Model Answer (AU-6565)

B. Pharm. (Seventh Semester) Examination, 2014

PHARMACEUTICAL BIOTECHNOLOGY

Max Marks : 80

Section A

- (i) Change in genetic structure of an organism by incorporation of foreign DNA. Transformation of genetic information in an individual organism's genetic make up, from two or more organism.

(ii) The mutagenesis is an essential requisite employed in any genetic manipulation as well as in the study of gene structure function relationship. The isolation and study of single single gene has become not only possible but a routine paving way for precise and defined mutagenesis, as incorporation of special changes in any given base in a cloned DNA sequence is now possible. This is referred as site directed mutagenesis *in -vitro.* The technique work as an effective tool in gene manipulations.

(iii)APCs(non lymphocytic) are one among the two types of mononuclear cells. Their role is to present antigen to specific antigen sensitive lymphocytes. These are rich in class II MHC antigens and are important for presenting antigens to T cells. some of the cell involved in antigen presentation are- follicular dendritic cells, interdigitating dendritic cell.

(iv) Hypersensitivity type –II

(v) Interferons are proteinaceous substances produced inside the body for defense against virus infections.

(vi) Limitation of monoclonal antibodies

The first and foremost limitation is the initial cost involved in the technique.

- Precipitate formation
- Complement fixation
- Antibody specificity
- Antibody avidity

(vii) DNA polymerase- the primary enzyme which carries out the condensation of nucleotides to form polynucleotide chain is the DNA polymerase. It catalyses the synthesis of DNA from 5' to 3' direction i.e. starts coping the template DNA from its 3' end. It has three distinct

catalytic properties.

- A 5’ 3’ polymerase is responsible for the esterification of nucleotides to form the DNA chain.
- A 5’ 3’ exonuclease activity which is responsible for the removal of the RNA primer from 5’ end of the newly synthesized chain.
- A 3’ 5’ exonuclease activity is in proof reading which helps in removing any mismatched nucleotide and is essential for maintaining the accuracy of replication.

(viii) Selectable marker genes- they are used to isolate the transformed cells/ tissue. There are certain selectable marker genes present in vectors that facilitate the selection process. In transformed cells the selectable marker genes are introduced through vector. The transformed genes are cultured on medium containing high amount of toxic level of substrate.

(ix) Passive immunity – the immunity that a non-immune individual acquires by receiving antibodies or sensitizing white blood cells from another immune individual is known as passive immunity.

(x) Toxoids may be defined as modified toxins, detoxified by the use of moderate heat and chemical treatment so that their antigenic property is retained. Toxoids are the toxins whose toxicity has been removed. They are employed for the development of active immunity.

(xi) Approaches for Gene therapy

Various approaches have been tried for the effective transfer of genes to appropriate target sites. The approaches broadly fall into four categories. They are:

- Gene modification
    - Replacement therapy
    - Corrective gene therapy
- Gene transfer
    - Physical (Microinjection, Gene gun, ‘naked DNA’, EPD, Electroporation, etc.)
    - Chemical (Liposomes, Cationic liposomes, Oligonucleotides, etc.)

- Biological (Viral vectors, mammalian artificial chromosomes, etc.)

- Gene transfer in specific cell lines
  - Somatic gene therapy
  - Germline gene therapy
- Eugenic approach (gene insertion)

(xii) Hepatitis Surface Antigen (HBsAg)

Section B

Long Answer Type Questions

Ans.2 (a) Cystic fibrosis is a complex, multi-system disease which is inherited in an autosomal recessive pattern. The goal in CF patients with established lung disease is to prevent a further loss of lung function. CF is due to a mutation in the CFTR gene, which perturbs the salt and water composition of secretions, slows the mucocilliary clearance of airways and promotes infection. CF is dominated by involvement of the respiratory tract which is characterized by the airways obstruction caused by accumulation of thick purulent secretions and progressive deterioration of lung functions. Majority of patients acquire bronchopulmonary infections due to *pseudomonas aeruginosa*. The current therapy involves the use of antibiotics and approaches to improve mucous clearance.

A large number of studies have established that many gene transfer vectors are highly efficient *in vitro*, it has been found that transformed and primary cultures of human airways epithelia in non-polarized and polarized culture conditions have been relatively easy to transduce with the help of adenoviral vectors containing CFTR, with correction of the CF Cl^- transport defect.

New generation modalities in the treatment of CF utilize recombinant human deoxyribonuclease 1 (rhDNase) which reduce the viscoelasticity of respiratory tract.

Problems arises in gene therapy

1. For CF gene therapy is the functional efficiency of the system *invivo.*
2. safety profile of the various vectors that certain vectors like adeno viral vector can slow the cell cycle and induce apoptosis.

(b) Growth hormone is an anabolic hormone secreted by the anterior pituitary which stimulates tissue growth and anabolism.It is polypeptide of 191 amino acids. Segment of gene for first 24 amino acids of the peptide is constructed chemically from block of nucleotide; second segment

of gene is produced by extracting mRNA from, human pituitary gland and then converted into cDNA by treatment with reverse transcriptase and restriction endonuclases. These two fragments are then legated to get hGH gene with ATG sticky end, to be legated with modified plasmid pBR322 vector, having Lac operator for expression control. They are further expressed in bacterial cell to produce hGH. Fermentation in E.coli host is followed by isolation of the product from intact cell.

Ans.3 Replication of DNA

DNA replication is the process of DNA duplication and its distribution in two daughter cells two possible scenarios.
One cell received the pre-existing DNA, while the other cell receives the newly synthesized DNA. This type of DNA replication will be called as the conservative mode of DNA replication.

Two strands of original DNA are separated, each is duplicated by the synthesis of the complementary strand, thus producing two exact replica of the parent DNA. This mode of replication is known as semiconservative mode of replication.
DNA replication continuous on 5'-3' strands while it is discontinuous on 3'-5' strands. DNA polymerase I capable of step-by- step addition of deoxyribonucleotide units to a DNA chain.

Unbinding is must for replication
The bacterial chromosome is circular in appearance therefore mode of replication is generally called Θ-replication. Since two chain of replicating molecules must undergo full rotation to unwinding of entire structure. The axis of rotation for the process of unwinding is provided by nicks made in the back bone of one the strands of the double helix during the course of replication. After unwinding each cut is repaired rapidly.
An enzyme that could generate cuts and nicks and repairs them quickly is called topoisomerase. The position just adjacent to cut or nicks is called replication origin and the region where parent strand tend to separate allowing the synthesis of new one is referred to as **replication fork.** The process responsible for generating new fork is termed as initiation.

DNA contains multiple origin of replication
The eukaryotic DNA is linear molecule that replicates bidirectionally. The replication could be initiated at a time at many sites in DNA. This result into multiple loops. This process reduced the replication time significantly.

DNA polymerase responsible for new DNA Strands
DNA polymerase is enzymes that form the sugar phosphate bond between adjacent nucleotides in a nucleic acid chain. DNA polymerase provides:

The 5'-triphosphate are dATP,dGTP, dTTP, dCTP
Single strand of DNA
The primer segment
The DNA polymerase possessing nuclease activities these are:
Exonucleases and Endonucleases

DNA synthesis proceeds in 5'to 3' Direction

DNA polymerase can add nucleotide only to the 3'-OH group. The 3' –OH terminals of continuously replicating strands proceed to be ahead of discontinuous strand hence referred as leading strand while in order to 5'-3' oriented growth one of the parent strand is replicating in small precursor fragment as lagging strand.

RNA as an initiator of new strand

This short stretch of RNA provides a primer over which DNA polymerase can subsequently add deoxy- nucleotides. The DNA primer producing RNA polymerase is called primase.
Percursor fragment
Finally combined to produce a continuous strand of DNA . Joining of fragmented DNA is accomplished by DNA ligase.

Proteins also participate in DNA replication

During replication nearly twelve different types of proteins participate in the activity.

Ans 4. A single type of antibodies having the same antigenic determinant produced by a single hybridoma clone, is called monoclonal antibody.

By fusing a normal B cell (Plasma cell) with a myeloma cell (a cancerous plasma cell), called a hybridoma or heterokaryons., that possessed the proliferating growth properties of the myloma cells but secreted the antibody product of the B cell.

Four basic steps are involved in the production

- Immunization
- Generation of B cell hybridomas by fusing primed B cell and myeloma cell.
- Selection and the screening of the resulting cloned for those secretes antibody with the desired specificity.
- Cloning of propagating the desired hybridomas.

Ans 5 (1) Hypersensitivity

Hypersensitivity is defined as the violent reaction of the immune system. Immune response is always directed towards the protection towards the protection of host but in hypersensitivity the immune response becomes injurious to the host. Hence the immune response becomes a destructive process in hypersensitivity. In protective immune response, the antigen or bacterium or virus is killed or neutralized. But in hypersensitivity, the cells of host are killed or the host itself is damaged or killed. Hypersensitivity is the changed reactivity of the immune system. It is a beneficial protective system gone out of order. The factors causing hypersensitivity are called allergens. In clinical terms, hypersensitivity is called allergy.

Common hypersensitivity reactions

1. anaphylaxis
2. transfusion reactions
3. erythroblastosis foetalis
4. arthus reaction
5. mantoux reaction
6. serum sickness
7. contact dermatitis
8. graves disease

Type of hypersensitivity

Classified in two ways

A. based on the time taken for the reaction.

B. based on the different mechanisms of pathogenesis.

A.Classification based on the time taken for reactions

a. immediate hypersensitivity

- this immune reaction appears and disappears rapidly.
- it produces urticaria, wheal and granulocyte accumulation.
- it involves the interaction of antigen and antibody.

b. delayed hypersensitivity

- this immune response appears slowly and lasts longer.
- inflammatory response manifests only after 24 to 48 hrs.
- it produces erythema, induration and lymphocyte infilteration.

B. Classification based on the different mechanisms of pathogenesis

Coombs and gell proposed 5 types of hypersensitivity based on the different mechanisms of pathogenesis. They are the following:

a. Type I : Anaphylactic hypersensitivity
b. Type II : Antibody- dependent cytotoxic hypersensitivity.
c. Type III: Immune complex- mediate hypersensitivity
d. Type IV: Cell-mediated hypersensitivity
e. Type V: Stimulatory hypersensitivity
of these 5 types, type IV alone is delayed type of hypersensitivity and the other types are immediate type.

Type I : Anaphylactic hypersensitivity (Anaphylaxis)- Anaphylaxis is defined as exaggerated reactions of an organism to a foreign substance to which it has previously become sensitized resulting from the release of histamine, serotonin and other vasoactive substances. It is immediate type hypersensitivity..

Symptoms of anaphylaxis- death, diarrhoea and vomiting, urticaris, atopy

Prevention and treatment – avoiding contact with allergens, desensitization, hyposensitivity, stabilizing mast cells, inhibition of histamine receptors, blocking the release of histamines.

Type II: Antibody dependent cytotoxic hypersensitivity- Type II hypersensitivity is due to the interactions of antibodies and cell associated antigens.

Types of cytotoxic hypersensitivity

These are classified in two types

1. Isoimmune reactions. 2. Autoimmune reactions

1. Isoimmune reactions- the reactions brought about by the antigen and antibody of two individuals belonging to the same species are called isoimmune reactions.

These include

a. Transfusion reactions
b. Erythroblastosis foetalis
c. Transplant rejection reactions

2. Autoimmune reaction

These reaction brought about by the interactions of antigen and antibody of the same individual is called autoimmune reaction. Examples of this reactions are

a. Autoimmune haemolytic anaemia
b. Autoimmune thrombocytopaenic purpura
c. Autoimmune thyroiditis
d. Autoimmune glomerulonephritis

Type III : Immune complex mediated hypersensitivity- In certain occasions, enormous amount of antigens enter the body. In response, the body produces higher concentration of antibodies. These antigens and antibodies combine together to form an insoluble precipitate called antigens- antibody complex or immune complexes. These complexes get attached in and around minute blood vessels in the regions of glomerulus, synovium, skin, choroid plexus.

The antibodies involved in this reaction belong to the class of IgG or IgM. Examples for immune complex mediated immunity are Arthus reaction, Serum sickness, etc.

Type IV Cell- mediated delayed hypersensitivity- Type IV hypersensitivity is caused by the interaction between antigens and sensitized T cells. This reaction leads to inflammatory reaction and causes tissue damage. Antibodies are not involved in type IV hypersensitivity. As T cells are involved in this reaction, it is called cell- mediated hypersensitivity.
Example- tuberculin reaction (Mantoux reaction).

(ii) **Transgenic plants**
The plants, in which a functional foreing gene has been incorporated by and any biotechnological methods that generally not present in plants, called transgenic plants. There are several method used in gene transfer . these includes: i) electroporation ii) particle bombardment iii) microinjection, iv) agro bacterium-mediated gene transfer, v) protoplast transformation, vi) leaf disc transformation, vii) virus mediated transformation, viii) pollen- mediated transformation, ix) liposome mediated transformation. These transgenic plants contain certain selected traits such as herbicide resistance, insect resistance, virus resistance, seed storage protein, modified ripening. The transgenic plant are being looked up as bioreactor for molecular forming.
Examples: **Genes for Bt toxins-** The use of pesticides and insecticides is a common measure in plant protections programmes, since pests and insects cause appreciable damage to our crops. Most of these pesticides and insecticides are chemically synthesized. However, an exception is the Bt toxins produced by a bacterial species (*Bacillus thuringiensis*), so that a spore preparation of this bacterium has been used as a biological insecticide during the last 20 years. Insecticidal activity of this species depends on the protein (delta endotoxins) synthesized during sporulation. Since these toxins are very specific in their action, they are safe insecticides, but their use is limited due to high production cost and due to instability of crystal protein when exposed in the field.
The above toxin gene from B. thuringiensis has been isolated and used for Agrobacterium Ti plasmid mediated transformation of tobacco, cotton and tomato plants.

Genes for protease inhibitors- In cowpea (Vigna unguiculata), trypsin inhibitor (CpTI) level was shown to be responsible for its resistence to attack by the major storage pest of its seed(i.e. bruchid beetle= Callosobruchus maculates). CpTI gene was joined with CaMV 35S promoter, and one or more marker genes.

Gene for other insecticidal secondary metabolites –Secondary metabolites produced by plants have also been implicated in the resistance to insect attack. However, biosynthesis of each of these metabolites involves a series of steps(more than one biosynthetic pathway), each

controlled by a separate gene. Furthermore, these genes are tissue specific in expression.

Ans 6. Purification

- In monoclonal antibodies, to remove contaminants from production process such as protein nucleic acid, endotoxin and adventitious agent
- The process employ must reproducibly remove contaminants to approve level, maintaining immunogenicity of MAbs
- Various chromatographic techniques are available
- Initial purification is performed by affinity chromatography for purification of IgG
- Endotoxins & DNA are removed by anion exchange chromatography
- Gel filteration is used to remove both high & low molecular form of MAbs. Such as aggregates & small fragments
- By product removed by
- Controlled precipitation with salt
- Ultrafilteration

Quality control

Mabs are produced from living cells in animals or by *invitro* fermentation

Testing must be perform at every stage of the manufacturing process

1. at the cell bank stage to determine the cell line stability, identify and presence of adventitious agent such as viruses & mucoplasma.

2. Testing during downstream processing removal of impurities derived from cell line & fermentation medium, to remove or inactivate viruses.

3. Testing of the final product to check the sterility, safety, a pyrogenicity & biochemical or immunological characterization of MAbs for identify purity & potency.

Storage

for desired specificity, store in liquid nitrogen. Monitoring genetic stability of cell line
-various metabolic & growth characteristic in culture.

Physical chemical stability- different storage condition.

- **(a) Preparation of antisera**.

Antibody containing preparation obtained from immune person called human sera or homologus ; animal hourse called heterologous sera.

They are classified into

- Antitoxic sera called antitoxins
- Antibacterial antisera
- Antiviral antisera

Antitoxins

Diptheria antitoxin preparaed to produce antibodies againstexotoxins of C. diptheriae.

Method- Horses are preferred

Gradually increasing amount of toxoid are injected into neck muscles at regular interval for several mounths, dose initial 5ml- upto 600ml satisfactory antitoxin titre has been attained.

Almost 8 lit of blood withdrown aseptically from the juglar vein into bottle containing anticoagulant solution .

Repeated twice over next 8days.

After 10day rest(for horses), again inject short course of antigen to horses and blood is withdrawn after confirming antitoxin titre. Allowing cell to settle (in refrigerator). Add cacl2 to induce clotting, filter (take serum) . serum contain high conc. Of several proteins, remove by:

Proteolytic digestion

Trating dil serum with pepsin at ph-4, incubated 37°C for abou 2days. Pepsin digest albumin, ϒ globulin are partly digested. Bglobulin is remove by filterationand filter is treated with more amm.sulfate to ppt active fragment. Separate, dialysed, adjusted to isotonicity blended with preservative and pass thr. Sterilizing filter.

Fractional ppt.- Add amm. Sulfate to remove ϒ globulin liquid protin contain albumin , separated by filter press, employed dialysis, to remove amm.sulfate by suspending

cellophane bage of the liquid into chlorinated water.

In solution adjust isotonically, blended with preservative pass thr. Sterilizing filter.

(b) Gene synthesis by using mRNA

mRNA are the transcript of genes which translate proteins and to identify a gene on a chromosome. It is a difficult process. However, it is easier to pick up the mRNA and synthesize a gene. Total population of mRNA in a cell is high and contain long polyadenylated tract at 3' end. long polyadenylated tract at 3' end is used to separate mRNA from rest of the RNA population. mRNA pass through poly dT column (10-20 nucleotide). Adenine bind to thymine, oligo dT provide primer at poly (A) region with a free 3'-OH end.The reverse transcriptase use the free end and synthesized a single stranded c DNA, in the presence of dCTP, dGTP, dATP , dTTP which results in mRNA- cDNA hybrid. At the end of enzyme forms a loop by using last few bases as the template which result in synthesis of a short hairpin loop at 3' end of the cDNA. mRNA degraded from mRNA – cRNA by alkali, single strand DNA act as template for the synthesis of double strand in presence of poly I and 4- deoxynucleotide. S1 nuclease use to cleave hairpin loop.