 to 5 per cent of cloning attempts succeed, said Randall Prather, a cloning expert. That is, for every 100 eggs, one to five clones are born.

Nuclear transfer from cultured cell populations:

Until 1995 development of mammalian nuclear transfer reconstructed embryos was restricted to the use of donor genetic material from early embryos. Although this technique has a number of applications both on a scientific and a technological level, it was the goal of many scientists to obtain development of nuclear transfer embryos from cells which could be maintained in culture. Many reports suggested that specific 'pluripotent' cell types (e.g. embryonic stem cells (ES)) would be required; however, in farm animal species such cell populations have as yet not been identified. In the mouse, development to term of embryos reconstructed using ES cells as nuclear donors has recently been reported, but problems with epigenetic instability occur during *in vitro* culture (Humpherys et al. 2001).

Previous reports had demonstrated that offspring can be obtained by nuclear transfer using inner cell mass cells of blastocyst-stage embryos in both cattle and sheep (Sims & First, 1994). Rather than try to isolate a 'pluripotent' ES-like cell line we followed the ability of blastocyst-derived cells to produce live offspring when placed into culture and used as donors of genetic material for nuclear transfer. Owing to the seasonal nature of ovine reproduction, these experiments were initiated over the winter of 1993–1994. To avoid the need for synchronization of the donor cell cycle, nuclear transfer embryos were reconstructed using pre-activated enucleated MII oocytes as recipients. During early passages (P1–P3) live offspring were obtained; however, on continued culture (P6–P11) and subsequent embryo reconstruction during the winter of 1994–1995, no further offspring were obtained. Immunofluorescent analysis of this cell population demonstrated the presence of A type lamins, cytokeratin and vimentin, which are associated with differentiated cells (Campbell et al. 1996b).

An alternative to selecting a cell type which may be successful at controlling development in nuclear transfer embryos is to modify the donor chromatin structure prior to embryo reconstruction. Previously, this paper has discussed the cell cycle in relation to growing cells: an additional cell cycle phase termed G0 is found in cells which have exited the cell cycle in response to a number of conditions. Such cells are said to be quiescent and the G0 stage has been implicated in cellular differentiation. G0 cells are arrested in a post M pre S-phase state with a diploid DNA content and may therefore be transferred to MII oocytes with high MPF activity.

In our experiments, cells were induced into a quiescent state by serum starvation. Quiescent donor nuclei were transferred to MII oocytes at the time of activation, prior to activation and following activation. Live lambs (five in total) were obtained from all combinations, but unfortunately two of these died within minutes of birth and a third at 10 days following birth with a range of congenital abnormalities. The remaining two lambs remain healthy and both have proved to be fertile (Campbell et al. 1996b).

To confirm and extend these studies they were subsequently repeated using a male day 9 embryo-derived cell population, primary fetal fibroblasts from a day 26 fetus and a mammary epithelial cell line isolated from a 6-year-old ewe. Live offspring were obtained from each of these cell populations, the adult cell giving rise to the birth of 'Dolly' (Wilmut et al. 1997). Since this time we have seen a number of reports in cattle, sheep and mouse reporting the birth of live offspring from fetal and adult cells which have been induced to enter a quiescent state, or exist in a quiescent state *in vivo*.

The potential role of quiescence in successful development of nuclear transfer reconstructed embryos using cultured cell populations is presently unclear. The use of a diploid cell line allows co-ordination of donor and recipient cycles, the use of MII oocytes maximizes the number of mitotic events which the donor nucleus passes

through in the absence of transcription, changes in the donor cell including a reduction in transcription, a reduction in translation, active degradation of unnecessary mRNA and chromatin condensation are factors which may facilitate interaction of the donor chromatin with maternal factors in the recipient oocyte cytoplasm.

Today there are a number of independent reports of live offspring produced by nuclear transfer from embryonic, fetal and adult cell cultures in sheep (Wells et al. 1998), cattle (Wells et al. 1999), mice (Wakayama et al. 1998), goats (Baguisi et al. 1999) and pigs (Polejaeva et al. 2000). In many of these reports, primary cell populations have been established in culture. Such populations are not clonally derived and therefore it is difficult to compare or predict the behaviour of the population. In cultured primary cell populations the number of cells able to complete the cell cycle and divide decreases as a function of age in culture. Individual cells will enter a non-growing but viable condition, which has been termed senescence. *In vivo*, many senescent cells complete DNA replication but do not go on to divide, arresting in the G2 phase of the cell cycle and therefore tetraploid (i.e. liver). During culture *in vitro* it has been reported that on the way to true senescence (i.e. G2 arrested) individual cells can enter a quiescent or G0 state. In any primary cell population in culture one can hypothesize that at any one time a percentage of the cells will be non-growing and diploid. Whether this is a result of suboptimal growth conditions or cells entering a 'presenescent' G0 phase is yet to be determined (Campbell, 1998a).

The only true measure of the efficiency of the NT process is the production of viable offspring. The development of reconstructed embryos is influenced by many factors, including quality of the recipient oocyte, method of activation and culture methods. Similarly, induction and maintenance of pregnancy is dependent upon a range of factors influenced both by the quality of the transferred embryo and the age, seasonality, nutritional and hormonal status of the surrogate recipient. Studies on the optimal cell cycle stage of donor and recipient are underway in many laboratories, but to date no clear answer to the problem has surfaced.

Problems and abnormalities:

The development of offspring produced by nuclear transfer is an inefficient process, with the majority of studies reporting between 0.5 and 5.0% development to term. Losses occur throughout gestation, at birth and following birth, and a range of developmental abnormalities have been reported. The reasons for these abnormalities are unknown but may reflect incomplete or inappropriate reprogramming possibly related to problems associated with imprinted genes (Young et al. 2000; Young & Fairburn, 2000). A greater understanding of the control of normal development may help elucidate the mechanisms involved in these processes.

Implications of nuclear transfer from cultured cells:

Nuclear transfer using embryonic blastomeres as nuclear donors has a number of applications in agriculture and research for multiplication of elite embryos or for the production of multiple copies for research purposes. However, these applications are limited by the number of donor cells available and the efficiency of the process. The use of cultured cell populations can increase the number of animals which may be produced from an elite embryo, fetus or adult. In addition, the storage of frozen cell populations may prove useful in the preservation of genetic resources in a number of species. However, in the short term, the major implication of the use of cell populations that may be maintained in culture prior to their use as nuclear donors is the provision of a route for the precise genetic modification of farm animal species. Previously transgenic farm animals were produced by pronuclear injection; this route to genetic modification will be discussed in relation to nuclear transfer, subsequently specific applications of genetic modification for human therapeutic use will be cited.

Advantages of nuclear transfer for genetic modification of farm animal species

The addition of genetic material or production of a transgenic animal can be achieved by the injection of the required gene into the pronucleus of a zygote. Although this technique has been applied successfully in a number of species including mice, rabbits, pigs, sheep, goats and cattle (for review see Wall & Seidel, 1992) there are a number of disadvantages. (1) Integration does not always occur during the 1st cell cycle, resulting in the production of mosaic embryos (Burdon & Wall, 1992). (2) Integration occurs at random within the genome resulting in variable expression of the gene product. (3) At present only simple gene additions may be performed. (4) The selection of transgenic embryos prior to their transfer is hampered by mosaicism (Rusconi, 1991). (5) The production of the required phenotype coupled to germ line transmission may require the generation of several transgenic lines. (6) Multiplication of the required phenotype or its dissemination into the population is restricted by breeding programmes.

In contrast, the production of offspring from a single cell or cloned population offers significant advantages. Genetic modification can be performed in culture and the modified cells selected prior to animal production. It will be possible to remove (knockout) as well as to add genes, and precise modification of control regions or addition of genes to specific regions of the genome (knockin) will be facilitated. The production of an animal from a single nucleus removes the problems associated with mosaicism; all of the cells within the resultant animal will contain the modification which will be transmitted through the germ line. All of the animals produced will be transgenic and flock or herd generation can be accelerated by producing multiple copies

from the cultured cells. The experiments which led to 'Dolly' involved the use of mammary epithelial cells. The use of this cell line was related to the potential screening of transgenic cells for milk production *in vitro* prior to animal production. Thus it may be possible to predict expression level and select the highest expressing cell populations prior to animal production.

In order to carry out these modifications the cultured cell populations must be amenable to transfection and selection in culture and maintain their ability to be used for successful nuclear transfer. It has been demonstrated that fetal fibroblasts are suitable for this purpose with the production of 'Polly', a nuclear transfer lamb transgenic for human factor IX derived from a transfected, selected cell population (Schnieke et al. 1997). These experiments also demonstrated that the efficiency of animal production was increased over two-fold, in terms of total animals used, as compared to pronuclear injection. More recently, live lambs have been produced which carry a deletion of a single allele of the collagen gene (McCreath et al. 2000) as well as targeted addition of a gene to the collagen locus. In addition, fetuses and offspring have been produced with knockouts of the alpha 1–3 galactosyl transferase and PrP genes (Denning et al. 2001).

The generation of animals carrying multiple genetic modifications requires the sequential addition, removal or modification of specific genes. In culture, primary cell populations have a finite lifespan; however, by re-deriving cell populations from embryos, fetuses or offspring produced by nuclear transfer it will be possible to extend the period that cells can be maintained in culture to carry out these modifications.

Role of nuclear transfer in stem cell therapies

The ability to produce animals by nuclear transfer demonstrates the ability to de-differentiate and subsequently re-differentiate the genetic material contained within a range of somatic cell types. The use of embryonic stem cells as a source for the production of specific differentiated cell lineages provides numerous opportunities for human therapies. At the present time stem cell populations have been isolated from embryonic fetal and adult tissues and studies are underway in numerous laboratories on the controlling lineage-specific differentiation. The practical aspects of cell transplantation with regard to function, longevity and rejection may differ dependent upon the cell origin. The establishment of cell banks of numerous tissue types may be required to reduce the problems that may be associated with cell rejection. Although stem cells derived from cord blood or adult tissues may provide a possible route for autologous transplantation, nuclear transfer followed by embryonic stem (ES) cell isolation may prove to be a valuable tool. The production of ES cells following dedifferentiation of somatic cells by nuclear transfer in the mouse has now been

demonstrated (Munsie et al. 2000). The application of nuclear transfer in humans will provide valuable information on embryonic development and cell differentiation.

Probable Questions:

1. Discuss the methodology of nuclear transfer technology.
2. How animal cloning is done?
3. How Dolly was created?
4. What are the problems and abnormalities observed in nuclear transfer?
5. State the Implications of nuclear transfer from cultured cells.
6. State Role of nuclear transfer in stem cell therapies.

Suggested Readings:

1. Biotechnology by P.K. Gupta

2. Gene Cloning by T. Brown.

3. Biotechnology by N. Kumarsen.

4. Biotechnology by B.D. Singh

UNIT-VI

Gene Replacement and Transgenic Technology

Objective: In this unit you will learn about different techniques related to Transgenic Technology which are used in animal science.

Features of Transgenic Technology:

Important points related to gene technology are briefly presented as follows:

i. Direct Gene Transfer:
Gene technology permits direct gene transfer into the recipient parent bypassing sexual process. In other words, there is no need of union of male and female gametes in gene technology. The gene of interest can be directly inserted into the cell of recipient parent.

ii. Single Gene Transfer:
Gene technology permits transfer of one or two genes from donor species or organism to the recipient organisms. It hybridization method hundreds of genes are transferred to the recipient parent which are eliminated by repeated back, crossing from the recipient or recurrent parent.

iii. Rapid and Accurate Technique:
Gene technology is a rapid method of crop improvement. It takes 4-5 years for release of new cultivar against 10-12 years by conventional breeding method. Moreover, it is highly accurate and reliable technique.

iv. Free Gene Transfer:
Gene technology permits gene transfer between two totally unrelated organisms i.e. from bacteria to higher plant cell and even from animals to plants. Thus gene technology has overcome the natural barriers of gene transfer.

Steps in Transgenic Technology:

Development of transgenic (genetically engineered) plant is a lengthy process which consists of following important steps:

i. Identification of useful Genes:
The desirable genes may be located in wild species, unrelated plant species, unrelated organisms i.e., micro-organisms (bacteria, viruses or fungi) and animals. This work is carried out in the research laboratory.

ii. Designing Gene for Insertion:
The gene of interest is isolated from the donor source and cloned in the laboratory. The cloning is done generally using plasmids.

Introduction to Transgenesis:

Conventional animal husbandry involves the proper feeding, caring, management and breeding methods for the increased production of their yield which may be in the form of meat, milk, eggs, etc. In animal husbandry, selective breeding aims to increase the frequency of desired genes and the desired phenotype. For many farm animals, the conventional breeding has already achieved high yielding animals but by this procedure, it seems that the productivity would soon be approaching a plateau. To sustain an ever increasing world population, new methods must be developed to meet this increasing demand for animal products. Secondly, selective breeding is a painfully slow process and, especially with larger animals with long gestation period, can take many years to establish desired phenotypic changes.

The advent of technology of transgenesis also called transfection and its application to animal breeding programmes may greatly increase the speed and range of selective breeding. The transgenesis involves the transfer of desired isolated gene or gene fragments or individual chromosome or chromosomal fragments, or isolated nuclei from one organism to another organism. The first recorded examples of the transfer of a foreign gene into an animal by recombinant DNA technology was the insertion and expression of a rat gene for growth hormone (rGH) into the mouse metallothionein (mMT) gene in 1882. The subsequent progeny were all much larger than, the parents and the transgenic mouse was called **"super mouse"** (Fig. 12.11). Since then, many transgenic animals including cattie, goats, pigs, rabbits, chickens and fish have been produced (Table 12.2).

Table 12.2. Examples of transgenic animals.

Transgenic animals	Genes transferred
1. Mouse	mMT/rGH; mMT/bGH (b=bovine) ; mMT/oGH (o= ovine); mMT/hGH(h=human); mMT/hGRF; mMT/hf IX(factor IX).
2. Chickens	ALV (Avian Leukosis Virus); REV (Raticulo Endotheliosis Virus)
3. Cow	BPV (Bovine Papilloma Virus);Lactoferin.
4. Fish	hGH; Cd-crystallin(c= chicken); AFP(Anti-Freeze Protein).
5. Pig	hMT/pGH (p= porcine); MLV(Moloneymurine Leukemia Virus)/ rGH; bPRL (prolactin)/bGH.
6. Rabbit	hMT/hGH; rbEu (Immunoglobulin heavy chain)/rb.
7. Sheep	mMT/TK (Thymidine kinase); oMT/ oGH; oBLG (β-Lactoglobulin)/hf IX.
8. Goat	A varient of tPA gene (human tissue-type plasminogen activator).

About 95 % of the existing transgenic animals are mice.

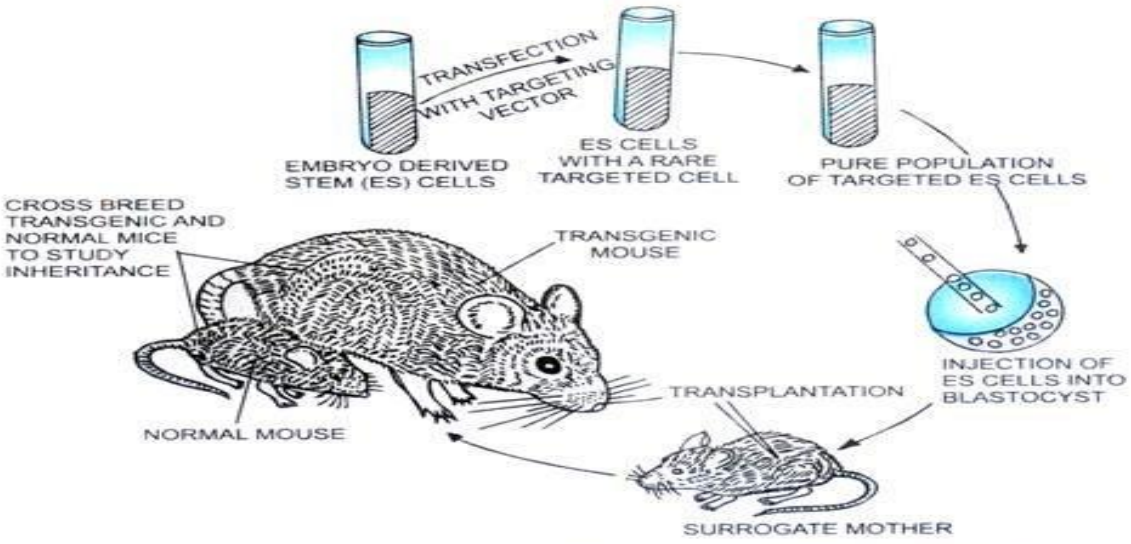


Fig. 12.11. Gene targeting using embryonic stem cells.

Mechanism of Transgenesis in Animals:

Transgenesis involved a number of methods like:

1. Transfer of whole nucleus from a somatic cell of a superior donor into the enucleated egg of recipient animal.

2. Transfer of a part of dissected embryo into the enucleated unfertilized egg.

3. Transfer of a chromosome or chromosomal fragments.

4. DNA microinjection technique.

5. Gene targeting using embryonic stem cells.

Frequency of Success of Transgenesis:

Now the transgenic pigs, sheep and cattle have been produced, although the frequency of success is only about 1% compared to 2-5% in mice while successful fish transgenics can be as high as 70%.

Significance of Transgenesis:

1. Role of Transgenesis in Molecular Farming:

Molecular farming involves the extraction of useful proteins and drugs from the milk, food and urine of hansgenic animals which may be used as bioreactors e.g:

(i) Transgenic goats with LAtPA protein were produced by microinjecting murine whey acid promotor (WAP) carrying a c-DNA with LAtPA protein coding gene. The LAtPA protein dissolves the blood clot and is useful for treating coronary thrombosis.

(ii) (ii) Transgenic sheep with human antihaemophillic factor IX gene was produced with the help of BLG-gene (β-Lactoglobulin gene), which acts as vector gene Transgenic ewes secrete human factor IX in their milk.

(iii) Transgenic sheep having human α_1 antitrypsin gene (hα_1 AT) was produced with the help of ovine β- lactoglobulin gene promoter. Transgenic ewes produce h α_1 AT protein in their milk and can be used against the emphysema.

Thus by transgenesis the animals are genetically modified in such a way that they start acting bioreactors producing useful products in abundance and continuously.

Study of Diseases and Gene Therapy :

Many transgenic animals help us to understand the following facts:

(i) How do genes contribute to the development of disease?

(ii) These act as models for human diseases like cancer, cystic fibrosis, rheumatoid arthritis, Alzheimer's disease, etc., and their possible new methods of their treatment.

(iii) Transfections of cultured mammalian cells have been used extensively for detecting the cancer genes (oncogenes) and their gene therapy. In these retroviruses, adeno-associated virus (AAVs) and naked DNA have been used as vectors and gene therapy.

(iv) In 1991, the transgenic cow with a bovine alpha- Si casein promotor driving a c-DNA was produced. This foreign gene encodes for the lactoferrin (a iron- binding protein) which has the antibacterial properties.

Increased Production of Biological Products:

(i) Transgenic sheep with genes like cysE and cysM (coding for two enzymes—serine acetyl transferase and o-acetyl serine sulphhydrylase) have been produced. In the transgenic sheep, wool production was found to much more than the non-transgenic sheep because these two enzymes are essential for the biosynthesis of the amino acids involved in the formation of wool.

(ii) In 1985, the transgenic fishes of many species like common carp, catfish, goldfish, salmon, Tilapia, rainbow trout and zebra fish have been produced by microinjection of genes coding for rat or human growth hormone (rGH or hGH). It was found that transgenic fish with hGH gene was found to be twice in size than the non- transgenic fish.

(iii) In 1997, first transgenic cow, named Rosie, with human alpha-lactalbumin gene was produced. The milk of transgenic cow contained about 2.4 grams of human protein per litre of milk and was found to be more nutritionally balanced product for human babies than that of natural cow milk. Such human milk proteins can be extracted and used pharmaceutically.

For Study of Normal Physiology and Development:

Transgenic animals have been successfully utilized to understand:

(i) Mechanism of regulation of genes.

(ii) Mode of effects of genes on the normal functions of the body and its development e.g. study of biological role of insulin-like growth factor in regulating the body's growth.

Vaccine Safety Testing:
Transgenic mice are first to be used as laboratory animals to test the efficacy of a newly discovered vaccine before it is used on human beings e.g. polio vaccine. If such vaccines are found satisfactory and reliable on mice, then these are tested on the monkeys much closely related to man.

Chemical Safety Testing:
For this transgenic animals with foreign genes are produced so that the transgenic animals become more sensitive to the toxic chemicals of them, the non-transgenic animals. Then these animals are exposed to toxic chemicals and their effects are observed. The time required to obtain the results is less.

So contrary to some popular view points, transgenic animal studies are not about producing animal monsters but rather introducing specific and economically significant

traits into livestock that will have benefits to mankind. These encouraging results have made transfection and production of transgenic animals a fascinating thrust of research.

Importance of Transgenic Animals-General:

Trans-genesis has now become a powerful tool for studying the gene expression and developmental processes in higher organisms, besides the improvement in their genetic characteristics. Transgenic animals serve as good models for understanding the human diseases.

Further, several proteins produced by transgenic animals are important for medical and pharmaceutical applications. Thus, the transgenic farm animals are a part of the lucrative world-wide biotechnology industry, with great benefits to mankind. Trans-genesis is important for improving the quality and quantity of milk, meat, eggs and wool production, besides creating drug resistant animals.

Milk as the Medium of Protein Production:

Milk is the secretion of mammary glands that can be collected frequently without causing any harm to the animal. Thus, milk from the transgenic animals can serve as a good and authenticated source of human proteins for a wide range of applications. Another advantage with milk is that it contains only a few proteins (casein, lactalbumin, immunoglobulin etc.) in the native state, therefore isolation and purification of a new protein from milk is easy.

Commonly used Animals for Trans-genesis:

The first animals used for trans-genesis was a mouse. The 'Super Mouse', was created by inserting a rat gene for growth hormone into the mouse genome. The offspring was much larger than the parents. Super Mouse attracted a lot of public attention, since it was a product of genetic manipulation rather than the normal route of sexual reproduction. Mouse continues to be an animal of choice for most transgenic experiments. The other animals used for trans-genesis include rat, rabbit, pig, cow, goat, sheep and fish.

Position Effects:

Position effect is the phenomenon of different levels of gene expression that is observed after insertion of a new gene at different position in the eukaryotic genome. This is commonly observed in transgenic animals as well as plants. These transgenic organisms show variable levels and patterns of transgene expression. In a majority of cases, position effects are dependent on the site of transgene integration. In general, the defective expression is due to the insertion of transgene into a region of highly packed chromatin. The transgene will be more active if inserted into an area of open chromatin.

The positional effects are overcome by a group of DNA sequences called insulators. The sequences referred to as specialized chromatin structure (SCS) are known to perform

the functions of insulators. It has been demonstrated that the expression of the gene is appropriate if the transgene is flanked by insulators.

Animal Bioreactors:

Trans-genesis is wonderfully utilized for production proteins of pharmaceutical and medical use. In fact, any protein synthesized in the human body can be made in the transgenic animals, provided that the genes are correctly programmed. The advantage with transgenic animals is to produce scarce human proteins in huge quantities. Thus, the animals serving as factories for production of biologically important products are referred to as animal bioreactors or sometimes pharm animals. Frankly speaking, transgenic animals as bioreactors can be commercially exploited for the benefit of mankind. Once developed, animal bioreactors are cost-effective for the production of large quantities of human proteins. Routine breeding and healthful living conditions are enough to maintain transgenic animals.

Transgenic Animals in Xenotransplantation:

Organ transplantation (kidney, liver, heart etc.) in humans has now become one of the advanced surgical practices to replace the defective, nonfunctional or severally damaged organs. The major limitation of transplantation is the shortage of organ donors. This often results in long waiting times and many unnecessary deaths of organ failure patients.

Xeno transplantation refers to the replacement of failed human organs by the functional animal organs. The major limitation of xeno transplantation is the phenomenon of hyper acute organ rejection due to host immune system.

The organ rejections is mainly due to the following two causes:

i. The antibodies raised against the foreign organ.

ii. Activation of host's complement system.

Pigs in Xenotransplantation?

Some workers are actively conducting research to utilize organs of pigs in xenotransplantation. It is now identified that the major reason for rejection of pig organs by primates is due to the presence of a special group of disaccharides (Gal-α 1, 3-Gal) in pigs, and not in primates.

The enzyme responsible for the synthesis of specific disaccharides in pigs has been identified. It is α 1, 3-galactosyltransferase, present in pigs and not in primates. Scientists are optimistic that knockout pigs lacking the gene encoding the enzyme α 1, 3-galactosyltransferase can be developed in the next few years. Another approach is to introduce genes in primates that can degrade or modify Gal-α 1, 3-Gal disaccharide

groups (of pigs). This will reduce immunogenicity. Besides the above, there are other strategies to avoid hyperactive organ rejection by the hosts in xenotransplantation.

i. Expression of antibodies against the pig disaccharides.

ii. Expression of complement— inactivating protein on the cell surfaces.

By the above approaches, it may be possible to overcome immediate hyperactive rejection of organs. The next problem is the delayed rejection which involves the macrophages and natural killer cells of the host.

Another concern of xenotransplantation is that the endogenous pig retroviruses could get activated after organ transplantation. This may lead to new genetic changes with unknown consequences.The use of transgenic animals in xenotransplantation is only at the laboratory experimental stages, involving animals. It is doubtful whether this will become a reality in the near future. There is a vigorous debate concerning the ethics of xenotransplantation and the majority of general public are against it.

Transgenesis in large animals:

In general, trans-genesis in large animals is more difficult than with mice. There are several factors for the lower efficiency of trans-genesis in large animals. These include less number of eggs they produce and technical difficulties in handling, besides long gestational periods to get the offspring (It takes about 2 years to produce a calf from a fertilized egg).

Some of the early experiments to produce transgenic large animals were far from satisfactory. For instance, transgenic sheep overproducing growth hormone grow leaner with increased feed efficiency. But they are more susceptible to infection, become infertile and tend to die at young age. All this might be due to ineffective control of gene regulation. Several improvements have been made to produce transgenic animals with desirable characters. Biotechnologists are particularly interested to improve the quality of animals, with improved resistance to diseases, besides enhancing their ability produce foods. ‘Building a better animal’, being the motto. Further, production of commercial and pharmaceutical compounds by transgenic animals is also gaining importance in recent years. The protocol adopted for producing other transgenic animals is comparable with that already described for transgenic mice, with certain modifications.

Transgenic Cattle:

The mammary gland of the dairy cattle is an ideal bioreactor for producing several new proteins (of pharmaceutical importance), besides improving the quality and quantity of the existing ones. For instance, a transgenic cow, with an over-expressed casein transgene, can give milk with higher content of casein.

If lactase transgene is introduced and expressed in the mammary gland, milk free from lactose will be secreted. Such a milk will be a boon for lactose intolerant people who experience indigestion and other complications, after consuming normal milk and milk products. Some success has been achieved in creating transgenic cattle with improved resistance to viral, bacterial and parasitic diseases. However, this is not an easy job due to the complexity of genetic control to combat the disease-producing organisms.

Attempts have been made in recent years to produce cattle with inherited immunological protection by trans-genesis. Introduction of genes that code for heavy and light chains of monoclonal antibodies has met with some success in this direction .In vivo immunization of an animal although not yet fully successful, is ideal for disease protection. In vivo immunization primarily involves the insertion of a transgene for an antibody that specifically binds to an antigen.

Transgenic Sheep and Goats:

Trans-genesis experiments in sheep and goats mostly involve the development of mammary glands as bioreactors for the production of proteins for pharmaceutical use. This is possible despite the fact that quantity of milk produced by sheep and goats is less than that of dairy cattle (cow, buffalo). Some proteins produced by sheep and goats have good pharmaceutical use (Table 41.2).

TABLE 41.2 Transgenic animals as bioreactors for the production of therapeutically important proteins

Transgenic animal	*Protein product*	*Biological importance*
Cow	Lactoferrin	Promotes intestinal iron absorption and hence can be used to overcome iron-deficiency anemias. Possesses antibacterial activity
Cow	Interferon	Provides resistance against viral infections
Sheep	α_1-Antitrypsin	Used in the treatment of emphysema (promotes the exchange of gases in lungs)
Goat	Cystic fibrosis transmembrane regulator (CFTR)	For the treatment of patients suffering from cystic fibrosis (promotes transport of ions)
Goat	Tissue plasminogen activator (tPA)	Used in treating the patients of myocardial infarction (dissolves blood clots)
Goat	Antithrombin III	Regulates blood clotting
Rabbits	α-Glucosidase	Treatment of Pompe's disease (a genetic disorder characterized by block in glycogen degradation)
Mouse	Urokinase	For dissolving blood clots
Mouse	Immunoglobulins (antibodies)	Administration enhances immunity
Pig	Hemoglobin	Blood transfusion
Goat and other animals	Vaccines (?)	To immunize against various diseases

Transgenic sheep with increased wool production:

Keratin is the wool protein with highly cross- linked disulfide bridges. For good production of quality wool, the amino acid cysteine (or its precursor methionine) is required in large quantities. However, cysteine supply to sheep is always inadequate, since the microbes harboring the rumen utilize it and release in the form of sulfide. This problem can be overcome by producing transgenic sheep containing bacterial genes for the synthesis of cysteine. The two enzymes, synthesized by the transgenes, are capable of trapping the hydrogen sulfide liberated in the intestine to produce cysteine. Thus, good supply of cysteine to the sheep improves the quality and quantity of wool.

Transgenic Pigs:

Transgenic pigs that can produce human haemoglobin have been successfully developed. This human haemoglobin can be separated from pig haemoglobin by simple analytical techniques. Hemoglobin, the oxygen carrying protein of RBC, can be used as a substitute in blood transfusion experiments.

In fact, haemoglobin can be stored for longer period (a few months) than whole blood (weeks only). Further, there is no problem of contamination (like HIV) as is the case with whole blood. However, the free haemoglobin (naked haemoglobin) cannot transport oxygen as effectively as the haemoglobin of RBC. In addition, naked haemoglobin is easily degraded and the breakdown products cause damage to kidney. There also exists a risk of contamination by pig viruses and other compounds to cause allergic reactions. With these limitations, the initial enthusiasm for substituting blood transfusion with free haemoglobin has remained short-lived. It is now advised not to use naked haemoglobin for transfusion, when there is a heavy blood loss. However, it can be used during major surgeries for supplementing the whole blood transfusion.

Pig in organ farms:

The human organs such as heart, liver, pancreas, kidney and lungs are in great demand for transplantation surgery. The shortage of these transplantable organs can be overcome by developing them in animals. Pig is a favourite animal for harvesting human organs. This is because the physiology of pigs is close to that of humans.

Further, pigs do not carry any major infectious diseases transmissible to humans. The use of pigs in organ farming is still at the experimental stages. In the preliminary experiments, organ transplantation from transgenic pigs into primates showed some promising results. The day may not be very far for utilizing transgenic pigs as donors of human organs.

Transgenic Chickens:

The production of transgenic chickens (or other birds) is rather complicated. This is mainly because during fertilization in chickens, several sperms enter the ovum instead of one. This is in contrast to mammals where usually only one sperm enters the egg. The

identification of male pronuclei that will fuse with female pronuclei is quite difficult. Further, embryonic stem (ES) cells have not been identified in chicken. Despite all these limitations, transgenic chickens have been developed.

The blastoderm cells (from an egg) can be removed from a donor chicken. They are transfected with transgenes (usually by lipofection with liposomes). The so modified blastoderm cells are reintroduced into the sub-germinal space of irradiated blastoderm of freshly laid eggs. Some of the resulting chickens may carry the transgene. Transgenic lines of chickens can be established.Trans-genesis in chicken can be used to develop low fat and cholesterol, and high protein containing eggs. Transgenic chickens that are resistant to viral and bacterial diseases have also been developed. Some attempts have also been made to develop pharmaceutical proteins in the eggs of transgenic chickens.

Transgenic Fish:

Several transgenic fish (catfish, salmon, trout etc.) have been developed with increase in their growth and size. This was carried out by introducing growth hormone transgene (by microinjection or electroporation). The fertilized eggs with inserted transgene are incubated in temperature-regulated holding tanks. (Note: The fish egg development is external in contrast to the mammalian embryogenesis). The efficiency of fish trans-genesis is as high as 70%. It was found that the transgenic salmon fish (with growth hormone transgene) were 10 times heavier than the normal ones, at the end of one year.

Aquatic animals are being engineered to increase aquaculture production, for medical and industrial research, and for ornamental reasons (Fig. 18.7). Genes inserted to promote disease resistance may allow transgenic fish to absorb higher levels of toxic substances, including heavy metals. In turn, consumers of these fish may be ingesting higher amounts of substances such as mercury and selenium.

Transgenic fish that have genes from species such as peanuts or shellfish that are common causes of allergic reactions in humans may prompt allergic reactions in an unsuspecting consumer. Transgenic species may behave much like invasive species when interacting with the natural environment. They may compete with native species for resources and pose a threat to the genetic diversity of native populations, especially when genetic modifications such as a rapid growth rate offer advantages over slower-developing native species. Despite industry assurances that transgenic fish would be unable to naturally reproduce or significantly threaten the environment, some scientists are far more doubtful.

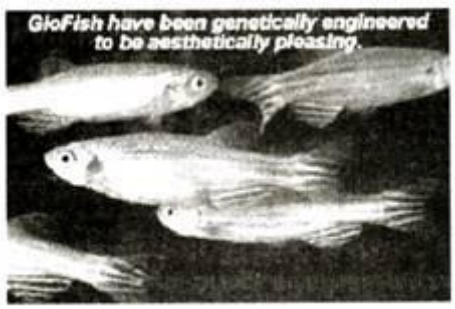


Fig. 18.7. Transgenic fish.

The sample bill included in this package addresses these concerns by banning the importation, transportation, possession, spawning, incubation, cultivation, or release of aquatic transgenic animals except under a permit.

Fluorescent Cat:

Recently South Korean scientist produced transgenic white Turkish angora cats to glow red under ultraviolet light these cats contain a fluorescent gene for flu protein and expressed under skin. Subsequently they produced a number of cloned cats from the skin cells of transformed mother cat. They proposed that such cat could be beneficial in diagnosis of genetic diseases and also showed a way to produce endangered animal by cloning.

Use of DNA in the Diagnosis of Infectious and Genetic Diseases

I. DNA in the Diagnosis of Infectious Diseases :

The use of DNA analysis (by employing DNA probes) is a novel and revolutionary approach for specifically identifying the disease-causing pathogenic organisms. This is in contrast to the traditional methods of disease diagnosis by detection of enzymes, antibodies etc., besides the microscopic examination of pathogens.Although at present not in widespread use, DNA analysis may soon take over the traditional diagnostic tests in the years to come. Diagnosis of selected diseases by genetically engineered techniques or DNA probes or direct DNA analysis is briefly described.

Tuberculosis:

Tuberculosis is caused by the bacterium Mycobacterium tuberculosis. The commonly used diagnostic tests for this disease are very slow and sometimes may take several weeks. This is because M. tuberculosis multiplies very slowly (takes about 24 hrs. to double; E. coli takes just 20 minutes to double).

A novel diagnostic test for tuberculosis was developed by genetic engineering, and is illustrated in Fig. 14.3. A gene from firefly, encoding the enzyme luciferase is introduced

into the bacteriophage specific for M. tuberculosis. The bacteriophage is a bacterial virus, frequently referred to as luciferase reporter phage or mycophage.

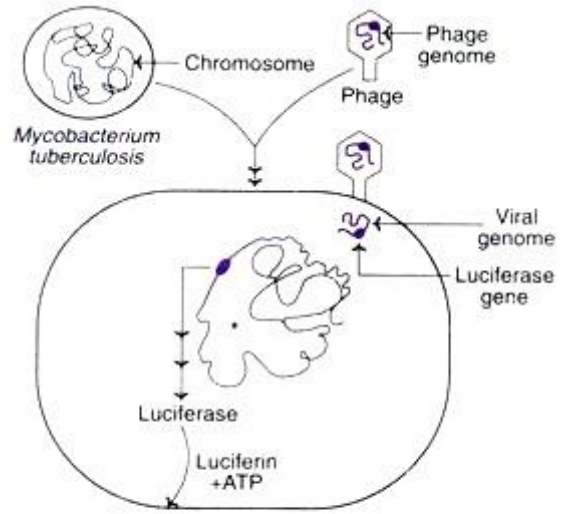


***Fig. 14.3** : Diagnosis of tuberculosis by using a genetically engineered bacteriophage (phage).*

The genetically engineered phage is added to the culture of M. tuberculosis. The phage attaches to the bacterial cell wall, penetrates inside, and inserts its gene (along with luciferase gene) into the M. tuberculosis chromosome. The enzyme luciferase is produced by the bacterium.

When luciferin and ATP are added to the culture medium, luciferase cleaves luciferin. This reaction is accompanied by a flash of light which can be detected by a luminometer. This diagnostic test is quite sensitive for the confirmation of tuberculosis. The flash of light is specific for the identification of M. tuberculosis in the culture. For other bacteria, the genetically engineered phage cannot attach and enter in, hence no flash of light would be detected.

Malaria:

Malaria, mainly caused by *Plasmodium falciparum* and *P. vivax*, affects about one-third of the world's population. The commonly used laboratory tests for the diagnosis of malaria include microscopic examination of blood smears, and detection of antibodies in the circulation. While the former is time consuming and frequently gives false-negative tests, the latter cannot distinguish between the past and present infections.

A specific DNA diagnostic test for identification of the current infection of *P. falciparum* has been developed. This is carried out by using a DNA probe that can bind and hybridize with a DNA fragment of *P. falciparum* genome and not with other species of

Plasmodium. It is reported that this DNA probe can detect as little as 1mg of *P. falciparum* in blood or 10 pg of its purified DNA.

Chagas' Disease:

The protozoan parasite *Trypanosoma cruzi* causes Chagas' disease. This disease is characterized by destruction of several tissues (liver, spleen, brain, lymph nodes) by the invading parasite. Chagas' disease is diagnosed by the microscopic examination of the fresh blood samples. Immunological tests, although available, are not commonly used, since they frequently give false-positive results.

Scientists have identified a DNA fragment with 188-base pair length present in *T. cruzi* genome. This is however, not found in any other related parasite. A PCR technique is employed to amplify the 188 bp DNA fragment. This can be detected by using polyacrylamide gel electrophoresis. Thus, PCR-based amplification can be effectively used for the diagnosis of Chagas' disease.

Acquired Immunodeficiency Syndrome (AIDS):

AIDS is caused by the virus, human immunodeficiency virus (HIV). The commonly used laboratory test for detection of AIDS is the detection of HIV antibodies. However, it might take several weeks for the body to respond and produce sufficient HIV antibodies. Consequently, the antibodies test may be negative (i.e., false-negative), although HIV is present in the body. During this period, being a carrier, he/she can transmit HIV to others.

DNA probes, with radioisotope label, for HIV DNA are now available. By using PCR and DNA probes, AIDS can be specifically diagnosed in the laboratory. During the course of infection cycle, HIV exists as a segment of DNA integrated into the T-lymphocytes of the host. The T-lymphocytes of a suspected AIDS patient are isolated and disrupted to release DNA. The so obtained DNA is amplified by PCR, and to this DNA probes are added. If the HIV DNA is present, it hybridizes with the complementary sequence of the labelled DNA probe which can be detected by its radioactivity. The advantage of DNA probe is that it can detect the virus when there are no detectable antibodies in the circulation.

HIV diagnosis in the newborn:

Detection of antibodies is of no use in the newborn to ascertain whether AIDS has been transmitted from the mother. This is because the antibodies might have come from the mother but not from the virus. This problem can be solved by using DNA probes to detect HIV DNA in the newborn.

Human Papilloma Virus:

Human papilloma virus (HPV) causes genital warts. HPV is also associated with the cervical cancer in women. The DNA probe (trade name Virapap detection kit) that

specifically detects HPV has been developed. The tissue samples obtained from woman's cervix are used. HPV DNA, when present hybridizes with DNA probe by complementary base pairing, and this is the positive test.

Lyme Disease:

Lyme disease is caused by the bacterium, Borrelia burgdorferi. This disease is characterized by fever, skin rash, arthritis and neurological manifestations. The diagnosis of Lyme disease is rather difficult, since it is not possible to see B. burgdorferi under microscope and the antibody detection tests are not very reliable. Some workers have used PCR to amplify the DNA of B. burgdorferi. By using appropriate DNA probes, the bacterium causing Lyme disease can be specifically detected.

Periodontal Disease:

Periodontal disease is characterized by the degenerative infection of gums that may ultimately lead to tooth decay and loss. This disease is caused by certain bacteria. At least three distinct species of bacteria have been identified and DNA probes developed for their detection. Early diagnosis of periodontal disease will help the treatment modalities to prevent the tooth decay.

DNA Probes for Other Diseases:

In principle, almost all the pathogenic organisms can be detected by DNA probes. Several DNA probes (more than 100) have been developed and many more are in the experimental stages. The ultimate aim of the researchers is to have a stock of probes for the detection of various pathogenic organisms—bacteria, viruses, parasites. The other important DNA probes in recent years include for the detection of bacterial infections caused by E. coli (gastroenteritis) Salmonella typhi (food poisoning), *Campylobacter hyoitestinalis* (gastritis).

Diagnosis of tropical diseases:

Malaria, filariasis, tuberculosis, leprosy, schistosomiasis, leishmaniasis and trypanosomiasis are the tropical diseases affecting millions of people throughout the world. As already described for the diagnosis of malaria caused by P. falciparum, a DNA probe has been developed. A novel diagnostic test, by genetic manipulations, has been devised for the diagnosis of tuberculosis. Scientists are continuously working to develop better diagnostic techniques for other tropical diseases.

B. DNA in the Diagnosis of Genetic Diseases:

Traditional laboratory tests for the diagnosis of genetic diseases are mostly based on the estimation of metabolites and/or enzymes. This is usually done after the onset of symptoms. The laboratory tests based on DNA analysis can specifically diagnose the inherited diseases at the genetic level. DNA-based tests are useful to discover, well in advance, whether the individuals or their offspring's are at risk for any genetic disease;

Further, such tests can also be employed for the prenatal diagnosis of hereditary disorders, besides identifying the carriers of genetic diseases.

By knowing the genetic basis of the diseases, the individuals can be advised on how to limit the transmission of the disease to their offspring's. It may also be possible, in due course of time, to treat genetic diseases by appropriate gene therapies. Theoretically, it is possible to develop screening tests for all single-gene diseases. Some of the important genetic diseases for which DNA analysis is used for diagnosis are briefly described.

Cystic Fibrosis:

Cystic fibrosis (CF) is a common and fatal hereditary disease. The patients produce thick and sticky mucus that clogs lungs and respiratory tract. Cystic fibrosis is due to a defect in cftr gene that encodes cystic fibrosis trans membrane regulator protein, CFTR gene is located on chromosome 7 in humans, and a DNA probe has been developed to identify this gene.

The genetic disease cystic fibrosis is inherited by a recessive pattern, i.e., the disease develops when two recessive genes are present. It is now possible to detect CF genes in duplicate in the fetal cells obtained from samples of amniotic fluid. As the test can be done months before birth, it is possible to know whether the offspring will be a victim of CF. One group of researchers have reported that CF gene can be detected in the eight-celled embryo obtained through in vitro fertilization.

Sickle-Cell Anaemia:

Sickle-cell anaemia is a genetic disease characterized by the irregular sickle (crescent like) shape of the erythrocytes. Biochemically, this disease results in severe anaemia and progressive damage to major organs in the body (heart, brain, lungs, and joints).Sickle-cell anaemia occurs due to a single amino acid change in the β-chain of haemoglobin. Specifically, the amino acid glutamate at the 6th position of β-chain is replaced by valine. At the molecular level, sickle-cell anaemia is due to a single-nucleotide change (A → T) in the β-globin gene of coding (or antisense) strand.

In the normal β-globin gene the DNA sequence is CCTGAGGAG, while in sickle-cell anaemia, the sequence is CCTGTGGAG. This single-base mutation can be detected by using restriction enzyme Mstll to cut DNA fragments in and around β-globin gene, followed by the electrophoretic pattern of the DNA fragments formed.

The change in the base from A to T in the β-globin gene destroys the recognition site (CCTGAGG) for Mstll (Fig. 14.4). Consequently, the DNA fragments formed from a sickle-cell anemia patient for β-globin gene differ from that of a normal person. Thus, sickle-cell anaemia can be detected by digesting mutant and normal β-globin gene by restriction enzyme and performing a hybridization with a cloned β-globin DNA probe.

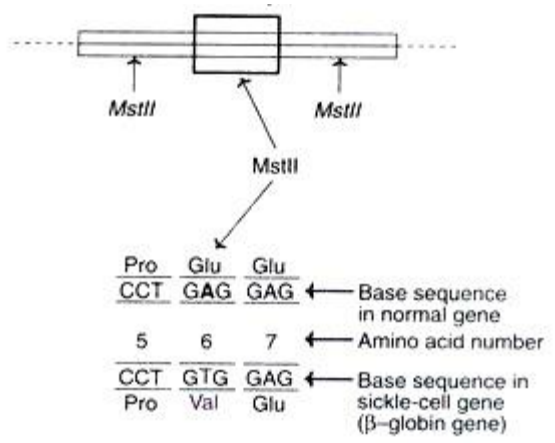


*Fig. 14.4 : Single-base change resulting in sickle-cell anemia. (**Note** : A small and relevant DNA fragment of β-globin gene magnified and shown with encoded amino acids).*

Single-nucleotide polymorphisms:

The single base changes that occur in some of the genetic diseases (e.g., sickle-cell anaemia) are collectively referred to as single-nucleotide polymorphisms (SNPs, pronounced snips). It is estimated that the frequency of SNPs is about one in every 1000 bases. Sometimes SNPs are associated with amino acid change in the protein that is encoded. A point mutation in α_1-antitrypsin gene is also a good example of SNPs, besides sickle-cell anaemia.

Duchenne's Muscular Dystrophy:

Duchenne's muscular dystrophy (DMD) is a genetic abnormality characterized by progressive wasting of leg and pelvic muscles. It is a sex-linked recessive disease that appears between 3 and 5 years of age. The affected children are unsteady on their feet as they lose the strength and control of their muscles. By the age of ten, the victims of DMD are confined to wheel chair and often die before reaching 20 years age.

The patients of DMD lack the muscle protein, namely dystrophin which gives strength to the muscles. Thus, DMD is due to the absence of a gene encoding dystrophin. For specific diagnosis of Duchenne's muscular dystrophy, a DNA probe to identify a segment of DNA that lies close to defective gene (for dystrophin) is used. This DNA segment, referred to as restriction fragment length polymorphism (RFLP), serves as a marker and can detect DMD with 95% certainty.

In the DNA diagnostic test using RFLP for DMD, DNA samples must be obtained from as many blood relatives (parents, grand-parents, uncles, aunts etc.) as possible. The RFLP patterns, constructed for the entire family are thoroughly checked for the affected and unaffected relatives. This is required since there is a wide variation in RFLPs from

family to family. Thus, there is no single identifying test for the diagnosis of genetic diseases based on RFLPs analysis.

Huntington's Disease:

Huntington's disease is a genetic disease (caused by a dominant gene) characterized by progressive deterioration of the nervous system, particularly the destruction of brain cells. The victims of this disease (usually above 50 years of age) exhibit thrashing (jerky) movements and then insanity [older name was Huntington's chorea; chorea (Creek) means to dance]. Huntington's disease is invariably fatal.

The molecular basis of Huntington's disease has been identified. The gene responsible for this disease lies on chromosome number 4, and is characterized by excessive repetition of the base triplet CAG. The victims of Huntington's disease have CAG triplet repeated 42-66 times, against the normal 11-34 times. The triplet CAG encodes for the amino acid glutamine. It is believed that the abnormal protein (with very high content of glutamine) causes the death of cells in the basal ganglia (the part of the brain responsible for motor function).

Huntington's disease can be detected by the analysis of RFLPs in blood related individuals. The clinical manifestations of this disease are observed after middle age, and by then the person might have already passed on the defective gene to his/ her offspring's.

Fragile X Syndrome:

Fragile X syndrome, as the name indicates, is due to a genetic defect in X chromosome (a sex chromosome) and affects both males and females. The victims of this disease are characterized by mental retardation. Researchers have found that sufferers of fragile X syndrome have the three nucleotide bases (CGG) repeated again and again.

It is believed that these tri-nucleotide repeats block the transcription process resulting in a protein deficiency. This protein is involved in the normal function of the nerve cells, and its deficiency results in mental retardation. A DNA probe has been developed for the detection of fragile X syndrome in the laboratory.

Other Triple Repeat Diseases:

Excessive repetition of triplet bases in DNA are now known to result in several diseases which are collectively referred to as triple repeat diseases. Besides Huntington's disease and fragile X syndrome, some more triple repeats are given below.

Friedreich's ataxia:

The tri-nucleotide GAA repeats 200 to 900 times on chromosome 9 in Friedreich's ataxia. This disease is associated with degradation of spinal cord. Spinocerebellar ataxia

is another triplet disease, characterized by neuromuscular disorder, and is due to tri-nucleotide repeats of CAG by 40 to 80 times on chromosome 6.

There are a few triple repeat diseases in which the repeats tend to increase with each generation and the diseases become more severe. This also results in the onset of clinical manifestations at early ages. Kennedy's disease, also called spinobulbar muscular atropy (CAG repeat) and myotonic dystrophy (CTG) are good examples.

Are triple repeat diseases confined to humans?

Triple repeat diseases have so far not been detected in any other organisms (bacteria, fruit flies, other mammals) except in humans. More studies however, may be needed to confirm this. The occurrence of triple repeat diseases indicates that the structure of DNA may be rather unstable and dynamic. This is in contrast to what molecular biologists have been thinking all along.

Alzheimer's Disease:

Alzheimer's disease is characterized by loss of memory and impaired intellectual function (dementia). The victims of this disease cannot properly attend to their basic needs, besides being unable to speak and walk. The patients of Alzheimer's disease were found to have a specific protein, namely amyloid in the plaques (or clumps) of dead nerve fibers in their brains. A group of researchers have identified a specific gene on chromosome 21 that is believed to be responsible for familial Alzheimer's disease.

A DNA probe has been developed to locate the genetic marker for Alzheimer's disease. The present belief is that many environmental factors and a virus may also be responsible for the development of this disease. It may be possible that in the individuals with genetic predisposition, the outside factors may be stimulatory for the onset of the disease.

Amyotrophic Lateral Sclerosis:

Amyotrophic lateral sclerosis (ALS) is characterized by degenerative changes in the motor neurons of brain and spinal cord. A gene to explain the inherited pattern of ALS was discovered. The gene, known as sodl, encoding for the enzyme superoxide dismutase is located on chromosome 21. This gene was found to be defective in families suffering from amyotrophic lateral sclerosis. In fact, certain point mutations in the sodl resulting in single amino acid changes in superoxide dismutase have been identified.

Superoxide dismutase is a key enzyme in eliminating the highly toxic free radicals that damage the cells (free radicals have been implicated in aging and several disease e.g. cancer, cataract, Parkinson's disease, Alzheimer's disease). On the basis of the function of superoxide dismutase, it is presumed that ALS occurs as a result of free radical accumulation due to a defective enzyme (as a consequence of mutated gene sodl). The

deleterious effects of free radicals can be reduced by administering certain compounds such as vitamins C and E.

Another group of workers have reported that the defective superoxide dismutase cannot control a transporter protein responsible for the removal of the amino acid glutamate from the nerve cells. As a result, large quantities of glutamate accumulate in the nervous tissue leading to degenerative changes.

Cancers:

It is now agreed that there is some degree of genetic predisposition for the occurrence of cancers, although the influence of environmental factors cannot be underestimated. In fact, cancer susceptible genes have been identified in some families e.g., genes for melanoma susceptibility in humans are located on chromosomes 1 and 9.

p^{53} Gene:

The gene p^{53} encodes for a protein with a molecular weight 53 kilo Daltons (hence the name). It is believed that the protein produced by this gene helps DNA repair and suppresses cancer development. Certain damages that occur in DNA may lead to unlimited replication and uncontrolled multiplication of cells.

In such a situation, the protein encoded by p^{53} gene binds to DNA and blocks replication. Further, it facilitates the faulty DNA to get repaired. The result is that the cancerous cells are not allowed to establish and multiply. Thus, p^{53} is a cancer-suppressor gene and acts as a guardian of cellular DNA.

Any mutation in the gene p^{53} is likely to alter its tumor suppressor function that lead to cancer development. And in fact, the altered forms of p^{53} recovered from the various tumor cells (breast, bone, brain, colon, bladder, skin, lung) confirm the protective function of p^{53} gene against cancers.

It is believed that the environmental factors may cause mutations in p^{53} gene which may ultimately lead to cancer. Some of the mutations of p^{53} gene may be inherited, which probably explains the occurrence of certain cancers in some families.

Genes of breast cancer:

Two genes, namely BRCAI and BRCAII, implicated in certain hereditary forms of breast cancer in women, have been identified. It is estimated that about 80% of inherited breast cancers are due to mutations in either one of these two genes — BRCAI or BRCAII. In addition, there is a high risk for ovarian cancer due to mutations in BRCAI.

It is suggested that the normal genes BRCAI and BRCAII encode proteins (with 1863 and 3418 amino acids respectively) that function in a manner comparable to gene p^{53} protein (as described above). As such, BRCAI and BRCAII are DNA- repair and tumor-suppressor genes. Some researchers believe that these two proteins act as gene regulators. Diagnostic tests for the analysis of the genes BRCAI and BRCAII were

developed. Unfortunately, their utility is very limited, since there could be hundreds of variations in the base sequence of these genes.

Genes of colon cancer:

The occurrence of colon cancer appears to be genetically linked since it runs in some families. Some researchers have identified a gene linked with hereditary non-polyposis colon cancer or HNPCC (sometimes called Lynch syndrome). This gene encoded a protein that acts as a guardian and brings about DNA repair whenever there is a damage to it.However, as and when there is a mutation to this protective gene, an altered protein is produced which cannot undo the damage done to DNA. This leads to HNPCC. It is estimated that the occurrence of this altered gene is one in every 200 people in general population.

Microsatellite marker genes:

Microsatellites refer to the short repetitive sequences of DNA that can be employed as markers for the identification of certain genes. For colon cancer, microsatellite marker genes have been identified on chromosome 2 in humans. There is a lot of variability in the sequence of microsatellites.

Early detection of the risk for colon cancer by DNA analysis is a boon for the would be victims of this disease. The suspected individuals can be periodically monitored for the signs and treated appropriately. Unlike many other cancers, the chances of cure for colon cancer are reasonably good.

Gene of retinoblastoma:

Retinoblastoma is a rare cancer of the eye. If detected early, it can be cured by radiation therapy and laser surgery or else the eyeball has to be removed. Scientists have identified a missing or a defective (mutated) gene on chromosome number 13, being responsible for retinoblastoma. The normal gene when present on chromosome 13 is anticancer and does not allow retinoblastoma to develop.

Diabetes:

Diabetes mellitus is a clinical condition characterized by increased blood glucose level (hyperglycemia) due to insufficient or inefficient (incompetent) insulin. In other words, individuals with diabetes cannot utilize glucose properly in their body.

A rare form of type II diabetes (i.e., non-insulin dependent diabetes mellitus, NIDDM) is maturity onset diabetes of the young (MODY). MODY, occurring in adolescents and teenagers is found to have a genetic basis. A gene, synthesizing the enzyme glucokinase, located on chromosome 7, is found to be defective in MODY patients. Glucokinase is a key enzyme in glucose metabolism. Besides its involvement in the metabolism, glucokinase in the pancreatic cells serves as a detector for glucose concentration in the blood. This detection stimulates β-cells of the pancreas to secrete insulin. A gene

modification that results in a defective or an altered glucokinase hampers pancreatic insulin secretion. Later work has shown that glucokinase gene is defective in the common form of type II diabetes.

DNA probes for type II diabetes:
The glucokinase genes from normal and type II diabetes patients were cloned and scanned with DNA probes. It was found that a single base mutation of the gene led to a defective glucokinase production that is largely responsible for MODY, and also a majority of individuals with type II diabetes. Later, some workers reported a possibility of at least a dozen mutations in glucokinase gene for type II diabetes.

Genes responsible for type I diabetes:

Type I diabetes or insulin-dependent diabetes mellitus (IDDM) mainly occurs in childhood, particularly between 12-15 years of age. IDDM is characterized by almost total deficiency of insulin. Researchers have identified at least 18 different chromosome regions linked with type I diabetes. These DNA sequences are located on chromosomes 6, 11 and 18.

Obesity:

Obesity is an abnormal increase in the body weight due to fat deposition. Men and women are considered obese if their weight due to fat, respectively exceeds more than 20% and 25% of the body weight. Obesity increases the risk of high blood pressure, diabetes, atherosclerosis and other life-threatening conditions.

Although many believed that obesity could be genetically inherited, the molecular basis was not known for long. It was in 1994, a group of workers identified a mutated gene that caused obesity in mice. Later, a similar gene was found in humans also.

The gene designated ob (for obese) is located on chromosome 6 in mouse. The DNA of ob gene contains 650 kb and encodes a protein with 167 amino acids in adipose tissue. This protein is responsible to keep the weight of the animals under control. The genetically obese mice have mutated ob gene and therefore the weight-control protein is not produced. It is believed that this protein functions like a hormone, acts on the hypothalamus, and controls the site of hunger and energy metabolism (these two factors are intimately linked with obesity).

With the discovery of ob gene, the treatment for inherited obesity may soon become a reality. In fact, one multinational biotechnology company has started producing ob protein that can be used for weight reduction in experimental mice. Besides the ob gene, a few other genes (fat gene, tub gene) that might be associated with obesity have also been discovered.

DNA Analysis for Other Human Diseases:

There is a continuous search for the identification of more and more genes that are responsible for human diseases. Such an approach will ultimately help in the specific diagnosis of these diseases before their actual occurrence. In addition to human diseases described above, some more are given below.

Deafness:

The deafness, inherited in some families, has genetic basis. A team of workers have identified a gene on chromosome 5, encoding a protein that facilitates the assembly of actin (protein) molecules in the cochlea of inner ear. The association of actin is very essential for the detection of sound waves by the ear.A mutation of the gene on chromosome 5 results in a defective protein synthesis and non- assembly of actin molecules which cause deafness. Some other genes, besides the one described here, have also been found to be associated with deafness.

Glaucoma:

Glaucoma is a disease of the eye that may often lead to blindness. It occurs as a result of damage to the optic nerve due to pressure that builds up in the eye. A gene responsible for the hereditary glaucoma in teenagers has been detected on chromosome 1. Another group of researchers have found a gene on chromosome 3 which is linked with the adult-onset glaucoma.

Baldness:

There is an inherited form of baldness, called alopecia universals. This is found to be associated with a gene located on chromosome 12.

Parkinson's disease:

Parkinson's disease is a common disorder in many elderly people, with about 1% of the population above 60 years being affected. It is characterized by muscular rigidity, tremors, expressionless face, lethargy, involuntary movements etc. In the victims of Parkinson's disease, there is degeneration of brain cells, besides a low concentration of dopamine (a neurotransmitter).

Researchers have identified that a gene-encoded protein namely α-synuclein plays a significant role in the development of Parkinson's disease. An altered form of α-synuclein (due to a mutation in the gene) accumulates in the brain as Lewy bodies. This is responsible for nerve cells degeneration and their death in the Parkinson's disease.

Hemochromatosis:

Hemochromatosis is an iron-overload disease in which iron is directly deposited in the tissues (liver, spleen, heart, pancreas and skin). An abnormal gene on chromosome 6 is linked with hemochromatosis. The amino acid tyrosine, in the normal protein encoded

by this gene is replaced by cysteine. This abnormal protein is responsible for excessive iron absorption from the intestine which accumulates in the various tissues leading to their damage and malfunction.

Menke's disease:

Menke's disease, a copper deficiency disorder, is characterized by decreased copper in plasma, depigmentation of hair, degeneration of nerve cells and mental retardation. A gene located on X-chromosome, encoding a transport protein, is linked with Menke's disease. A defect in the gene, consequently in the protein, impairs copper absorption from the intestine.

Gene Banks—A Novel Concept:

As the search continues by scientists for the identification of more and more genes responsible for various diseases, the enlightened public (particularly in the developed countries), is very keen to enjoy the fruits of this research outcome. As of now, DNA probes are available for the detection a limited number of diseases. Researchers continue to develop DNA probes for a large number of genetically predisposed disorders.

Gene banks are the centres for the storage of individual's DMAs for future use to diagnose diseases. For this purpose, the DNA isolated from a person's cells (usually white blood cells) is stored. As and when a DNA probe for the detection of a specific disease is available, the stored DNA can be used for the diagnosis or risk assessment of the said genetic disease.

In fact, some institutions have established gene banks. They store the DNA samples of the interested customers at a fee (one firm was charging $ 200) for a specified period (say around 20-25 years). For the risk assessment of any disease, it is advisable to have the DNAs from close relatives of at least 2-3 generations.

Gene Therapy:

Gene therapy is a novel treatment method which utilizes genes or short oligonucleotide sequences as therapeutic molecules, instead of conventional drug compounds. This technique is widely used to treat those defective genes which contribute to disease development. Gene therapy involves the introduction of one or more foreign genes into an organism to treat hereditary or acquired genetic defects. In gene therapy, DNA encoding a therapeutic protein is packaged within a "vector", which transports the DNA inside cells within the body. The disease is treated with minimal toxicity, by the expression of the inserted DNA by the cell machinery. In 1990 FDA for the first time approved a gene therapy experiment on ADA-SCID in the United States after the treatment of Ashanti DeSilva. After that, approximately 1700 clinical trials on patients have been performed with various techniques and genes for numerous diseases.

Gene therapy is the process of inserting genes into cells to treat diseases. The newly introduced genes will encode proteins and correct the deficiencies that occur in genetic diseases. Thus, gene therapy primarily involves genetic manipulations in animals or humans to correct a disease, and keep the organism in good health. The initial experiments on gene therapy are carried out in animals, and then in humans. Obviously, the goal of the researchers is to benefit the mankind and improve their health.

An overview of gene therapy strategies is depicted in Fig. 13.1. In gene augmentation therapy, a DNA is inserted into the genome to replace the missing gene product. In case of gene inhibition therapy, the antisense gene inhibits the expression of the dominant gene.

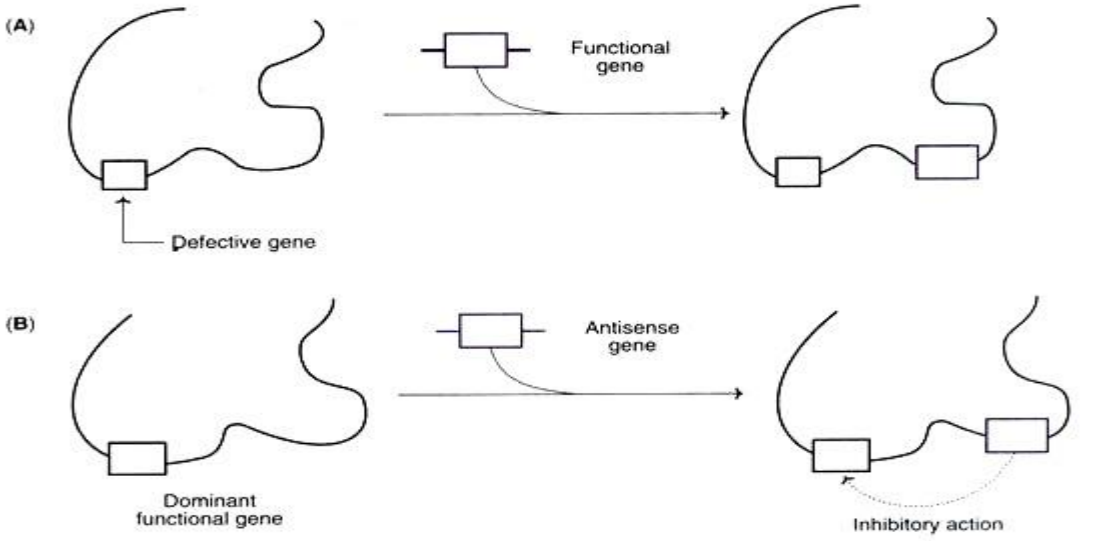


Fig. 13.1 : Overview of two major gene therapy strategies (A) Gene augmentation therapy (B) Gene inhibition therapy.

I. General gene therapy strategies

a. Gene Augmentation Therapy (GAT):

For diseases caused by loss of function of a gene, introducing extra copies of the normal gene may increase the amount of normal gene product to a level where the normal phenotype is restored (see Fig. 23.1). As a result GAT is targeted at clinical disorders where the pathogenesis is reversible.

It also helps to have no precise requirement for expression levels of the introduced gene and a clinical response at low expression levels. GAT has been particularly applied to autosomal recessive disorders where even modest expression levels of an introduced gene may make a substantial difference.

Dominantly inherited disorders are much less amendable to treatment; gain-of-function mutations are not treatable by this approach and, even if there is a loss-of- function

mutation, high expression efficiency of the introduced gene is required: individuals with 50% of normal gene product are normally affected, and so the challenge is to increase the amount of gene product towards normal levels.

b. Targeted Killing of Specific Cells:
This general approach is popular in cancer gene therapies. Genes are directed to the target cells and then expressed so as to cause cell killing. Direct cell killing is possible if the inserted genes are expressed to produce a lethal toxin (suicide genes), or a gene encoding a pro drug is inserted, conferring susceptibility to killing by a subsequently administered drug. Indirect cell killing uses immunostimulatory genes to provoke or enhance an immune response against the target cell.

c. Targeted Mutation Correction:
If an inherited mutation produces a dominant-negative effect, gene augmentation is unlikely to help. Instead, the resident mutation must be corrected. Because of practical difficulties, this approach has yet to be applied but, in principle, it can be done at different levels: at the gene level (e.g. by gene targeting methods based on homologous recombination); or at the RNA transcript level (e.g. by using particular types of therapeutic ribozymes — or therapeutic RNA editing).

d. Targeted Inhibition of Gene Expression:
If disease cells display a novel gene product or inappropriate expression of a gene (as in the case of many cancers, infectious diseases, etc.), a variety of different systems can be used specifically to block the expression of a single gene at the DNA, RNA or protein levels. Allele-specific inhibition of expression may be possible in some cases, permitting therapies for some disorders resulting from dominant negative effects.

II. Approaches for Gene Therapy:

There are two approaches to achieve gene therapy.

1. Somatic Cell Gene Therapy:

The non- reproductive (non-sex) cells of an organism are referred to as somatic cells. These are the cells of an organism other than sperm or eggs cells, e.g., bone marrow cells, blood cells, skin cells, intestinal cells. At present, all the research on gene therapy is directed to correct the genetic defects in somatic cells. In essence, somatic cell gene therapy involves the insertion of a fully functional and expressible gene into a target somatic cell to correct a genetic disease permanently.

2. Germ Cell Gene Therapy:

The reproductive (sex) cells of an organism constitute germ cell line. Gene therapy involving the introduction of DNA into germ cells is passed on to the successive

generations. For safety, ethical and technical reasons, germ cell gene therapy is not being attempted at present.

The genetic alterations in somatic cells are not carried to the next generations. Therefore, somatic cell gene therapy is preferred and extensively studied with an ultimate objective of correcting human diseases. Development of gene therapy in humans for any specific disease involves the following steps. In fact, this is a general format for introducing any therapeutic agent for human use.

a. In vitro experiments and research on laboratory animals (pre-clinical trials).

b. Phase I trials with a small number (5-10) of human subjects to test safety of the product.

c. Phase II trials with more human subjects to assess whether the product is helpful.

d. Phase III trials in large human samples for a final and comprehensive analysis of the safety and efficacy of the product.

As such, gene therapy involves a great risk. There are several regulatory agencies whose permission must be sought before undertaking any work related to gene therapy. Recombinant DNA Advisory Committee (RAC) is the supervisory body of the National Institute of Health, U.S.A. that clears proposals on experiments involving gene therapy. A large number of genetic disorders and other diseases are currently at various stages of gene therapy trials. A selected list of some important ones is given in Table 13.1.

TABLE 13.1 Human gene therapy trials

Disease	*Gene therapy*
Severe combined immunodeficiency (SCID)	Adenosine deaminase (ADA).
Cystic fibrosis	Cystic fibrosis transmembrane regulator (CFTR).
Familial hypercholesterolemia	Low density lipoprotein (LDL) receptor.
Emphysema	α_1-Antitrypsin
Hemophilia B	Factor IX
Thalassemia	α- or β-Globin
Sickle-cell anemia	β-Globin
Lesch-Nyhan syndrome	Hypoxanthine-guanine phosphoribosyltransferase (HGPRT).
Gaucher's disease	Glucocerebrosidase
Peripheral artery disease	Vascular endothelial growth factor (VEGF)
Fanconi anemia	Fanconi anemia C
Melanoma	Tumor necrosis factor (TNF)
Melanoma, renal cancer	Interleukin-2 (IL-2)
Glioblastoma (brain tumor), AIDS, ovarian cancer	Thymidine kinase (herpes simplex virus)
Head and neck cancer	p^{53}
Breast cancer	Multidrug resistance I
AIDS	*rev* and *env*
Colorectal cancer, melanoma, renal cancer	Histocompatability locus antigen-B_7 (HLA-B_7)
Duchenne muscular dystrophy	Dystrophin
Short stature*	Growth hormone
Diabetes*	Glucose transporter-2, (GLUT-2), glucokinase
Phenylketonuria*	Phenylalanine hydroxylase
Citrullinemia*	Arginosuccinate synthetase

III. Methods of gene therapy:

There are mainly two approaches for the transfer of genes in gene therapy:

1. Transfer of genes into patient cells outside the body (ex vivo gene therapy)

2. Transfer of genes directly to cells inside the body (in vivo).

1. Ex vivo gene therapy:

The ex vivo gene therapy can be applied to only selected tissues (e.g., bone marrow) whose cells can be cultured in the laboratory. The technique of ex vivo gene therapy involves the following steps (Fig. 13.2).

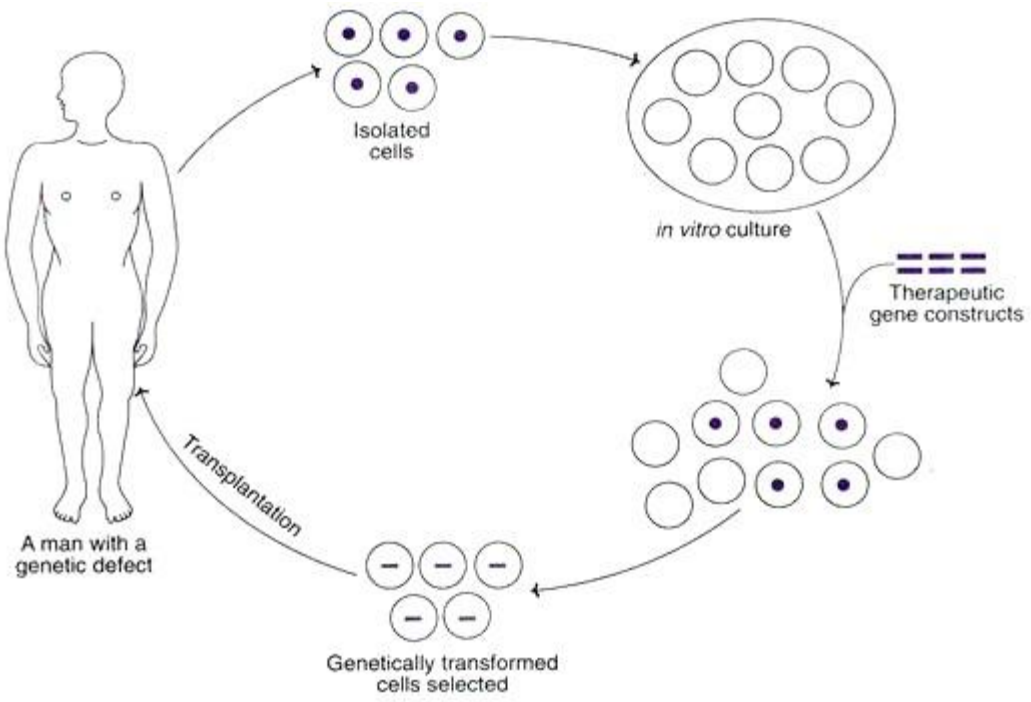


Fig. 13.2 : The procedure for ex vivo gene therapy.

1. Isolate cells with genetic defect from a patient.

2. Grow the cells in culture.

3. Introduce the therapeutic gene to correct gene defect.

4. Select the genetically corrected cells (stable trans-formants) and grow.

5. Transplant the modified cells to the patient.

The procedure basically involves the use of the patient's own cells for culture and genetic correction, and then their return back to the patient. This technique is therefore, not associated with adverse immunological responses after transplanting the cells. Ex vivo gene therapy is efficient only, if the therapeutic gene (remedial gene) is stably incorporated and continuously expressed. This can be achieved by use of vectors.

Vectors in Gene Therapy:

The carrier particles or molecules used to deliver genes to somatic cells are referred to as vectors. The important vectors employed in ex vivo gene therapy are listed below and briefly described next.

i. Viruses

ii. Human artificial chromosome

iii. Bone marrow cells.

i. Viruses:

The vectors frequently used in gene therapy are viruses, particularly retroviruses. RNA is the genetic material in retroviruses. As the retrovirus enters the host cell, it synthesizes DNA from RNA (by reverse transcription). The so formed viral DNA (referred to as provirus) gets incorporated into the DNA of the host cell.

The proviruses are normally harmless. However, there is a tremendous risk, since some of the retroviruses can convert normal cells into cancerous ones. Therefore, it is absolutely essential to ensure that such a thing does not happen.

Making retroviruses harmless:

Researchers employ certain biochemical methods to convert harmful retroviruses to harmless ones, before using them as vectors. For instance, by artificially removing a gene that encodes for the viral envelope, the retrovirus can be crippled and made harmless. This is because, without the envelope, retrovirus cannot enter the host cell. The production of a large number (billions) of viral particles can be achieved, starting from a single envelope defective retrovirus (Fig. 13.3).

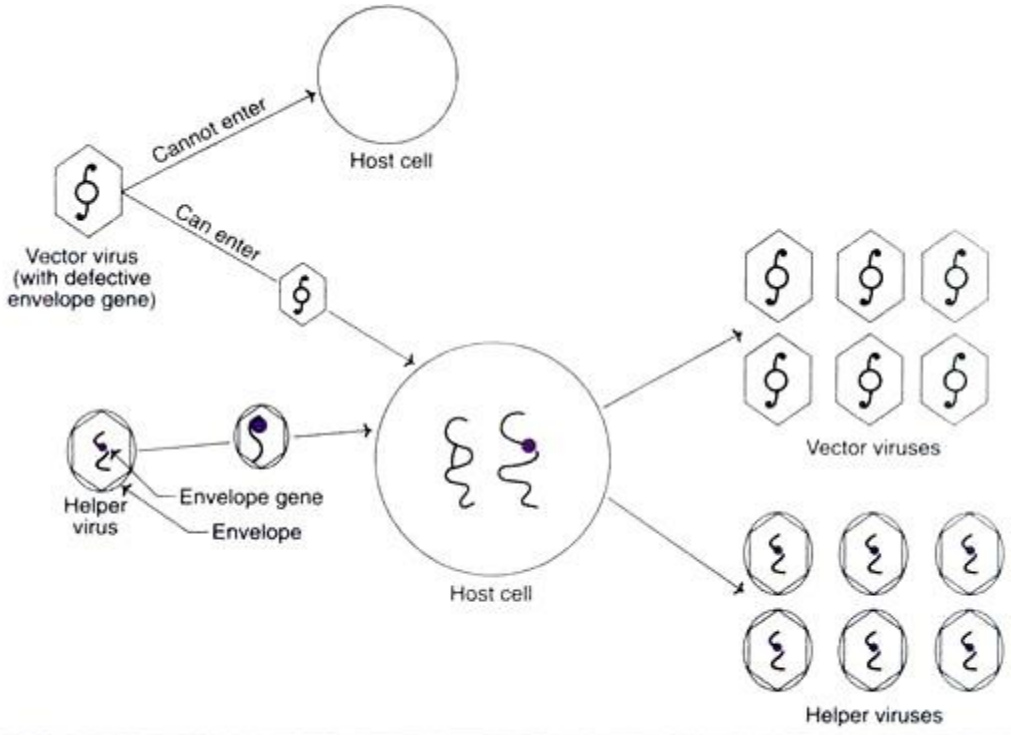


Fig. 13.3 : Large scale production of vector viruses by using helper viruses.

This is made possible by using helper viruses which contain normal gene for envelope formation. Along with the helper virus, the vector (with defective envelope gene) can enter the host cell and both of them multiply. By repeated multiplication in host cells, billions of vector and helper viruses are produced.

The vector viruses can be separated from the helper viruses and purified. Isolation of vector viruses, totally free from helper viruses, is absolutely essential. Contamination of helper viruses is a big threat to the health of the patients undergoing gene therapy.

Retroviruses in gene therapy:

The genetic map of a typical retrovirus is depicted in Fig. 13.4A. In general, the retrovirus particle has RNA as a genome organized into six regions. It has a 5′-long terminal repeat (5′-LTR), a non-coding sequence required for packaging RNA designated as psi (Ψ), a gene gag coding for structural protein, a gene pol that codes for reverse transcriptase, a gene env coding for envelope protein and a 3-LTR sequence.

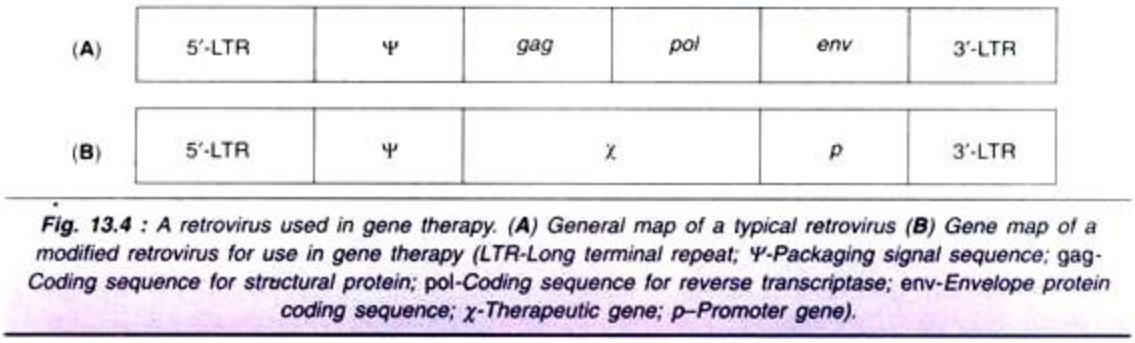


Fig. 13.4 : A retrovirus used in gene therapy. (A) General map of a typical retrovirus (B) Gene map of a modified retrovirus for use in gene therapy (LTR-Long terminal repeat; Ψ-Packaging signal sequence; gag-Coding sequence for structural protein; pol-Coding sequence for reverse transcriptase; env-Envelope protein coding sequence; χ-Therapeutic gene; p–Promoter gene).

For use of a retrovirus as a vector, the structural genes gag and pol are deleted. These genes are actually adjacent to Ψ region. In addition, a promoter gene is also included (Fig. 13.4B). This vector design allows the synthesis of cloned genes. A retroviral vector can carry a therapeutic DNA of maximum size of 8 kb.

A retroviral vector DNA can be used to transform the cells. However, the efficiency of delivery and integration of therapeutic DNA are very low. In recent years, techniques have been developed to deliver the vector RNA to host cells at a high frequency. For this purposes, packaged retroviral RNA particles are used. This technique allows a high efficiency of integration of pharmaceutical DNA into host genome.

Several modified viral vectors have been developed in recent years for gene therapy. These include oncoretrovirus, adenovirus, adeno associated virus, herpes virus and a number of hybrid vectors combining the good characters of the parental vectors.

Murine leukaemia viruses in gene therapy:

This is a retrovirus that causes a type of leukaemia in mice. It can react with human cells as well as the mouse cells, due to a similarity in the surface receptor protein. Murine leukaemia virus (MLV) is frequently used in gene transfer.

AIDS virus in gene therapy?

It is suggested that the human immunodeficiency virus (HIV) can be used as a vector in gene transfer. But this is bound to create public uproar. Some workers have been successful in creating a harmless HIV (crippled HIV) by removing all the genes related

to reproduction. At the same time, the essential genes required for gene transfer are retained. There is a distinct advantage with HIV when compared with MLV. MLV is capable of bringing out gene transfer only in dividing cells. HIV can infect even non-dividing cells (e.g., brain cells) and do the job of gene transfer effectively. However, it is doubtful whether HIV can ever be used as a vector.

ii. Human Artificial Chromosome:

The details of human artificial chromosome (HAC) are described elsewhere .HAC is a synthetic chromosome that can replicate with other chromosomes, besides encoding a human protein. As already discussed above, use of retroviruses as vectors in gene therapy is associated with a heavy risk. This problem can be overcome if HAC is used. Some success has been achieved in this direction.

iii. Bone Marrow Cells:

Bone marrow contains totipotent embryonic stem (ES) cells. These cells are capable of dividing and differentiating into various cell types (e.g., red blood cells, platelets, macrophages, osteoclasts, B- and T-lymphocytes). For this reason, bone marrow transplantation is the most widely used technique for several genetic diseases.

And there is every reason to believe that the genetic disorders that respond to bone marrow transplantation are likely to respond to ex vivo gene therapy also (Table 13.2). For instance, if there is a gene mutation that interferes with the function of erythrocytes (e.g., sickle-cell anaemia), bone marrow transplantation is done. Bone marrow cells are the potential candidates for gene therapy of sickle-cell anaemia. However, this is not as simple as theoretically stated.

TABLE 13.2 Selected list of genetic diseases that are likely to be cured by using bone marrow cells (potential candidates for gene therapy)

Severe combined immunodeficiency (SCID)
Sickle-cell anemia
Fanconi anemia
Thalassemia
Gaucher's disease
Hunter disease
Hurler syndrome
Chronic granulomatous disease
Infantile agranulocytosis
Osteoporosis
X-linked agammaglobulinemia

Selected Examples of Ex Vivo Gene Therapy:

a. Therapy for Adenosine Deaminase Deficiency:

The first and the most publicized human gene therapy was carried out to correct the deficiency of the enzyme adenosine deaminase (ADA). This was done on September 14, 1990 by a team of workers led by Blaese and Anderson at the National Institute of Health, USA (The girl's name is Ashanti, 4 years old then).

b. Severe combined immunodeficiency (SCID):

This is rare inherited immune disorder associated with T-lymphocytes, and (to a lesser extent) B-lymphocytes dysfunction. About 50% of SCID patients have a defect in the gene (located on chromosome 20, and has 32,000 base pairs and 12 exons) that encodes for adenosine deaminase. In the deficiency of ADA, deoxyadenosine and its metabolites (primarily deoxyadenosine 5′-triphosphate) accumulate and destroy T-lymphocytes.

T-Lymphocytes are essential for body's immunity. Besides participating directly in body's defense, they promote the function of B-lymphocytes to produce antibodies. Thus, the patients of SCID (lacking ADA) suffer from infectious diseases and die at an young age. Previously, the children suffering from SCID were treated with conjugated bovine ADA, or by bone marrow transplantation.

c. Technique of therapy for ADA deficiency:

The general scheme of gene therapy adopted for introducing a defective gene in the patient has been depicted in Fig 13.2. The same procedure with suitable modifications can also be applied for other gene therapies.

A plasmid vector bearing a pro-viral DNA is selected. A part of the pro-viral DNA is replaced by the ADA gene and a gene (G 418) coding for antibiotic resistance, and then cloned. The antibiotic resistance gene will help to select the desired clones with ADA gene.A diagrammatic representation of the treatment of ADP deficient patient is depicted in Fig. 13.5.

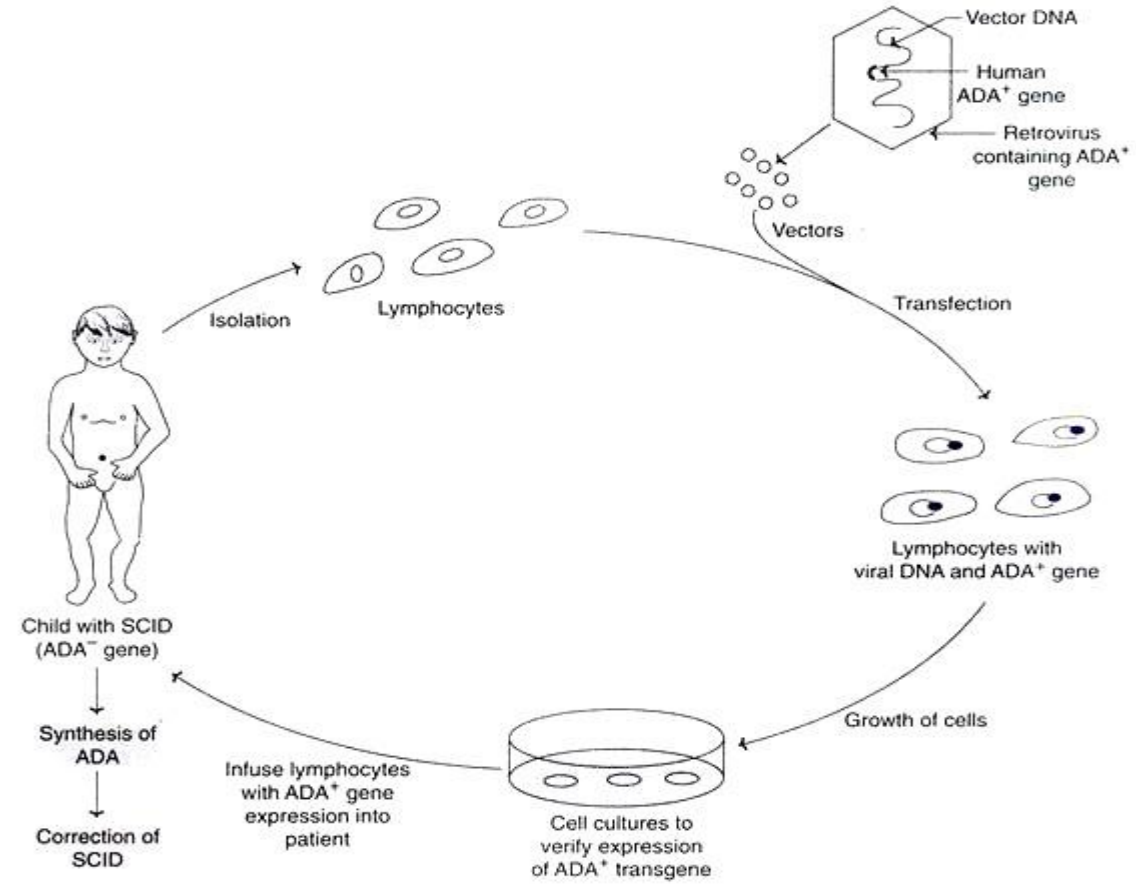


Fig. 13.5 : Treatment of adenosine deaminase (ADA) deficient patient by somatic ex vivo gene therapy (SCID-Severe combined immunodeficiency disease).

Circulating lymphocytes are removed from a patient suffering from ADA deficiency. These cells are transfected with ADA gene by exposing to billions of retroviruses carrying the said gene. The genetically-modified lymphocytes are grown in cultures to confirm the expression of ADA gene and returned to the patient. These lymphocytes persist in the circulation and synthesize ADA.

Consequently, the ability of the patient to produce antibodies is increased. However, there is a limitation. The lymphocytes have a short life span (just live for a few months), hence the transfusions have to be carried out frequently.

Transfer of ADA gene into stem cells:

In 1995, ADA gene was transferred into the stem cells, obtained from the umbilical cord blood, at the time of baby's delivery. Four days after birth, the infant received the modified cells back. By this way, a permanent population of ADA gene producing cells was established.

d. Therapy for Familial Hypercholesterolemia:

The patients of familial hypercholesterolemia lack the low density lipoprotein (LDL) receptors on their liver cells. As a result, LDL cholesterol is not metabolised in liver. The

accumulated LDL- cholesterol builds up in the circulation, leading to arterial blockage and heart diseases.

Attempts are being made by gene therapists to help the victims of familial hypercholesterolemia. In fact, there is some success also. In a woman, 15% of the liver was removed. The hepatocytes were transduced with retroviruses carrying genes for LDL receptors. These genetically modified hepatocytes were infused into the patient's liver. The hepatocytes established themselves in the liver and produced functional LDL-receptors. A significant improvement in the patient's condition, as assessed by estimating the lipid parameters in blood, was observed. Further, there were no antibodies produced against the LDL-receptor molecules, clearly showing that the genetically modified liver cells were accepted.

e. Therapy for Lesch-Nyhan Syndrome:

Lesch-Nyhan syndrome is an inborn error in purine metabolism due to a defect in a gene that encodes for the enzyme hypoxanthine-guanine phosphoribosyl transferase (HCPRT). In the absence of HGPRT, purine metabolism is disturbed and uric acid level builds up, resulting in severe gout and kidney damage. The victims of Lesch- Nyhan syndrome exhibit symptoms of mental retardation, besides an urge to bite lips and fingers, causing self-mutilation.

By using retroviral vector system, HGPRT producing genes were successfully inserted into cultured human bone marrow cells. The major problem in humans is the involvement of brain. Experiments conducted in animals are encouraging. However, it is doubtful whether good success can be achieved by gene therapy for Lesch-Nyhan syndrome in humans, in the near future.

f. Therapy for Haemophilia:

Haemophilia is a genetic disease due lack of a gene that encodes for clotting factor IX. It is characterized by excessive bleeding. By using a retroviral vector system, genes for the synthesis of factor IX were inserted into the liver cells of dogs. These dogs no longer displayed the symptoms of haemophilia.

g. Ex Vivo Gene Therapy with Non-Autologous Cells:

The ex vivo gene therapies described above are based on the transplantation of genetically modified cells for the production of desired proteins. However, there are several limitations in using the patient's own cells (autologous cells) for gene therapy. These include lack of enough cells from target tissues, defective uptake of genes and their inadequate expression. To overcome these problems, attempts are on to develop methods to use non-autologous cells (i.e., cells from other individuals or animals). The outline of the procedure is briefly described below.

Tissue-specific cells capable of growing in culture are selected. These include fibroblasts from skin, hepatocytes from liver, and myoblasts from muscle and astrocytes from brain. These cells are cultured and genetically modified with the therapeutic gene. They are then encapsulated in artificial membrane composed of a synthetic polymer (e.g., polyether sultone, alginase-poly L-lysine-alginate).The polymeric membranes are non-immunogenic, therefore the patient can accept non-autologous encapsulated cells. Further, being semipermeable in nature, these membranes allow the nutrients to enter in, and the encoded protein (by the therapeutic gene) to pass out.

Experiments conducted in animals have shown some encouraging results for using non-autologous cells in gene therapy. The encapsulated cells were found to proliferate and produce the required protein. However, the success has been very limited in human trials.

2. In Vivo Gene Therapy:

The direct delivery of the therapeutic gene (DNA) into the target cells of a particular tissue of a patient constitutes in vivo gene therapy (Fig. 13.6). Many tissues are the potential candidates for this approach. These include liver, muscle, skin, spleen, lung, brain and blood cells. Gene delivery can be carried out by viral or non- viral vector systems. The success of in vivo gene therapy mostly depends on the following parameters

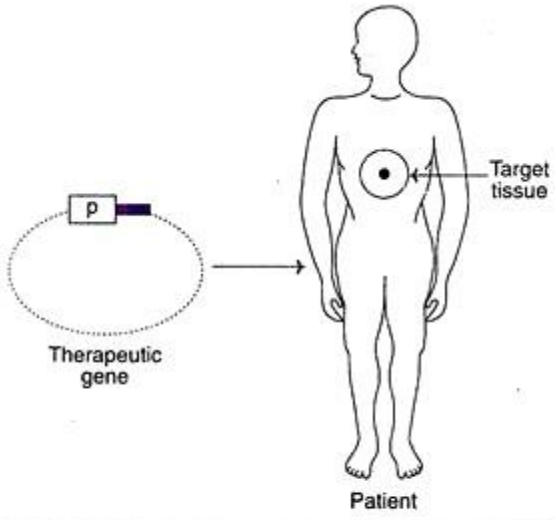


Fig. 13.6 : Diagrammatic representation of in vivo gene therapy. (p-Promoter gene specific for therapeutic gene)

i. The efficiency of the uptake of the remedial (therapeutic) gene by the target cells.

ii. Intracellular degradation of the gene and its uptake by nucleus.

iii. The expression capability of the gene.

In vivo gene therapy with special reference to gene delivery systems (viral, non-viral) with suitable examples is described.

Gene Delivery by Viruses:

Many viral vector systems have been developed for gene delivery. These include retroviruses, adenoviruses, adenoassociated viruses and herpes simplex virus.

Retrovirus vector system:

Replication defective retrovirus vectors that are harmless are being used. A plasmid in association with a retrovirus, a therapeutic gene and a promoter is referred to as plasmovirus. The plasmovirus is capable of carrying a DNA (therapeutic gene) of size less than 3.4 kb. Replication defective virus particles can be produced from the plasmovirus.

As such, for the delivery of genes by retroviral vectors, the target cells must be in a dividing stage. But majority of the body cells are quiescent. In recent years, viral vectors have been engineered to infect non-dividing cells. Further, attempts are on to include a DNA in the retroviral vectors (by engineering env gene) that encodes for cell receptor protein. If this is successfully achieved, the retroviral vector will specifically infect the target tissues.

Adenoviral vector system:

Adenoviruses (with a DNA genome) are considered to be good vectors for gene delivery because they can infect most of the non-dividing human cells. A common cold adenovirus is a frequently used vector. As the target cells are infected with a recombinant adenovirus, the therapeutic gene (DNA) enters the nucleus and expresses itself.

However, this DNA does not integrate into the host genome. Consequently, adenoviral based gene therapy required periodic administration of recombinant viruses. The efficiency of gene delivery by adenoviruses can be enhanced by developing a virus that can specifically infect target cells. This is possible by incorporating a DNA encoding a cell receptor protein.

Adeno-associated virus vector system:

Adeno-associated virus is a human virus that can integrate into chromosome 19. It is a single-stranded, non-pathogenic small DNA virus (4.7 kb). As the adeno-associated virus enters the host cell, the DNA becomes double- stranded, gets integrated into chromosome and expresses.

Adeno-associated viruses can serve as good vectors for the delivery of therapeutic genes. Recombinant viruses are created by using two plasmids and an adenovirus (i.e., helper virus) by a special technique. Some attempts were made to use therapeutic genes

for the treatment of the human diseases-haemophilia (for production of blood clotting factor IX) and cystic fibrosis (for synthesis of cystic fibrosis trans membrane regulator protein) by employing adeno-associated viruses.

Therapy for cystic fibrosis:

Cystic fibrosis (CF) is one of the most common (frequency 1: 2,500) and fatal genetic diseases. It is characterized by the accumulation of sticky, dehydrated mucus in the respiratory tract and lungs. Patients of CF are highly susceptible to bacterial infections in their lungs and most of them die before reaching the age of thirty. Cystic fibrosis can be traced in European folklore, the following statement used to be said **"Woe to that child which when kissed on the forehead tastes salty. He is be witched and soon must die".**

Biochemical basis:

In the normal persons the chloride ions of the cells are pushed out through the participation of a protein called cystic fibrosis trans membrane regulator (CFTR). In the patients of cystic fibrosis, the CFTR protein is not produced due to a gene defect. Consequently, the chloride ions concentrate within the cells which draw water from the surroundings. As a result, the respiratory tract and the lungs become dehydrated with sicky mucus, an ideal environment for bacterial infections.

Gene therapy for Cystic Fibrosis:

As the defective gene for cystic fibrosis was identified in 1989, researchers immediately started working on gene therapy for this disease. Adenoviral vector systems have been used, although the success has been limited. The major drawback is that the benefits are short-lived, since the adenoviruses do not integrate themselves into host cells. Multiple administration of recombinant adenovirus caused immunological responses that destroyed the cells.

By using adeno-associated virus vector system, some encouraging results were reported in the gene therapy of CF. In the phase I clinical trials with CF patients, the vector persisted for about 70 days and some improvement was observed in the patients. Some researchers are trying to insert CF gene into the developing foetal cells (in experimental animals such as mice) to produce CFTR protein. But a major breakthrough is yet to come.

Herpes simplex virus vector system:

The retroviruses and adenoviruses employed in in vivo gene therapy are engineered to infect specific target cells. There are some viruses which have a natural tendency to infect a particular type of cells. The best example is herpes simplex virus (HSV) type I, which infects and persists in non-dividing nerve cells. HSV is a human pathogen that causes (though rarely) cold sores and encephalitis.

These are a large number of diseases (metabolic, neurodegenerative, immunological, tumours) associated with nervous system. HSV is considered as an ideal vector for in vivo gene therapy of many nervous disorders. The HSV has a double-stranded DNA of about 152 kb length as its genome. About 30 kb of HSV genome can be replaced by a cloned DNA without loss of its basic characteristics (replication, infection, packaging etc.). But there are some technical difficulties in dealing with large-sized DNAs in genetic engineering experiments. Some modified HSV vectors with reduced genomic sizes have been developed.Most of the work on the gene therapy, related to the use of HSV as a vector, is being conducted in experimental animals. And the results are quite encouraging. HSV vectors could deliver therapeutic genes to the brain and other parts of nervous system. These genes are well expressed and maintained for long periods. More research, however, is needed before going for human trials. If successful, HSV may help to treat many neurodegenerative syndromes such as Parkinson's disease and Alzheimer's disease by gene therapy.

Gene Delivery by Non-Viral Systems:

There are certain limitations in using viral vectors in gene therapy. In addition to the prohibitive cost of maintaining the viruses, the viral proteins often induce inflammatory responses in the host. Therefore, there is a continuous search by researchers to find alternatives to viral vector systems.

a. Pure DNA constructs:

The direct introduction of pure DNA constructs into the target tissue is quite simple. However, the efficiency of DNA uptake by the cells and its expression are rather low. Consequently, large quantities of DNA have to be injected periodically. The therapeutic genes produce the proteins in the target cells which enter the circulation and often get degraded.

b. Lipoplexes:

The lipid-DNA complexes are referred to as lipoplexes or more commonly liposomes. They have a DNA construct surrounded by artificial lipid layers. A large number of lipoplexes have been prepared and used. They are non-toxic and non-immunogenic.

The major limitation with the use of lipoplexes is that as the DNA is taken up by the cells, most of it gets degraded by the lysosomes. Thus, the efficiency of gene delivery by lipoplex is very low. Some clinical trials using liposome-CFTR gene complex showed that the gene expression was very short-lived.

c. DNA-molecular conjugates:

The use of DNA-molecular conjugates avoids the lysosomal breakdown of DNA. Another advantage of using conjugates is that large-sized therapeutic DNAs (> 10 kb) can be delivered to the target tissues. The most commonly used synthetic conjugate is poly-L-

lysine, bound to a specific target cell receptor. The therapeutic DNA is then made to combine with the conjugate to form a complex (Fig. 13.7).

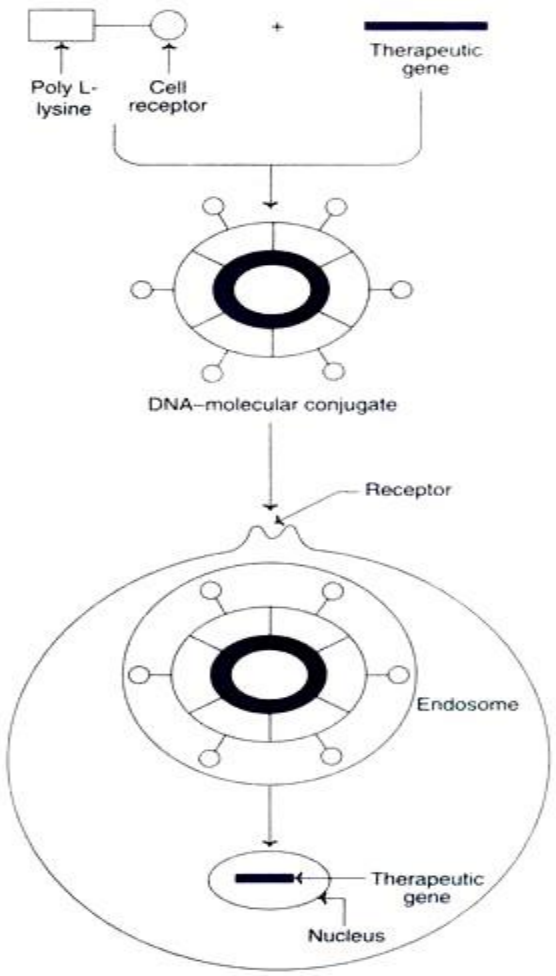


Fig. 13.7 : DNA-molecular conjugate in the delivery of therapeutic gene.

This DNA molecular conjugate binds to specific cell receptor on the target cells. It is engulfed by the cell membrane to form an endosome which protects the DNA from being degraded. The DNA released from the endosome enters the nucleus where the therapeutic gene is expressed.

d. Human artificial chromosome:

Human artificial chromosome (HAC) which can carry a large DNA one or more therapeutic genes with regulatory elements is a good and ideal vector. Studies conducted in cell cultures using HAC are encouraging. But the major problem is the delivery of the large-sized chromosome into the target cells. Researchers are working to produce cells containing genetically engineered HAC. There exists a possibility of encapsulating and implanting these cells in the target tissue.

Efficiency of gene delivery by non-viral vectors:

Although the efforts are continuously on to find suitable non-viral vectors for gene delivery, the success has been very limited. This is mainly due to the following two reasons.

1. The efficiency of transfection is very low.

2. The expression of the therapeutic gene is for a very short period, consequently there is no effective treatment of the disease.

Gene Therapy Strategies for Cancer:

Cancer is the leading cause of death throughout the world, despite the intensive treatment strategies (surgery, chemotherapy, radiation therapy). Gene therapy is the latest and a new approach for cancer treatment. Some of the developments are briefly described hereunder.

Tumour necrosis factor gene therapy:

Tumour necrosis factor (TNF) is a protein produced by human macrophages. TNF provides defence against cancer cells. This is brought out by enhancing the cancer-fighting ability of tumour- infiltrating lymphocytes (TILs), a special type of immune cells.

The tumour-infiltrating lymphocytes were transformed with a TNF gene (along with a neomycin resistant gene) and used for the treatment of malignant melanoma (a cancer of melanin producing cells usually occurs in skin). TNF as such is highly toxic, and fortunately no toxic side effects were detected in the melanoma patients injected with genetically altered TILs with TNF gene. Some improvement in the cancer patients was observed.

Suicide gene therapy:

The gene encoding the enzyme thymidine kinase is often referred to as suicide gene, and is used for the treatment of certain cancers. Thymidine kinase (TK) phosphorylates nucleosides to form nucleotides which are used for the synthesis of DNA during cell division. The drug ganciclovir (GCV) bears a close structural resemblance to certain nucleosides (thymidine). By mistake, TK phosphorylates ganciclovir to form triphosphate-GCV, a false and unsuitable nucleotide for DNA synthesis. Triphosphate-GCV inhibits DMA polymerase (Fig. 13.8).

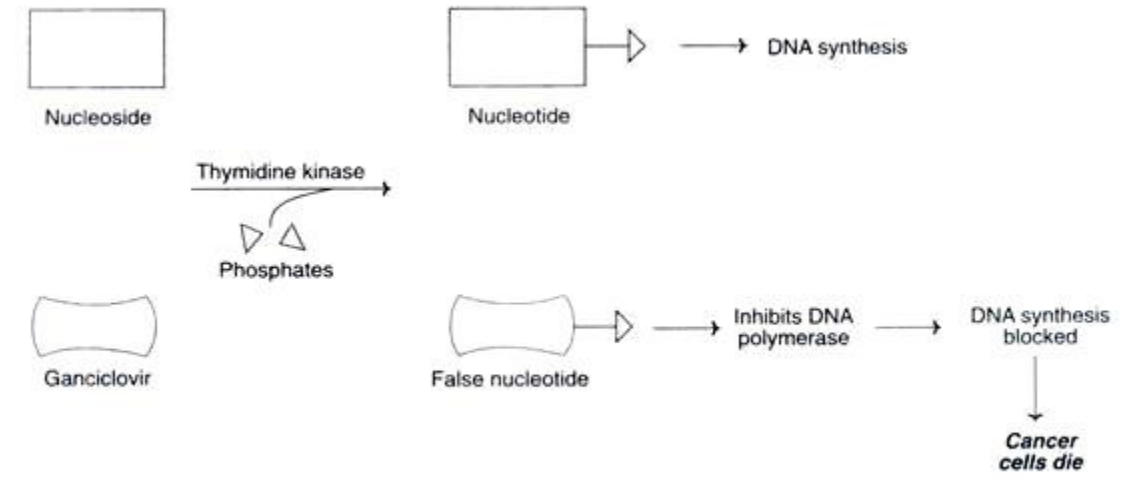


Fig. 13.8 : The action of ganciclovir mediated by thymidine kinase to inhibit the growth of cancer cells.

The result is that the elongation of the DNA molecule abruptly stops at a point containing the false nucleotide (of ganciclovir). Further, the triphosphate-GCV can enter and kill the neighbouring cancer cells, a phenomenon referred to as bystander effect. The ultimate result is that the cancer cells cannot multiply, and therefore die. Thus, the drug ganciclovir can be used to kill the cancer cells.

Ganciclovir is frequently referred to as a pro-drug and this type of approach is called pro-drug activation gene therapy. Ganciclovir has been used for treatment of brain tumours (e.g., glioblastoma, a cancer of glial cells in brain), although with a limited success.

In the suicide gene therapy, the vector used is herpes simplex virus (HSV) with a gene for thymidine kinase (TK) inserted in its genome. Normal brain cells do not divide while the brain tumour cells go on dividing unchecked. Thus, there is a continuous DNA replication in tumour cells. By using GCV-HSVTK suicide gene therapy, some reduction in proliferating tumour cells was reported. Several new strategies are being developed to increase the delivery of HSVTK gene to all the cells throughout a tumour.

Two-gene cancer therapy:

For treatment of certain cancers, two gene systems are put together and used. For instance, TK suicide gene (i.e., GCV-HSVTK) is clubbed with interleukin-2 gene (i.e. a gene promoting immunotherapy). Interleukin-2 produced mobilizes immune response. It is believed that certain proteins are released from the tumour cells on their death.

These proteins, in association with immune cells, reach the tumour and initiate immunological reactions directed against the cancer cells. Two-gene therapies have been carried out in experimental animals with colon cancer and liver cancer, and the results are encouraging.

Gene replacement therapy:

A gene named p^{53} codes for a protein with a molecular weight of 53 kilo Daltons (hence p^{53}). p^{53} is considered to be a tumour-suppressor gene, since the protein it encodes binds with DNA and inhibits replication. The tumour cells of several tissues (breast, brain, lung, skin, bladder, colon, bone) were found to have altered genes of p^{53}(mutated p^{53}), synthesizing different proteins from the original.

These altered proteins cannot inhibit DNA replication. It is believed that the damaged p^{53} gene may be a causative factor in tumour development. Some workers have tried to replace the damaged p^{53}gene by a normal gene by employing adenovirus vector systems .There are some encouraging results in the patients with liver cancer.

Gene Therapy for AIDS:

AIDS is a global disease with an alarming increase in the incidence every year. It is invariably fatal, since there is no cure. Attempts are being made to relieve the effects of AIDS by gene therapy. Some of the approaches are discussed hereunder.

a. rev and env genes:

A mutant strain of human immunodeficiency virus (HIV), lacking rev and env genes has been developed. The regulatory and envelope proteins of HIV are respectively produced by rev and env genes. Due to lack of these genes, the virus cannot replicate.

Researchers have used HIV lacking rev and env genes for therapeutic purposes. T-Lymphocytes from HIV-infected patients are removed, and mutant viruses are inserted into them. The modified T-lymphocytes are cultivated and injected into the patients. Due to lack of essential genes, the viruses (HIV) cannot multiply, but they can stimulate the production of CD_8 (cluster determinant antigen 8) cells of T-lymphocytes. CD_8 cells are the killer lymphocytes. It is proved in the laboratory studies that these lymphocytes destroy the HIV-infected cells.

b. Genes of HIV proteins:

Some genes synthesizing HIV proteins are attached to DNA of mouse viruses. These genetically-modified viruses are injected to AIDS patients with clinical manifestations of the disease. It is believed that the HIV genes stimulate normal body cells to produce HIV proteins. The latter in turn stimulate the production of anti-HIV antibodies which prevent the HIV replication in AIDS patients.

c. Gene to inactivate gp120:

gp120 is a glycoprotein (molecular weight 120 kilo Daltons) present in the envelope of HIV. It is absolutely essential for binding of virus to the host cell and to bring replication. Researchers have synthesized a gene (called F105) to produce an antibody that can inactivate gp120.

In the anti- AIDS therapy, HIV-infected cells are engineered to produce anti-HIV antibodies when injected into the organism. Studies conducted in experimental animals showed a drastic reduction in the synthesis of gp120 due to anti-AIDS therapy. The production of F1IV particles was also very reduced. There are some attempts to prevent AIDS by antisense therapy.

Advantages of Gene Therapy

Gene therapy can cure genetic diseases by addition of gene or by removal of gene or by replacing a mutated gene with corrected gene.

Gene therapy can be used for cancer treatment to kill the cancerous cells.

Gene expression can be controlled.

Therapeutic protein is continuously produced inside the body which also reduces the cost of treatment in long term.

The Future of Gene Therapy:

Theoretically, gene therapy is the permanent solution for genetic diseases. But it is not as simple as it appears since gene therapy has several inbuilt complexities. Gene therapy broadly involves isolation of a specific gene, making its copies, inserting them into target tissue cells to make the desired protein. The story does not end here.

It is absolutely essential to ensure that the gene is harmless to the patient and it is appropriately expressed (too much or too little will be no good). Another concern in gene therapy is the body's immune system which reacts to the foreign proteins produced by the new genes. The public, in general, have exaggerated expectations on gene therapy. The researchers, at least for the present, are unable to satisfy them. As per the records, by 1999 about 1000 Americans had undergone clinical trials involving various gene therapies.

Unfortunately, the gene therapists are unable to categorically claim that gene therapy has permanently cured any one of these patients. Some people in the media (leading newspapers and magazines) have openly questioned whether it is worth to continue research on gene therapy. It may be true that as of now, gene therapy due to several limitations, has not progressed the way it should, despite intensive research. But a breakthrough may come anytime, and of course, this is only possible with persistent research. And a day may come (it might take some years) when almost every disease will have a gene therapy, as one of the treatment modalities. And gene therapy will revolutionize the practice of medicine.

Probable Questions:

1. What are the main features of transgenic technology.
2. Write down the steps of transgenic technology.
3. What are the significance of transgenesis.
4. What are the importance of transgenic animals?
5. What are the implication of transgenic cattle?
6. What are the implication of transgenic pig?
7. What are the implication of transgenic chicken?
8. What are the implication of transgenic fish?
9. What are the implication of transgenic sheep?
10. What is fluorescent cat?
11. What is animal bioreactor?
12. define xenotransplantation.
13. Define Gene therapy.
14. Describe different strategies of gene therapy ?
15. Define somatic cell gene therapy and germ cell gene therapy?
16. What is ex vivo gene therapy and in vivo gene therapy?
17. How retroviruses are used in gene therapy?
18. How gene therapy is used in treatment of Cystic fibrosis ?
19. What is suicide gene therapy?
20. Describe gene therapy treatments for AIDS?
21. What is are the advantages of gene therapy?
22. Write about the future of gene therapy?
23. Describe the procedure of cloning in details with suitable diagram.
24. How DNA markers are used in disease diagnosis? Give examples.

Suggested readings:

1. Biotechnology by P.K. Gupta

2. Gene Cloning by T. Brown.

3. Biotechnology by N. Kumarsen.

4. Biotechnology by B.D. Singh

Disclaimer:

The study materials of this book have been collected from various books, e-books, journals and other e sources.