stimulating factor	transplantation	Institute
DNAse	Cystic fibrosis	Genentech
Recombinant Vaccines	Hepatitis	Merck, Chiron, Amgen
	Herpes	Chiron
	Malaria	Chiron
	Feline Leukaemia	Cambridge Biotech
	AIDS	MicroGenSys
Monoclonal antibodies	Cancer and immuno - suppression	Becton Dickinson and Oncogen

1. Insulin

Insulin is a ***hormone*** secreted by the beta cells of the Islets of Langerhans of Pancreas. It is essential for oxidation, utilization of blood sugar and for the maintenance of proper blood sugar level. Inadequate secretion of insulin leads to ***hyperglycemia*** (higher blood sugar level). This condition is said to be ***diabetes.***

Insulin reduces blood sugar level and promotes glycogen metabolism in liver and muscles. It also reduces the breakdown of cellular proteins. Further it increases the resistance towards infectious diseases. The treatment of diabetics by injecting insulin is named ***insulino-therapy.***

Structure of Insulin

The molecular weight of insulin is ***35,000 daltons***. Insulin molecule is made up of two polypeptide chains. They are ***A chain*** and ***B chain***. The ***A chain*** consists of ***21 amino acids*** and the ***B chain*** consists of ***30 amino acids***. These two chains are bound together by two ***disulphide linkages***. The thirtieth amino acid may be alanine or threonine.

Historical View

The antidiabetic role of insulin was first discovered by ***Sir F.G. Banting*** in 1922. Insulin was isolated from ***pancreas*** of *oxan, cattle, swines, dogs*, etc. and used to treat diabetes.

In 1982, ***Eli Lilly*** (USA) started to produce human insulin from genetically engineered bacteria. It is available in the name ***humulin.*** The Food and Drug Administration Department of USA approved it for therapeutic use.

Recombinant insulin is now available in the trade names ***Humulin, Recosulin*** (Shreya Life Science, Pune), ***Humanzinsulin*** (Sarabhai), ***Lentard 40*** (Torrent), ***Human insulatard*** (Novo Nordisk) and ***Huminsulin-R*** (Ranbax).

Genetic Manipulation of E.coli For Insulin Production

Genentech Company in USA chemically synthesized insulin gene. They synthesized A polynucleotide chain and B polynucleotide chain. These two single stranded polynucleotide chains were made into double-stranded DNAs.

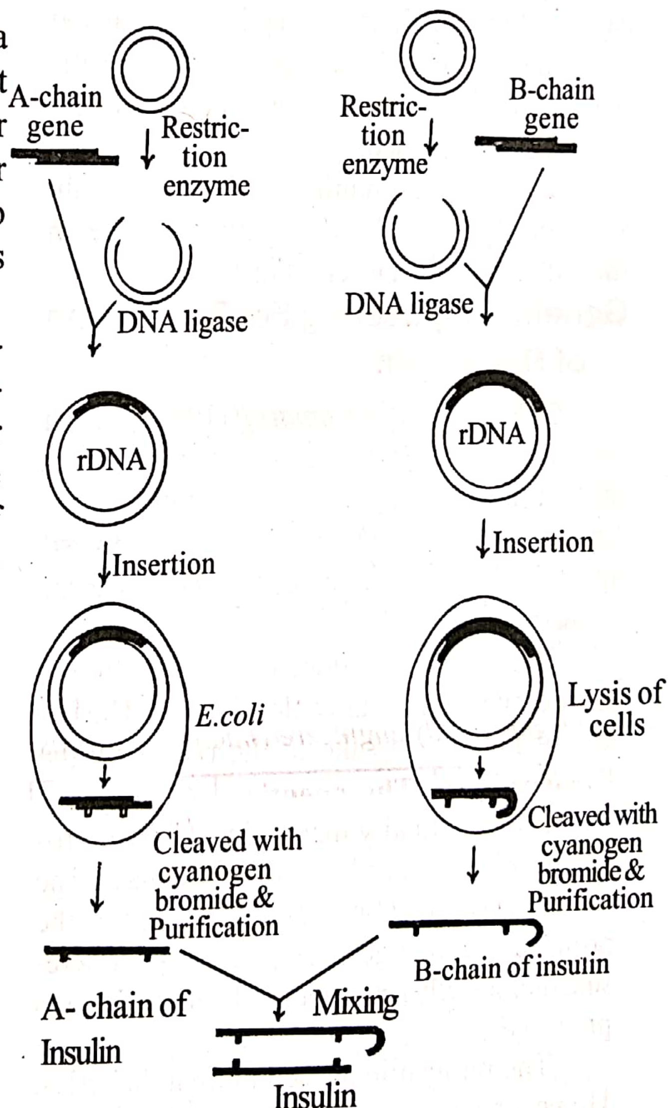

Fig.18.1: Genetic engineering for the production of human insulin from E.coli.

The long DNA fragment was inserted into a plasmid vector, especially at the upstream of its β-galactosidase gene. In the same way, the small DNA fragment was inserted into another vector to make rDNA.

The two rDNAs were introduced into *E.coli* cells separately. Recombinant *E.coli* cells were screened from these two *E.coli* cultures. One *E.coli* culture, produced long polypeptide of insulin bound with β-galactosidase enzyme and the other produced the short polypeptide bound with β-galactosidase.

The long and short polypeptides were isolated and purified separately from the cultures. They were then treated with ***cyanogen bromide*** to cleave the β-galactosidase from the insulin polypeptides. After removing the β-galactosidase, the long and short polypeptides were mixed together to form disulphide linkages between the two. As a result a biologically active insulin molecule was formed.

A genetically manipulated *E.coli* (strain k12) could produce 1 x 10^5 insulin molecules in the culture within a week. (Fig.18.1)

Genetic Engineering For Production of Rat Insulin

Gilbert and ***Villa-Komaroff*** (1980) isolated insulin mRNA from beta cells of pancreas of rat and synthesized duplex complementary DNA from it. The insulin DNA was inserted into PstI site of pBR322. As a result, rDNAs were formed.

The rDNA was added to *E.coli* culture in the presence of calcium chloride ions. The latter enhance the uptake of the rDNA by the *E.coli* cells.

The genetically manipulated *E.coli* produced proinsulin together with penicillinase. The penicillinase and C-polypeptide present in the proinsulin were digested with trypsin. As a result, biologically active rat insulin molecule was produced.

The rat insulin is used to treat diabetics. However, some patients are allergic to the rat insulin.

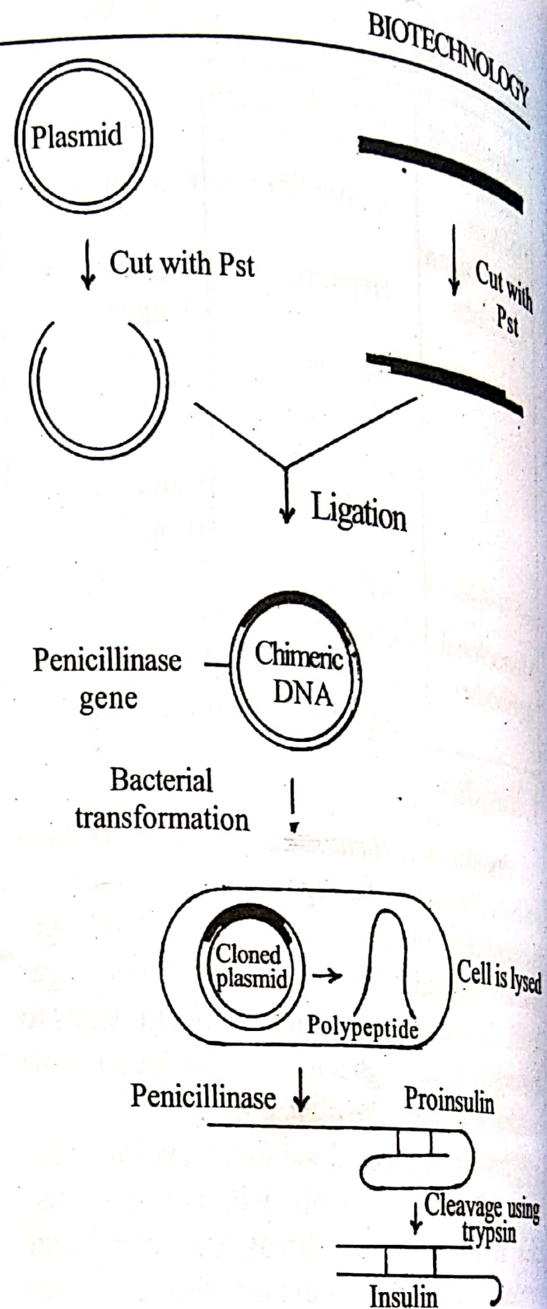

Fig.18.2: Genetic engineering for the production of rat insulin from E.coli.

Insulin Lispro

This is a ***human insulin analog*** that is identical to normal insulin in molecular weight and general structure. Its glucose lowering effect is more rapid than the normal insulin, but it works for a short duration. Insulin lispro has lysine as the 28th amino acid and proline as 29th amino acid in its structure instead of Lys (29) and Pro (28) in B-chain of normal insulin.

Insulin Lispro is mixed with protamine suspension and filled in ***AIR inhalers*** to treat diabetes. The aerosol preparation is inhaled through mouth. This is the latest therapy for diabetes.

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34

Monoclonal Antibodies (MCAs)

A single type of antibodies having the same antigenic determinant produced by a single hybridoma clone, is called ***monoclonal antibody.*** The hybridoma is made by fusing a lymphocyte (B cell) with a myeloma cell. Presence of a single antigenic determinant is the useful feature of the monoclonal antibodies. MCAs bind with only one type of epitope on the antigens.

Monoclonal antibodies were first made by ***Milstein*** *et al* in 1973. They are used in the diagnosis and treatment of severe diseases. They are also used in screening specific proteins. The antibodies produced against a single antigen but differing in antigenic determinants are called ***polyclonal antibodies***. They bind with different determinant sites on the antigen.

Important monoclonal antibodies and their uses are listed below:

Table 34.1: Monoclonal antibodies and their uses.

Monoclonal Antibody	Used to Treat
Gemtuzumab	Acute myeloid leukaemia
Rituximab	Non-Hodgkin's lymphoma
Trastuzumab	Metastatic breast cancer
Palivizumab	Paediatric respiratory syncytial virus
Infliximab	Crohn's disease and rheumatoid arthritis
Basiliximab	Acute organ rejection in transplants
Daclizumab	Acute kidney transplant rejection
Edrecolomab	Colorectal cancer
Abciximab	Prevention of blood clots
Muromomab	Kidney transplant rejection

Naming of MCA

The ***US Adopled Name*** (USAN) council has adopted a naming system while naming monoclonal antibodies. According to the USAN, the name of Mabs should end in the suffix-***mab.*** The suffix should be preceded by an abbreviation of animal source. The following abbreviations are used as organism identifiers:

a for rat
e for hamster
i for primate
o for mouse
u for human
xi for chimera
zu for humanized

The abbreviation of organism should be preceded by the abbreviation of disease state. The following abbreviations are used to designate the disease state:

vir for viral cause
bac for bacterial cause
lim for immunomodulator
col for colon tumour
mel for melanoma tumour

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mar for mammary tumour
got for testis tumour
gov for ovary tumour
pro for prostrate tumour
tum for miscellaneous tumour
cir for cardiovascular disease

The abbreviation of disease state should be preceded by a unique syllable as a prefix. It may be given by the inventor according to his choice.

For example, ***Basiliximab*** is a chimeric monoclonal antibody to treat organ rejection.

Production of Hybridoma

Hybridoma is a fusion product of a lymphocyte and myeloma cell. The production of hybridoma clones for monoclonal antibody production involves the following steps:

1. Isolation of B-Lymphocytes

B-lymphocytes are isolated from immunized mouse. It involves the following steps:

i. 2-4 weeks old mice are immunized with the known antigen by sub-cutaneous injection.

ii. A mouse is killed after 72 hours of immunization, especially within 4 days and its spleen is taken.

iii. The spleen is minced into small fragments and the fragments are sterilized.

iv. The fragments are then macerated into individual cells using enzymatic method.

v. The cell suspension so obtained is immediately suspended in a balanced salt solution.

vi. The suspension is washed 2 or 3 times with the balanced salt solution to get pure plasma cells (spleenocytes).

vii. Some of the spleenocytes are the antibody producing B-lymphocytes (B-cells). They are grown in fresh medium for cell fusion.

2. Isolation of Myeloma Cells

Myeloma cells are fast growing large cells of haematopoietic portion of bone marrow. Myeloma is taken from a bone and macerated to get a suspension of myeloma cells. The myeloma cells have the ability to produce a specific antibody in larger amount.

HGPRT- mutant myeloma cells are raised by inducing mutations using 8-azaguanine. This will help us to select hybridomas from fused and unfused cells.

3. Somatic Cell Fusion

The spleenocytes and myeloma cells are mixed together in the ratio of 2-5:1 and treated with PEG. The cell mix is shaken well for 3 minutes. The PEG brings the two cells together and induces cell fusion. As a result, spleenocyte-myeloma hybrids called ***hybridomas*** are formed. Sometimes, PVA is used as a fusagen to induce the cell fusion.

4. Selection of Hybrids

The selection of spleenocyte-myeloma hybrids is mentioned below:

i. After cell fusion, the cell suspension is treated with fresh medium lacking blood serum at a rate of 1 ml per minute.

ii. The diluted suspension is centrifuged to remove the fusagen along with the liquid medium.

iii. The cell suspension is then diluted slowly with a serum free medium containing hypoxanthine, aminopterin and thymidine.

iv. The diluted cell suspension is distributed into the wells of a multiwell plate and the plate is incubated at 25-29^{0}C for 2-3 weeks in an incubator.

v. Later, the wells of the multiwell plate is visualized for cell clumps.

The HGPRT$^-$ mutant myeloma cells fail to synthesize purines from hypoxanthine. Aminopterin blocks the metabolism of purines. So myeloma cells and myeloma -myeloma hybrids do not grow in the HAT medium.

Unfused spleenocytes and spleenocyte-spleenocyte hybrids never grow in the medium.

Spleenocytes contribute functionally active HGPRT enzyme to the spleenocyte-myeloma hybrids. Hence hybridomas synthesize purines from hypoxanthine and grow into cell clumps.

vi. The cell clumps are allowed to grow till each clone attains 500 or more cells by providing enough medium. Each of these cell lines is called ***hybridoma*** or ***clone***.

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5. Screening of the Hybridomas

i. The wells of a multiwell plate are coated with the antigen that was used to immunise the mouse.

ii. A small amount of medium was taken from each well having hybridoma and poured into the antigen coated well. Antibodies bind with epitope of the antigen and form antigen-antibody complex.

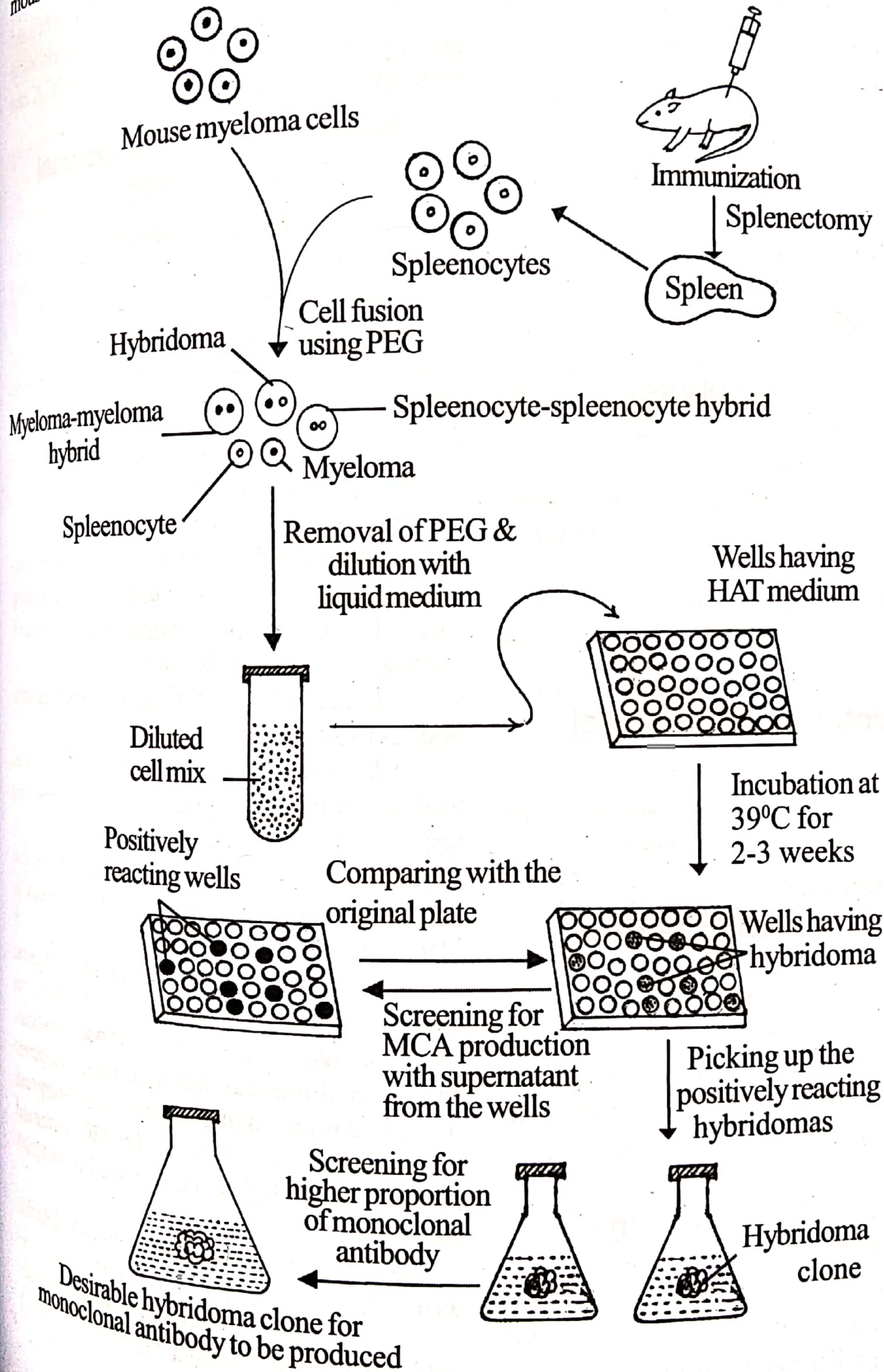

Fig.34.1: Important stages of hybridoma production.

iii. The unbound antibodies are washed out of the wells.

iv. Then a secondary antibody that specifically binds with mouse antibodies, is linked with an enzyme.

v. The enzyme coupled antibody is poured into the wells. The secondary antibody binds with the primary antibody.

vi. The unbound secondary antibody is washed out.

vii. The colourless compound on which the linked enzyme acts, is added to the wells.

viii. Those wells giving positive colour change indicate that the corresponding wells in the original plate have hybridomas.

ix. Hybridoma clones in the respective wells are taken and sub-cultured in fresh medium in small flasks for a week.

x. The supernatants are taken from these flasks and tested separately for the amount of antibody produced.

xi. The hybridoma clone producing monoclonal antibody in higher proportion is picked up and cultured in fresh medium in a flask.

Production of Monoclonal Antibodies

Monoclonal antibodies have been produced commercially in the following ways:

1. In Vivo Method

The hybridoma cell line that makes a desired monoclonal antibody is injected into the body of mice through intra-muscular injection. The mice are grown in the laboratory for 3 or 4 weeks. Their ascitic fluid or blood is taken and the monoclonal antibodies are isolated from it. By this method, a mouse can produce about 50 mg of MCA.

2. Suspended Cell Culture in Fermenters

A hybridoma clone is cultured in a large fermenter using chemically defined complex medium having all minerals, vitamins and co-factors. Airlift fermenter is of much use for this purpose. In this method, one litre of culture broth can yield 100 mg of MCA in 2 weeks. [For details refer to Animal cell culture].

3. Immobilized Cell Reactors

Cells of a hybridoma clone is immobilized in a hollow fibre reactor using polyacrylamide gel. By this method, a few grams of MCA can be produced within 2 weeks.

Applications of Monoclonal Antibodies

Monoclonal antibodies are very pure and specific to antigenic substances. Hence they have been put into many practical uses in research, clinical diagnosis of diseases and in the treatment of the diseases. Their overall applications are discussed briefly here under:

1. Monoclonal Antibodies As Research Tools

Monoclonal antibodies have been used as research tools in the fields of medicine, immunology, biochemistry, genetic engineering, carcinobiology and so on. Some of their research applications are given below:

a. In genetic engineering, monoclonal antibody is used to screen recombinants.

b. In immunological studies, MCAs are used to identify various cell types involving in immune responses and to detect interactions among them. For this purpose, MCAs are raised against the cell surface antigens and used as markers to identify the cells.

c. The rejection of transplanted organs, especially kidney transplants, is associated with OKT-3 antigen present on the surface of T-cells. Monoclonal antibody against OKT-3 antigen is made and injected into the patient to suppress the transplant rejection. The MCA named ***orthoclone OKT-3*** is mainly used for this purpose.

d. Monoclonal antibodies are used to determine the structure of cell membranes.

e. They are employed in serological classification of closely related bacteria, viruses and protozoans.

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f. They are used in radioimmunoassay, ELISA and immuno fluorescence assay in research to identify and detect some target products.

g. MCAs are used to detect the exact position of enzymes in the cells by using immunocytochemical methods.

h. MCAs are being used to detect and classify enzymes.

i. Monoclonal antibodies are used in affinity columns to pick up proteins from the solution. They help to isolate and purify proteins and enzymes from solutions.

2. Clinical Diagnosis

Since 1979, monoclonal antibodies have been put in the clinical diagnosis of certain severe diseases. MCAs are used as diagnostic kits for-

i. Childhood diarrhoea

ii. Malaria

iii. Cancers in gastro-intestinal tract, pancreas, breast, uterus, kidney, stomach, larynx, liver, bone and connective tissue.

iv. Leukaemias

vi. Venereal diseases caused by *Nisseria gonorrhoea* and *Chlamydias*.

vii. Infections by herpes virus 1 and 2.

Pregnancy can be detected in women as early as one or two weeks after conception using MCAs.

3. Treatment of Severe Diseases

The cancers of lungs, breast, pancreas, etc. are cured by using monoclonal antibodies. ***Marx*** in 1982, in the US National Cancer Institute, made monoclonal antibodies against some surface antigens of cancer cells. That institute has cured several cancer cases by injecting the MCAs.

Simpson and ***Cioli*** (1982) have prepared MCAs against the surface antigens of some parasites. These MCAs are used to control the spread of *Troponema pallidom*, *Mycobacterium leprae*, *Haemophilus influensae*, etc.

Hazards of MCAs

Most of the mouse myeloma cells contain certain genes related to retroviruses. If monoclonal antibodies were prepared from such myeloma cells, the MCAs may have the dangerous viruses. The hazards of the viruses should be eliminated before using the MCAs. In order to prevent the viral hazards, the regulatory authorities approve MCAs for medicinal use after confirming that they are virus free.

Chimeric Antibodies

Antibodies consisting of parts of polypeptide chains of two different species are called ***chimeric antibodies.*** It has a constant region from human gene and variable region from mouse gene.

The variable region of mouse origin determines the antigenic specificity to react with the concerned antigen. The constant region of human origin prevents the immune response of the patients against the antibody injected into the body. As a part of chimeric antibody is of human origin, chimeric antibodies are also known as ***humanized antibodies***.

Usually humanized antibodies are injected into patients to overcome the immune response against the mouse antibody. Therefore, they are known as ***human anti-mouse antibody*** (HAMA).

A complete gene that codes for light chain of an antibody is assembled from three DNA segments. A part of gene coding for constant region of human antibody is isolated from human lymphocytes. Two DNA segments coding for variable region of mouse antibody were isolated and linked with the human DNA. The complete DNA that codes for heavy chain of the antibody is also assembled. The genes for both light chain and heavy chain are introduced into *E.coli*. The recombinant *E.coli* is cultured in large fermenters so as to manufacture chimeric antibodies.

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Dabs / Single Domain Antibodies

The single strand of antibodies, that can bind with the target antigen as the whole antibodies do, is called ***single domain antibodies or domain antibodies (Dabs).*** Dabs are made up of either heavy chain or light chain.

The antigen binding site consists of two polypeptide chains. The two polypeptide chains are held together by a short polypeptide. As Dabs have a single domain structure, it is very easy to produce by genetic engineering.

A complete gene that codes either for the heavy chain or light chain is assembled by joining DNA fragments from mouse antibody gene and human antibody gene. It is then introduced into *E.coli* through a vector. The recombinant *E.coli* is cultured in large fermenters for producing Dabs in larger amount.

Researchers are now looking for ways to use Dabs in research, industries and therapeutics.

transgenic tobacco expressed the cloned phaseolin gene. The plant was named ***tobean***. However, tobean is of no value as a food crop.

The ***USA grigenetics corporation*** had introduced phaseolin gene of French bean into ***sunflower calli*** through Ti plasmid. The transgenic sunflower plants have been producing phaseolin in their seeds. The phaseolin producing sunflower plants are called ***sunbean***. ***Sunbean oil*** is rich in ***proteins***.

Grain *Amaranthus* contains a seed storage protein called ***AMA-I***. This protein is rich in all essential amino acids, but it is produced in least amount. ***Asis Datta*** and his colleagues in 1998 have made ***cDNA*** of ***AMA-I protein*** and introduced it into ***potato calli*** through ***Ti plasmid***. The transgenic potatoes contain higher proportion of essential amino acid than what the ordinary tubers contain. The ***starchy potato tubers*** are now a wholesome nutritive food. Field trials have been going on to release the transgenic potatoes for public use.

Sweet Proteins

Monellin is a ***sweet protein*** in the fruits of the African plant, *Dioscorephyllum cumminsii*. It is ***100,000*** times sweeter than ***sucrose***. It is a boon to diabetics as it is readily digestible as the normal dietary proteins. The gene coding for monellin was isolated from *D. cumminsii* and joined with ***35S promoter*** of ***CaMV***. It is introduced into ***lettuce*** through Ti plasmid; the transgenic lettuce produces ***monellin***.

The monellin gene is coupled with a ***tomato fruit*** specific ***promoter E8*** and introduced into tomato through Ti plasmid. Monellin content is low in the immature fruits and leaves of the transgenic tomato, but it is very high in ripe fruits. The transgenic tomatoes are just in field trials.

Thaumatin is a ***sweet protein*** present in the fruits of *Thaumatococcus danielli*, an endemic plant of the Western Africa. The vernacular name of the plant is ***Katemfe***. Thaumatin is 3000 times sweeter than sugar.

cDNA of ***thaumatin*** II was made from mRNA and cloned in *E.coli* and yeasts *(Saccharomyces cerevisiae)* The genetically engineered microbes produce ***thaumatin II***. Thaumatin -II has been manufactured in large fermenters and sold in the name ***Talin***. Talin is a boon to diabetics who could not taste sugars.

VI. Enriching the Carbohydrate Contents

Plants have been genetically manipulated in such a way as to have improved soluble sugars. ***Stark*** and others in 1992 introduced a mutant ***ADP glucose pyrophosphorylase (GP) gene*** of *E.coli* into potato. The transgenic potato has been richer in starch than the ordinary tuber.

Muller Robert introduced an antisense DNA construct into potato. The transgenic potato has been rich in glucose and sucrose, but poor in starch. It is used in the manufacture of glucose.

VII. Transgenic Rice with Vit-A

Vitamin A is a ***fat soluble*** vitamin formed in the human body from precursors, the yellow pigments of plants such as α, β and γ ***carotenes***. It is essential for normal growth and development, integrity of epithelial tissues and for proper development of teeth and bones. Deficiency of this vitamin causes disorders of ***skin, cornea, conjunctiva, hair follicles*** and ***renal pelvis***. It mainly interferes with ability of eye to adapt to darkness (night blindness). It is estimated that about 120 millions of children all over the world suffer from vitamin A deficiency. Every year about 20 million children are victimized due to vitamin A deficiency in areas where rice is used as major food crop.

Rice, that is a staple food in almost all countries, has neither vitamin A nor the precursor carotene. But it contains precursors ***geranylgeranyl diphosphate*** (GGDP) for the synthesis of ***β-carotene*** in the endosperm tissue. The ***GGDP*** cannot be converted into β-carotene because of the absence of three enzymes necessary for that carotene synthesis. The three enzymes are ***phytoene synthase, phytoene desaturase*** and ***lycopene β-cyclase***.

Ingro Potrykus and ***Peter Beyer***, in the year 2000, created a transgenic rice capable of

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producing β-carotene by using recombinant DNA technology. This plant is known as ***Golden rice.*** To create golden rice, ***two daffodil genes*** and ***one bacterial gene*** coding for ***β-carotene*** synthesis from GGDP are transferred to rice ***callus*** through ***transformation technique***. The plants regenerated from the transformed calli produce ***golden rice.***

Endosperm of golden rice contains all enzymes responsible for synthesis of ***β-carotene*** so that it accumulates β-carotene in it. Because of presence of β-carotene, dehusked grains appear in yellowish brown colour and hence the name golden rice.

When the golden rice is eaten, human body immediately synthesizes ***vitamin A*** from the β-carotene. It will reduce vitamin A deficiency in people suffering from under nutrition.

Fig.19.5: Grains of golden rice.

VIII. Improving the Quality of Oils and Fats

Oil quality can be improved by using genetic engineering.

Rape seed is poor in ***stearate*** and ***stearic acid***. ***Knutwom** et al.*, (1992) prepared an antisense DNA construct for the enzyme ***Δ9 stearoyl ACP desaturase*** and introduced it into embryos of rapeseed. The transgenic rapeseed produced oil with high level of stearate so that it is useful for the manufacture of margarine (a butter-like solid oil).

Arondel and others introduced Δ1... ***desaturase gene*** into the rape seed. The trans-genic plants produce oil rich in α-linoleic acid.

Monsanto (1994) developed transgenic rape seed varieties ***23-18-17*** and ***23-198*** which have improved seed fatty acids. Gene coding for ***thioesterase (TE)*** was transferred to the rape seed tissue through Ti based binary vectors. The transgenic plant is rich in ***laurate and myristic acid.***

The gene for the biosynthesis of oil in cocoa was transferred to soybean through Ti plasmid. The transgenic soybean produces cocoa oil that can be used for chocolate making.

A gene for ***acyl carrier protein*** of spinach was transferred to rapeseed through Ti plasmid. The transgenic rapeseed produces soybean oil-like oil.

IX. Male Sterility and Fertility Restoration

Plant that cannot produce viable pollen grains, is called ***male sterile plant.*** Male sterile plants are useful for plant breeding using hybridization method. They avoid the problem of emasculation.

The enzyme ribonuclease (RNase) of *Aspergillus oryzae* selectively acts on tapetal RNAs and digests it. So pollengrains do not develop from pollen mother cells. RNase of *Bacillus amyloliquefaciens* selectively acts on ribosomal RNA of anthers and hence stops pollen germination. It causes pollen sterility.

A synthetic ***Ti RNase gene*** of *A.oryzae* and a ***ba RNase*** gene of *B.amyloliquefaciens* are joined together to get a chimeric RNase gene. The chimeric RNase gene is linked with TA29 promoter of tobacco and is inserted into callus tissues through Ti plasmid. Transgenic plants regenerated from the recombinant calli produce Ti RNase and ba RNase. Therefore, neither tapetal layer nor pollen tube develops. So the transgenic plants remain sterile.

Monsanto company in the USA has developed male sterile *rape seed (Brassica napus), maize, cotton, groundnut, pumpkin, squash* ... ld the seeds to the farmers