REVIEW ARTICLE

Accepted 15 September 2001
Revised 25 July 2001
Received 14 August 2000
Indian J. Pharm. Sci., 2002, 64(2): 93-107

Monoclonal Antibody Technology: Trends and Prospectus

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A fantastic technological breakthrough was achieved by Milstein and Kohler who devised a technique for the production of immortal hybridoma cell lines making monoclonal antibody specific for a single epitope on a complex antigen. The availability of monoclonal antibodies has revived interest in immunotherapy. The ability to influence on individual's immune state by administering immunoglobulin of the appropriate specificity may provide a powerful approach to disease control and prevention. Monoclonal antibodies have tremendous applications in the field of diagnostics, therapeutics, targeted drug delivery systems, not only for infectious disease caused by bacteria, viruses and protozoa but also for cancer, metabolic and hormonal disorders. Looking at the pros and cons of traditional hybridoma technology for the production of monoclonal antibody, recombinant DNA technology has been used to engineer the chimeric monoclonal antibody, complementarity determining regions (CDR) grafted monoclonal antibodies, heteroconjugate antibodies and total recombinant monoclonal antibodies. Genetically engineered human antibody fragments can be derived by expanding the variable heavy (VH) and variable light (VL) chain genes from non-immunized, or preferably immunized donors and expressing them as completely randomised combinatorial libraries on the surface of bacteriophage. Recombinant antibodies can be expressed on a large scale in plants also.

The term monoclonal means antibody produced by a cell clone derived from a fusion of one antibody producing cell with one myeloma cell. Such a fused cell, which was originally described as hybrid-myeloma, was named as hybridoma[1]. Monoclonal antibodies (MAbs) are conventionally obtained by hybridoma technique and recently by recombinant DNA technology. These MAbs have tremendous applicability in bio-medical research and in diagnosis and management of human diseases.

THEORY AND PRINCIPLE

In a typical fusion experiment, for the production of monoclonal antibodies, spleen cells from hyperimmunised mouse are fused with myeloma cells in presence of polyethylene glycol[2]. An essential requirement of this technology is to allow the selective growth of hybrid cells. For this purpose, a mutant myeloma cell line, defective in the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT) is established by growing murine myeloma cell line in the presence of guanine analogue, 8-azaguanine. Proliferating eukaryotic cells normally utilize folic acid pathway for synthesis of DNA. Rescue pathway, which uses hypoxanthine/guanine is dependent on the presence of HGPRT enzyme in the cells. The fused cells are selected in a medium containing hypoxanthine, aminopterine, which is folic acid analogue and thymidine (HAT). The nonfused myeloma cells die in this medium, because aminopterine blocks the main pathway of DNA synthesis and the cells cannot use rescue pathway using exogenous hypoxanthine, as they lack the enzyme, HGPRT (fig. 1). The unfused spleen cells from immunized mice eventually die in the tissue culture as they have finite life. However the hybrid of the HGPRT negative my-

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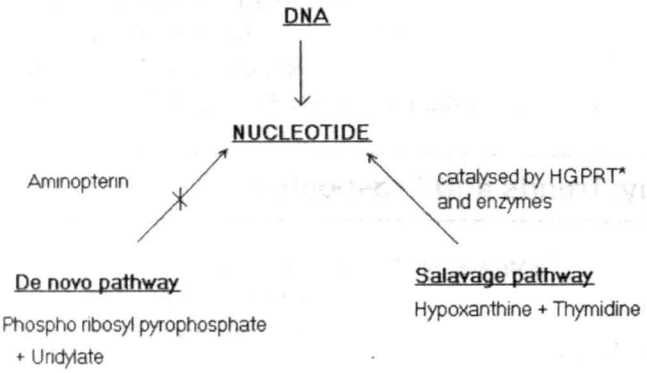

Fig. 1: Pathways for nucleotide synthesis.

HGPRT-Hypoxanthine Guanine Phosphoribosyl Transferase. X indicates inhibition of the step in forward direction.

eloma cell and the antibody producing splenic lymphocyte, selectively survives because the HGPRT enzyme is provided by spleen cell genome and immortality conferred by myeloma cells. The hybrids are subsequently cultured in normal medium and the supernatant tested to find out if a hybrid cell clone is producing the antibody of the right specificity.

The hybrids have a tendency to loose chromosomes randomly at every division for a few divisions, after which the chromosome complex gets stabilized. In this process the progeny of same positive hybrids sometimes becomes nonproducers resulting in a mix population. It is therefore essential to segregate the non-producers from producers by cloning in limiting dilutions, wherein single antibody producing hybrid cells are further segregated and are allowed to grow.

There are certain constraints in a successful fusion. In order to obtain stable hybrids, cell of same ontogeny need to be fused. A cell of similar phylogeny produced more stable hybrids. Therefore, for production of human monoclonal antibodies (hMAbs), human B cell must be fused with human myeloma. In case of mouse X human heterohybrids, genetic component is more unstable, because of loss of human chromosomes. For rescue of the fusion of antibody production, the B cell is fused with thymoma cell. Then the specific MAb producing hybrids are selected, cloned and expanded[3]. Details of conventional hybridoma technology used for the production of monoclonal antibody is described below[1,3,4] and illustrated in fig. 2.

IMMUNIZATION

To obtain a good number of hybrids secreting the specific antibody it is essential to have optimal immunization of the inbred mice. The choice of immunization schedule depends on the antigen whether it is soluble or particulate and the type of antibodies required. The antigen is given in Freund's complete adjuvant followed by two to three doses in incomplete adjuvant. When the concentration of antibodies is found to be nearly optimal, the animal is sacrificed and the spleen, which contains a large number of plasma cells, is dissociated into single spleenocytes by mechanical or enzymatic method. These spleenocytes are further used in the fusion experiment.

CELL FUSION

The characteristics for ideal myeloma fusion partner for producing monoclonal antibodies is that it should be rapidly growing, easily clonable and should not produce any antibody on its own. Initial fusion partner, which Kohler and Milstein[2] used was a mouse myeloma cell, which was not secreting the antibody (non-secretor). This was made HAT selective and is being used as the myeloma fusion partner of choice all over the world. These myeloma cells were from the inbred strain of the mouse and thus for fusion, immune spleen cells of inbred mice are used.

Originally Kohler and Meilstein used Sendai virus for fusion. Eventually the same investigators developed a method of fusion using polyethylene glycol (PEG)[2]. PEG binds to glycoproteins on surface of the cells and through complex events, dissolves the plasma membrane resulting in mixing of the contents of both cells to get hybrid cells. Fusion procedure is essentially by adding 50% PEG solution to the cell pellet containing both spleen cells and the myeloma cells in equal proportion. Then after appropriate dilution of PEG, cells are incubated for 1 h at 37° and then plated. Recently, instead of using virus and chemicals for fusion, another method of electrofusion has been developed. In principle, if a strong electrical pulse is passed for a second through the cells which are in contact with each other, plasma membrane at the contact points dissolves resulting in the fusion of the cells.

SELECTION OF HYBRID CELLS[1]

HAT selection method[1]:

The selection method for the hybrid should be such that it would allow growth of only hybrid of lymphocyte and myeloma cells and would not allow the growth of myeloma fusion partner or lymphocyte. This is usually done by selecting hybrids in HAT medium. Myeloma fusion partner is selected in such a way that it does not grow in this HAT medium. Aminopterine is an analogue of folic acid. Thus cells

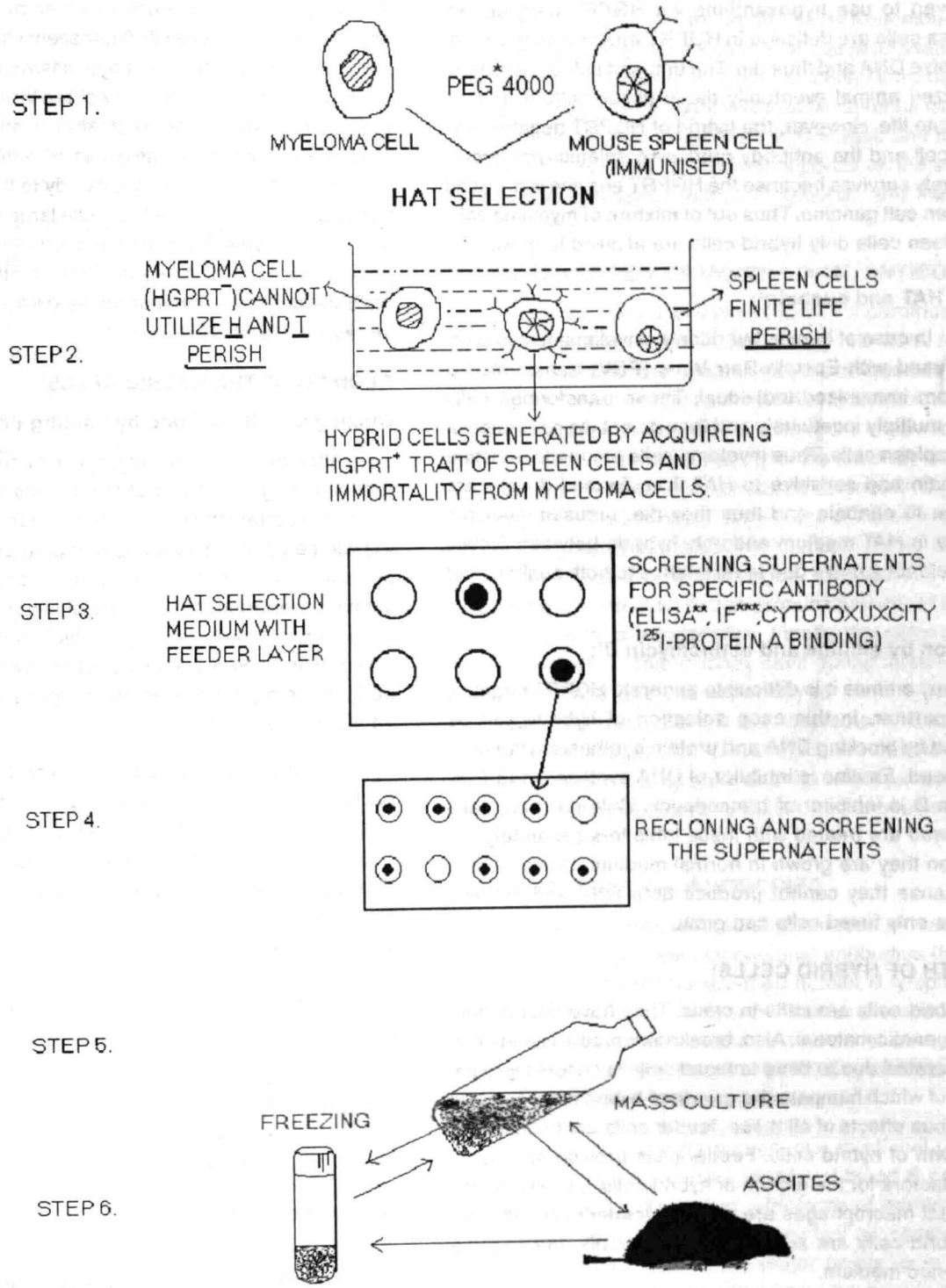

Fig. 2: Steps in preparation of monoclonal antibodies.

PEG, Polyethylene glycol; ELISA, Enzyme linked immunosorbent assay; IF, Immunofluorescence.

Step1. Cell Fusion, Step2. Selection, Step3. Screening, Step4. Purification, Step5. Mass Cultivation, Step6. Preservation.

are driven to use hypoxanthine via HGPRT pathway. As myeloma cells are deficient in HGPRT enzyme, they cannot synthesize DNA and thus die. The unfused spleen cells from immunized animal eventually die in tissue culture as they have finite life. However, the hybrid of HGPRT negative myeloma cell and the antibody producing splenic lymphocyte selectively survives because the HGPRT enzyme is provided by spleen cell genome. Thus out of mixture of myeloma cells and spleen cells only hybrid cells are allowed to grow.

Use of HAT and ouabain[1]:

In case of human hybridoma, myeloma fusion partner is fused with Epstein-Barr Virus (EBV) transformed B cells from immunized individual. These transformed cells have to multiply indefinitely and they do not die as in case of mouse spleen cells. Thus myeloma cells are made resistant to ouabain and sensitive to HAT. Transformed B cells are sensitive to ouabain and thus they die, unfused myeloma cells die in HAT medium and only hybrids between B cells and myeloma survive due to resistance to both ouabain and HAT.

Selection by emitine and actinomycin D[1]:

Many a times it is difficult to generate HGPRT negative fusion partner. In this case selection of hybrids can be achieved by blocking DNA and protein synthesis in the cells to be fused. Emitine is inhibitor of DNA synthesis and Actinomycin D is inhibitor of transcription. Before fusion, cells to be fused are treated with these inhibitors separately. After fusion they are grown in normal medium. Unfused cells die because they cannot produce both DNA and proteins and thus only fused cells can grow.

GROWTH OF HYBRID CELLS[1]

Hybrid cells are cells in crisis. They have double than normal genetic material. Also, breakdown products and debri are generated due to dead unfused cells in culture medium, toxicity of which hampers the growth of hybrid cells. To avoid deleterious effects of all these, feeder cells are provided for the growth of hybrid cells. Feeder cells provide necessary growth factors for the growth of hybrid cells. Usually mouse peritoneal macrophages are used as feeder cells. After fusion hybrid cells are added onto these cells retaining the conditioned medium.

Antibody detection methods[1]

Antibody to the antigen in question can be detected by various methods. In principle, antibody secreted by the hybrid cells is allowed to react with the antigen in question. The antigen bound antibody can then be detected by either enzyme, or radiolabelled or fluorescent chemical tagged anti-antibody. Many a times functional assays are also performed like neutralization of infectivity of the virus. The ideal detection method should be such that it should be sensitive enough to detect small amounts of antibody. It should be specific i.e. it should detect antibody to the antigen in question only. It should be able to handle large number of samples at a time. Enzyme linked immunosorbant assays (ELISA) is popular because of its ease. Other methods for detecting antibody are radio immuno assay (RIA), indirect immunofluorescence and Western blot.

CLONING OF THE HYBRID CELLS[1]

Cloning in soft agar and by limiting dilution analysis[1]:

After identifying hybrids, which secrete antibody to the required antigen, cloning of the hybrids is taken up. This is done to ascertain the monoclonality of the hybridoma. Cloning can be done in two ways, soft agar cloning and cloning by limiting dilution; the later is more popular. In soft agar cloning, heterogeneous mixture of hybrid cells is separated by localizing growth of single cells in soft agar. After colonies grow to sizes visible to the naked eye, they can be picked up from the agar with a Pasteur pipette and then grown in 24 well plates.

In limiting dilution technique, hybrid cells are diluted in such a way that 1 ml would contain one to three cells. One tenth of a millimeter of this is added to these feeder cells. Thus each well theoretically would contain 0.1 to 0.3 cells. This means that one third or one tenth of the wells will have single cells. These are allowed to grow into colonies. Antibody secretion by these clones is estimated by detection methods described earlier. These clones of hybrid cells can then be grown in large volumes to obtain monoclonal antibodies.

Cloning can also be done by the fluorescence activated cell sorter (FACS)[1] technique in which both B and T cell hybridomas may be selected for cell surface antigens or DNA content after cell fusion by FACS.

Cloning of human cells[1,9]:

Human cells are much harder to clone. For this, plates at higher cell density such as 10 cells in each microtitre well are usually included in the procedure as a precautionary measure. It may be anticipated that the majority of clones will be negative so that a very wide screen will have to be used to detect positive clones.

Failure of cloning:

Most hybridomas are lost at the cloning stages rather than the fusion stages. Sometimes this is unavoidable as the clone loses the appropriate chromosome at an early stage of development. In cross-species fusions, chromosome loss is clearly a complex and selective process[5] with different human chromosomes being retained.

STORAGE OF THE HYBRIDOMA CELLS BY CRYOPRESERVATION[1]

As hybrid cultures are needed for long term use, these cells are stored in liquid nitrogen. Usually hybridoma cells are suspended in 10% dimethyl sulphoxide in culture medium with serum. Cells are frozen to -80° slowly by various methods and then transferred to liquid nitrogen tanks having a temperature of -197°. Cells can be stored in this way without loss of viability for years and can be revived whenever required.

LARGE SCALE PRODUCTION OF MONOCLONAL ANTIBODIES[1]

Tissue culture method[1]:

Monoclonal antibody production can be scaled up using either tissue culture methods or growing hybridoma clones in mouse peritoneal cavities. Tissue culture scaling can be done by preparing many flasks of the antibody secreting clone. The culture volume can be increased by using spinner cultures, large layers of tissue culture trays, or with hollow fibre cartridges. As serum in the culture medium can interfere with the purification of monoclonal antibodies serum-free synthetic media can be used. In case of human hybridoma usually tissue culture methods are used for scaling up.

Mouse ascitic fluid[1]:

Inbred cells and the hybrid clones derived from these grow in the peritoneal cavity of inbred mouse as ascitic tumour. In the mouse peritoneal cavity, irritation is created by injecting an oil like pristane. This allows the growth of mouse myeloma cells or the hybrid in the peritoneal cavity. As hybrid cells grow, concentrated solution of monoclonal antibody is obtained which can be tapped by a needle. In case of rat-mouse hybrid immunodeficient mice which are further irradiated with gamma rays to destroy the immune system are used for the ascitic tumour growth.

PURIFICATION METHODS[1]

Antibodies obtained from the tissue culture fluids of the hybridoma culture or from the ascitic fluids from mouse can be purified by biochemical methods of salt precipitation. Alternatively, immunological methods of affinity chromatography with protein A or antiimmunoglobulin columns can also be used. Both methods have their advantages and the disadvantages. The choice of method depends on the stability of the antibody, original source of antibody and the purity grade required.

CHARACTERISATION OF MONOCLONAL ANTIBODIES[1]

Fused cells carry a tetraploid number of chromosomes. They therefore have to put more energy into DNA replication. A cell, which looses unnecessary chromosomes, might be thought to able to replicate its DNA and divide faster and be able to outgrow its neighbors. Among the chromosomes, which it loses, may be ones coding for the antibody required. Genetic stability of the cell lines can be determined by monitoring its properties during serial passage of the cells through culture. The properties include the number, shape and size of the chromosome, biochemical properties of the immunoglobin product and other proteins and various metabolic and growth characteristics. Finally the physical and chemical stability of the antibody itself during different conditions of storage and use especially for its intended purpose must be determined. It is also characterised to define its affinity for antigen, its immunoglobulin subclass, the epitopes for which it is specific and the effective number of binding site that it possesses by ELISA, Immunofluorescence and Western blot[1].

HUMAN MONOCLONAL ANTIBODIES

The most common method of generation of human hybridomas secreting human monoclonal antibodies (hMAbs) involves fusion of EBV transformed human B lymphocytes with appropriate fusion partners[6,7]. But for therapeutic use of these monoclonal antibodies, presence of contaminant EBV DNA can pose a problem of producing cancer and removal of EBV DNA is difficult and expensive[8].

In case of human hybridomas, due to ethical reasons and for ease, non-immunised peripheral blood B cells are used preferentially[9]. Therefore, efficiency of generation of human hybridomas is thus generally low as compared to mouse hybridomas. One of the major problems is that of more stringent need for growth factors by human B cells. While generating human hybridoma, the lack of Major

Histocompatibilty Complex (MHC) antigen compatibility may also lead to a lower efficiency[9]. In mouse hybridomas this situation does not exist as splenic B cells from inbred mice are fused with mouse myeloma. These deficiencies have prevented the development of appropriate and efficient fusion partners for human MAbs. Thus essentialities in generation of human hybridoma would include,

1. Vaccination of B-cell donor.
2. Prestimulation of B cells both to bring them in appropriate growth phase and to increase frequency of number of antigen specific B cells.
3. Generation of a fusion partner, which would provide necessary environment for fused human B, cells.

Currently available fusion partners for human hybridomas are based mainly on human non-secretor myeloma cell lines or mouse-human heterohybrids. Mouse human heterohybrids are generated by fusing mouse myeloma cells with stimulated human B cells. But, stability of human hybridomas is still a major problem[10].

Generally to increase frequency of stimulated B cells and to select B cells secreting desired antibody prior to fusion, EBV transformation of human B cells is done. As EBV is a cancerous mitogen for human B cells, a transforming mitogen like formalinised *Staphylococcus aureus* (FSTA) can be used for stimulating human B cells[9,11]. As a case study Singhania *et al.* have developed human monoclonal antibodies to rabies virus successfully by using FSTA stimulated human B cells[9].

HYBRID CHIMERIC MONOCLONAL ANTIBODIES

Many a times it is not possible to immunize a human with a particular antigen e.g. human immunodeficiency virus (HIV)[12]. In this case variable region from the murine monoclonal antibody can be attached to constant region of human antibodies[13-17]. This has been carried out using genetic engineering techniques[12,17] (fig. 3). Antibodies thus have the reactivity to the desired antigen and have human immunoglobulin constant regions[12,18]. Further refinement of these types of chimeric antibodies to delete possible immunogenic regions (framework antigens of mouse immunoglobulins) are undergoing[19,20]. Expression of these antibodies on eukaryotic cells will further improve the quality of these antibodies[21,22].

Chimeric monoclonal antibodies are prepared by cloning recombinant DNA from the mouse B cell containing the promoter, leader and variable region sequence coding for a mouse antibody and the constant region exons from the human B cell. Such type of recombinant antibody encoded by recombinant gene is a mouse human hybrid/chimera, also called as Humanized antibody[17]. The antigenic specificity of such antibody is determined by the variable region (Fab) which is derived from mouse DNA and the constant region (Fc) is derived from human DNA. Recently random recombinant mechanisms have been used to prepare humanized and human antibodies in bacteria[17].

Advantages and disadvantages of humanized antibody:

It is less immunogenic to humans than mouse monoclonal antibodies when it has to be used for therapeutic purposes. It retains the biological effector function of human antibody and therefore can trigger complement activation or Fc receptor binding. Apart form these advantages a major disadvantage is that though chimeric monoclonal antibody is less immunogenic to humans as compared with mouse monoclonal antibodies, it can induce some immune response in humans[23].

CDR GRAFTED HUMANISED MONOCLONAL ANTIBODY

The mouse variable region in the humanized antibodies can induce an immune response in humans[24] therefore chimeric antibodies containing only mouse CDRs have been developed[19,25,26]. This technique involves grafting of mouse antibody CDRs onto human framework regions to construct a variable region retaining the same human antibody framework (fig. 4). These antibodies are less immunogenic in humans than humanized antibodies containing the entire mouse variable region[27-31]. Since the CDRs compose the antigen-binding site, some CDR-grafted antibodies retain their ability to bind antigen. Often, however, CDR-grafted antibody exhibit reduced binding affinity[32-34]. In some cases, this can be corrected by introducing small mutations in the framework region that induce small changes in the three dimensional configuration of the CDRs resulting in improved antibody affinity[35]. CDR-grafted antibodies have many potential therapeutic uses[27-31] (Table 1).

IMMUNOTOXINS

Immunotoxins are chimeric molecules comprising a cell binding moiety, such as antibody or ligand, linked to a toxin or its subunit[36] (fig. 5a). In this case, the terminal constant region domain in a tumor specific monoclonal antibody is replaced with toxin chains. Because these immunotoxins lack the terminal Fc domain, they are not able to bind to cells bearing Fc receptors. The use of toxins bound to mono-

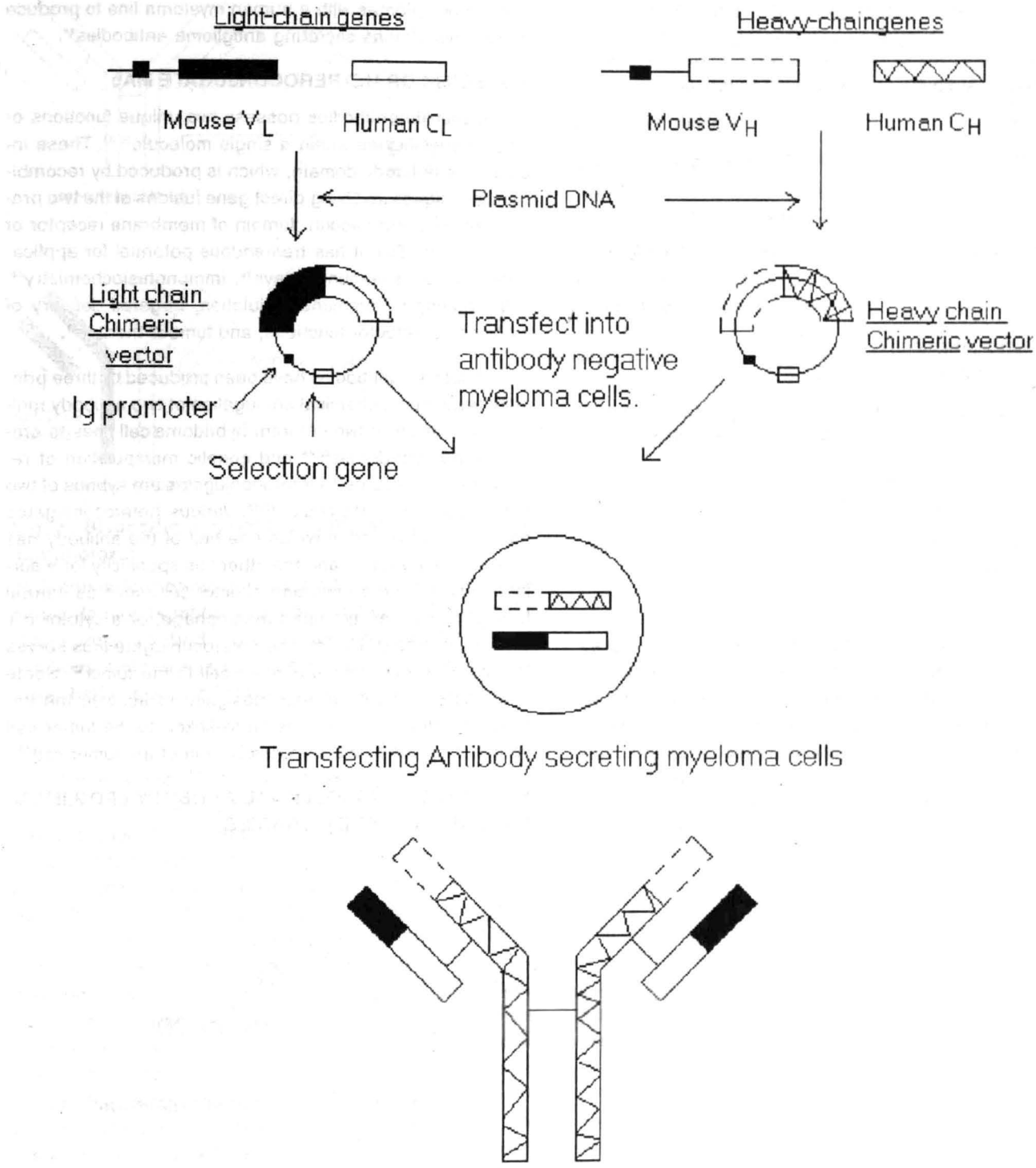

Fig.3: Production of chimeric mouse-human antibody.

Chimeric mouse-human heavy and light chain expression vectors are produced. These vectors are transfected into antibody negative myeloma cells. Culture in ampicillin containing medium selects for transfected myeloma cells, which secrete the chimeric antibody.

TABLE 1: USE OF CDR GRAFTED MONOCLONAL ANTIBODIES IN MEDICINE[17,87].

	Target Antigen	Clinical Potential
1	CD3*	Organ transplantation
2	CD4*	Organ transplantation, rheumatoid arthritis, Crohn's disease
3	Receptor for IL2**	Leukemia's and lymphomas, organ transplantation, graft-versus-host disease
4	Human immunodeficiency virus	AIDS***
5	Herpes simplex virus	Neonatal, genital and ocular herpes infection
6	Human epidermal growth factor	Cancer

* T-cell marker., ** Interleukin-2., ***Acquired Immuno Deficiency Syndrome.

clonal antibody in tumour destruction has gained wide attention[37]. Toxins could be of bacterial and plant origin. Bacterial toxins such as diphtheria toxin[38], pseudomonas exotoxin[39] and the plant toxin ricin, abrin and modecin are some representative examples of toxin[40]. These immunotoxins could bind specifically to tumor cells, making them more efficient as a therapeutic reagent[37,40,41]. Therapy of gliomas from brain therapy is achieved from fusion of lymphocytes obtained from gliomas with a human myeloma line to produce human hybridomas secreting antiglioma antibodies[41].

BISPECIFIC OR HETREROCONJUGATE MAb

Bispecific antibodies possess two unique functions or binding specificities within a single molecule[42,43]. These include one antibody domain, which is produced by recombinant techniques involving direct gene fusions of the two protein domains and second domain of membrane receptor or toxins[43,44] (fig. 5b). It has tremendous potential for applications, including immunoassays[35], immunohistochemistry[44], cell targeting[45,46], immunomodulation, targeted delivery of cytokines or effector functions, and tumour therapy[47].

Bispecific antibodies have been produced by three principal techniques: chemical conjugation of two antibody molecules[43], fusion of two different hybridoma cell lines to create hybrid hybridomas[48,49] and genetic manipulation of recombinant molecules[41]. Heteroconjugates are hybrids of two different antibody molecules[42,43]. Various heteroconjugates have been designed in which one half of the antibody has specificity for a tumor and the other has specificity for a surface molecule on an immune effector cell, such as natural killer (NK) cell, an activated macrophage, or a cytotoxic T cell lymphocyte (CTL)[45,46]. The heteroconjugate thus serves to cross-link the immune effector cell to the tumor[47]. Some heteroconjugates have been designed to activate the immune effector cell when it is cross-linked to the tumor cell so that it begins to mediate destruction of the tumor cell[48].

ENGINEERING MONOCLONAL ANTIBODY FROM IMMUNOGLOBULIN GENE LIBRARIES

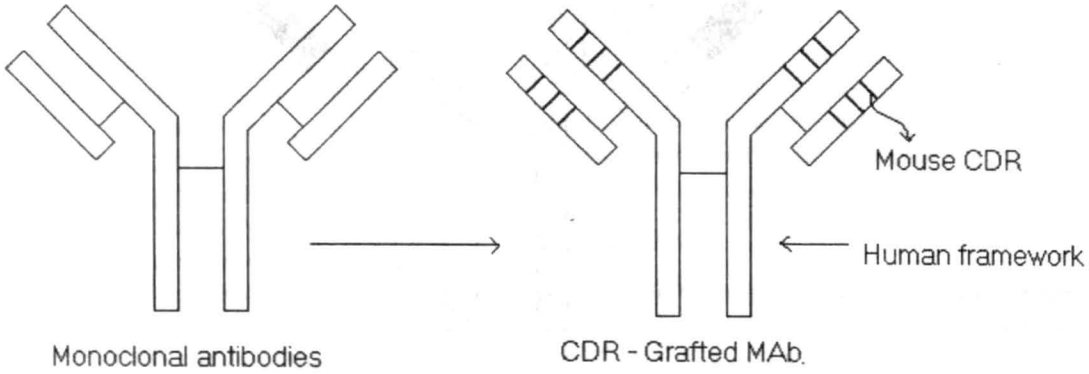

Fig. 4: CDR grafted monoclonal antibody.

A chimeric monoclonal antibody containing only the CDRs of a mouse monoclonal antibody (dark bands) grafted within the framework regions of a human monoclonal antibody.

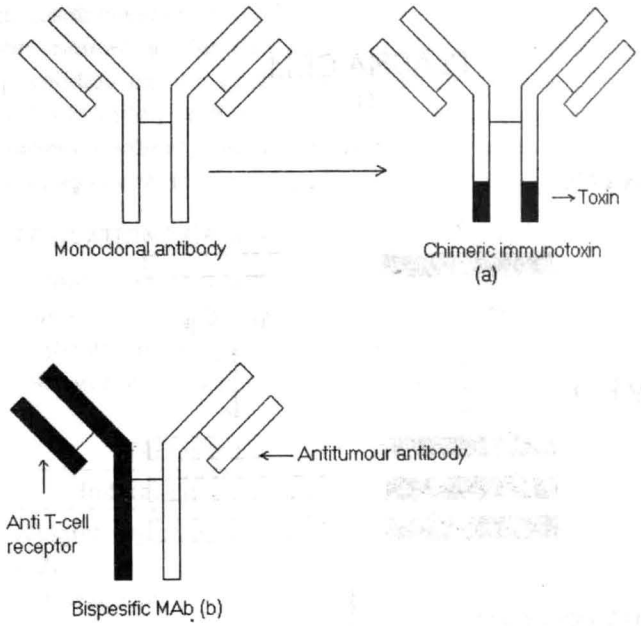

Fig. 5: Bispecific monoclonal antibody and Chimeric immunotoxin.

a) A chimeric monoclonal antibody in which the terminal Fc domain is replaced by toxin chain. b) A bispecific monoclonal antibody in which one half of the mouse antibody molecule is specific for a tumor antigen and other half is specific for CD3/T-cell receptor complex.

Antibody engineering evolved rapidly following the development of sophisticated techniques such as polymerase chain reaction (PCR) mediated amplification of genes; cloning and construction of gene library[50]. The generation of substantial database of antibody variable region sequences has not only permitted the design of variable region specific oligonucleotides for further PCR applications, but has also facilitated advances in the design, computer modeling and humanization of antibody derivatives. Generally, cDNAs from hybridomas or other cells producing a defined antibody are used to clone the sequences coding the V domains of antibodies. Methods for rapid and reliable cloning of hybrid V regions have been reported in the past two years[51,52]. A promoter region and a suitable restriction site e.g. *Eco*R1 are added to the amplified sequences, and the resulting constructs are inserted into the bacteriophage lambda, yielding separate heavy chain and light chain libraries. Cleavage with *Eco*R1 and random joining of heavy and light-chain genes yield numerous novel heavy-light constructs[53] (fig. 6). This procedure generates enormous diversity of antibody specificities; clones containing these random combinations of H+L chains can be rapidly screened for those secreting antibodies to a particular antigen. For example in one study a million clones were screened in just 2 days, with over 100 clones being identified that produced antibody specific for desired antigen. In the early 1980's expression of recombinant DNA products was often achieved in *E. coli*[54-56] or in yeast cells[57,58]. Subsequently, more efficient systems for expression in mammalian cells became available, including expression in CHO cells, which have been widely used for the production of other therapeutic proteins. These techniques have the potential of producing an enormous repertoire of antibody specificities without the limitation of antigen priming and hybridoma technology that currently complicate the production of monoclonal antibodies.

MONOCLONAL ANTIBODIES BY PHAGE DISPLAY TECHNOLOGY

Technologies have been emerging for making antibodies *in vitro* by mimicking the selection strategies of the immune system[59-61]. Repertoires of the antibody fragments are displayed on the surface of filamentous bacteriophage[62], each displaying a single antibody species; the phage are selected by binding to the antigen; and finally soluble antibody fragments are secreted from infected bacteria[63]. As in the immune system, the V genes can be subjected to random mutations, and mutants can be selected with higher binding affinities. This allows the isolation of human antibody fragments of defined specificity, against both foreign and self-antigens[64].

Phage display facilitates the construction of human antibodies of therapeutic value and of research reagents[64,65]. There is clearly a future for single pot libraries[66], as the same library can be selected with a range of different antigens, and without the need for immunization of animals. There may also be future for designer libraries[66]. As the potential antibody diversity is probably too large to be tapped in a single phase library, it may be advantageous to build libraries that shaped for complementarity to different antigen. As phage display cannot only exploit the principles of immune selection, but also cannibalise and improve on the antibody building blocks, it should increasingly be capable of outperforming the natural immune systems in making antibodies.

MINIBODIES

Drugs can be CDR's of minibodies. Minibodies are composed of a segment of heavy chain variable region containing three beta strands and H1 and H2 hypervariable loops.

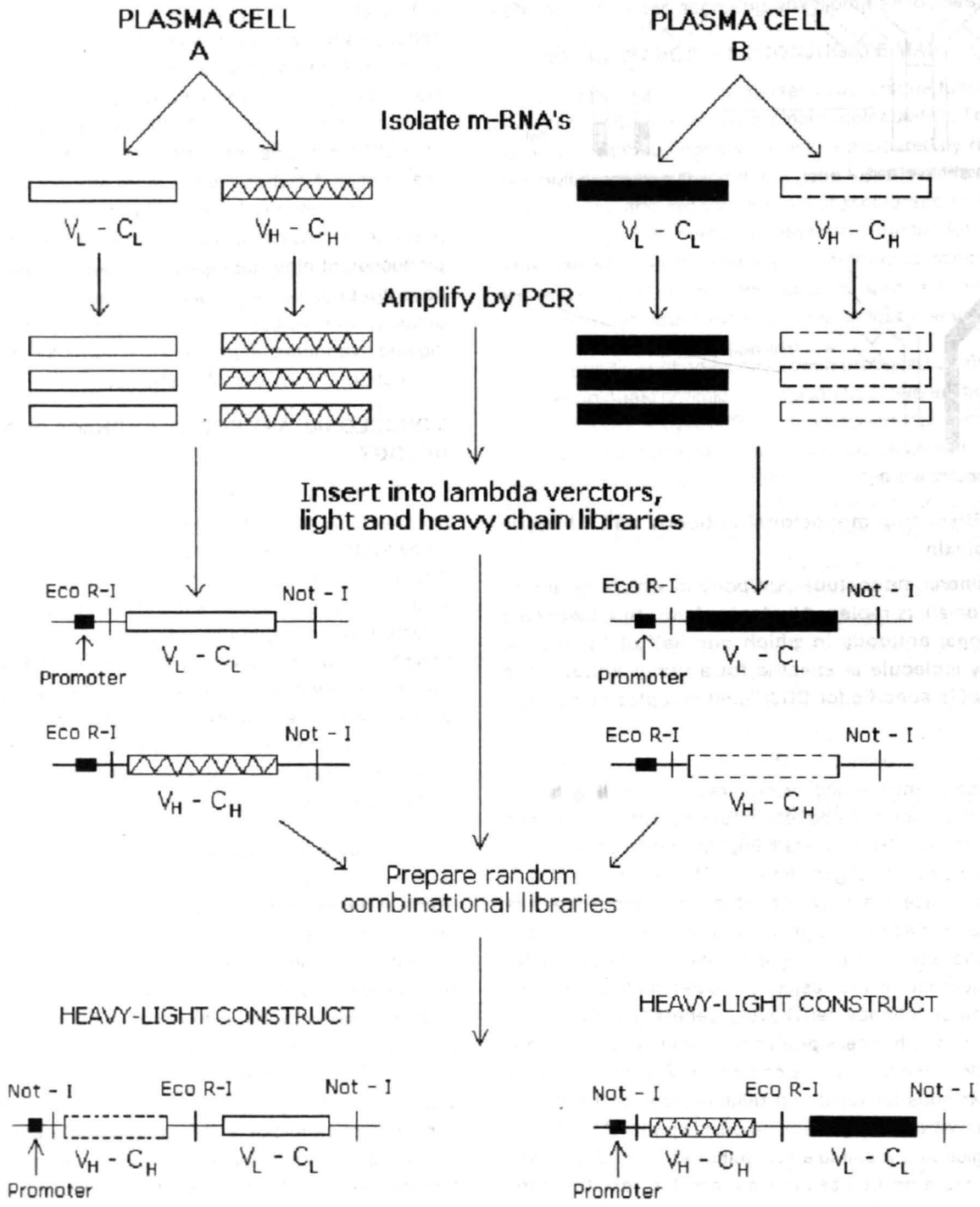

Fig. 6: General procedure for producing gene libraries encoding Fab fragments.

In this procedure isolated heavy and light chain m-RNA are amplified by PCR and cloned in lambda vectors. Random combinations of light and heavy chain genes generate an enormous number of heavy light-chain constructs encoding Fab fragments with different antigenic specificity.

By randomisation of CDR's and then expression of these recombinant molecules on bacteriophages millions of minibodies can be generated. These minibodies can be linked to variety of drugs and can be used for targeted drug delivery system[67]. Minibodies have proved to be excellent imaging agents in tumor-bearing mice[67].

APPLICATIONS OF MONOCLONAL ANTIBODY

Monoclonal antibodies have a remarkable range of applications in diagnostics[3,4,61], therapeutics[12,68-70] (Table 2), analytical[68], and drug targeting[71,72]. Detailed discussions on all the applications of monoclonal antibody is beyond the scope of this article. However, a brief list of applications is as follows:

1. Applications of Mab as a diagnostic reagents in diagnostic kits for the diagnosis of various infectious diseases[68], pregnancy, various tumors[73], HLA typing[71], monitoring therapeutic drug levels[73] and detecting diabetes[68]. Also for diagnostic imaging in cardiovascular diseases[72], cell surface markers[71], detection of circulating antigens[3,4], cancer[71-73] and hormones[74].
2. Monoclonal antibodies can be used as a therapeutic agent for the treatment of autoimmune diseases[75], in

TABLE 2: LIST OF MONOCLONAL ANTIBODIES, WHICH ARE CURRENTLY IN CLINICAL TRIAL[70].

Indication	Antibody name	Sponsors	Trial status
Allograft rejection	Orthoclone, Zenapax	Orthobiotech, Protein design lab	FDAapproved
AML	Smart M195	Protein design lab/Kanebo	III
Anticoagulant	Corsevin M	Centocor	I
Asthma/Allergy	RhuMAb-E25	Genetech/Norvartis/IDEC pharma.	III
Autoimmune diease	Smart anti-CD3	Protein design lab	I/II
Cancer (general)	Anti-VEGF	Genetech	III
Ovarian	OvaRex	Altarex	II/III
Colorectal	Panorex	Glaxo Wellcome/Centocor	Germany
Lung	BEC2	Merck LgaA	Approved
Head and Neck	IMC-225	Imclone Sys	III
Neck	Herceptin	Genetech	III
Breast			FDA approved
Crohn's disease	Infliximab	Centocor	FDA approved
Glaucoma surgery	CAT-152	Cambridge-Ab Tech	II
GVHD	ABX-CBL	Abgenix	III
Multiple Sclerosis	Antegren	Elan	II
Myocardial infarction	anti-CD 18	Genetech	II
NHL	Rituxan	IDECpharma/Genetech/Roche	FDA approved
Psoriasis	ABX-IL8	Abgenix	II
RA	D2E7	CAT/BASF	III
Sarcoma	Vitaxin	Medimmune	II
SLE	Antova	Biogen	II
Stroke	LDP-01	LeukoSite	I/IIa
Ulcerative colitis	LDP-02	Leukosite/genetech	II
Virus			
RSV	Syngis	Medimmune	FDA approved
HIV	PRO542	Progeneics/Genzyme	II
Hep B	Ostovir	transgenics	II
CMV	Protovir	Protein design lab/Novartis	III
		Protein design lab/Novartis	

AML, Acute myeloid leukemia; CD, cluster of differentiation; CMV, cytomegalovirus; FDA, Food and Drug Administration; GVHD, graft-versus-host-disease; Hep. B, hepatitis B; NHL, Non-Hodgkin's lymphoma; RA, rheumatoid arthritis; RSV, rous sarcoma virus; SLE, systemic lupus erythromatous.

bone marrow and organ transplant[76], toxin-drug conjugate[77,78], radioisotope immunoconjugates[79], as enzymes[80], antiinflammatories[75], and for certain infectious diseases[81-83].

3. In drug targeting monoclonal antibodies can be used as immunotoxins[77,84],

4. Monoclonal antibodies are also used for investigational and analytical purposes such as radioimmunoassay, lymphocyte phenotyping, ELISA, autoantibody fingerprinting and purification of proteins[73].

5. Catalytical monoclonal antibodies (Abzymes[85]): As the name implies these are antibodies, which exhibit catalytic activity. Their design is a form of protein engineering, and they can be considered designer catalysts. It has long been known that there are similarities between enzymes and antibodies, both are proteins, which bind other molecules with great specificity. The fundamental difference was that antibodies bind molecules in their ground state whereas enzymes bind their transition states (TS), thus leading to catalysis. Antigen is not altered by antibody binding but enzymes alters the binding by catalysing a chemical modification. The similarity in the binding is that both involve weak, noncovalent interactions and exhibit high specificity and high affinity[85].

   Inspite of sporadic previous attempts it has only been since the mid-eighties that successful attempts have been made to generate antibodies with catalytic properties. The general approach for this is to generate monoclonal antibodies to transition state analogs for a hydrolytic reaction. The phosphonate or phosphate analogs of transition states are used. Subsequently a number of catalytic antibodies were prepared in which the specificity has been greatly increased, in that a wide variety of different types of reactions have now been made to occur.

   The basic idea is that antibodies are extremely efficient at binding ground states of the target molecule (the hapten or antigen). Enzymes, as mentioned, obtain much of their catalytic efficiency from tight binding of the transition state for the reaction. Thus, if antibodies could be made for the reaction transition state then they would be expected to be potentially very efficient catalysts[86].

   The catalytic antibodies are made by generating monoclonal antibodies to a transition state analog, and screening for antibodies with catalytic activity[86]. Enzymes make use of acid/base, nucleophile/electrophile catalysts, and often have transient covalent intermediates. Current attempts are aimed at incorporating such functionalities into the active site of putative catalytic antibodies[86,87]. Two major approaches have been used to generate desired catalytic properties in antibodies: (1) the use of transition state analogs to generate the desired reaction-specific, molecule-specific catalysis and (2) to use genetic or chemical modification to change the structure of existing antibodies and introduce catalytic groups. Of course, the latest development is to combine both approaches, using a transition state analog to generate the initial specificity and then site-directed mutagenesis to provide the catalytic groups. Lerner *et al.*[85] produced hapten-carrier complex. The hapten structurally resembled the transition state of an ester undergoing hydrolysis. Using this as conjugate they generated antihapten monoclonal antibodies. The hydrolysis of the ester substrate increased 1000 fold after incubation. The catalytic activity of these antibodies was highly specific towards esters whose transition state resembled that of hapten in the system. Another example[87] is of 28B4 abzyme which catalyzes periodate oxidation of p-nitrotoluene-methyl sulfide to sulfoxide (fig. 7), where electrons from the sulfur atom are transferred to the more electronegative oxygen atom. The rate of this reaction is promoted by enzyme catalysts that stabilize the transition state of this reaction, thereby decreasing the activation energy and allowing for more rapid conversion of substrate to product. In this case, the transition state is thought to involve a transient positive charge on sulfur atom and a double-negative charge on the periodate ion (fig. 7c). In order to generate abzymes complementary in structure to this transition state, mice are immunized with an aminophosphoric acid hapten (fig. 7d). Obviously, its structure mirrors the structure and electrostatic properties of the sulfoxide transition state. Of the hapten-binding monoclonal antibodies produced with this hapten, many were found to catalyze sulfide oxidation but with a wide range of binding affinities and catalytic efficiencies. In particular, abzyme 28B4 binds hapten with high affinity and exhibits a correspondingly high degree of catalytic efficiency. Highly specific structural and electrostatic interactions create a remarkable degree of structural complementarity between the antigen-binding site and the sulfoxide transition state analog.

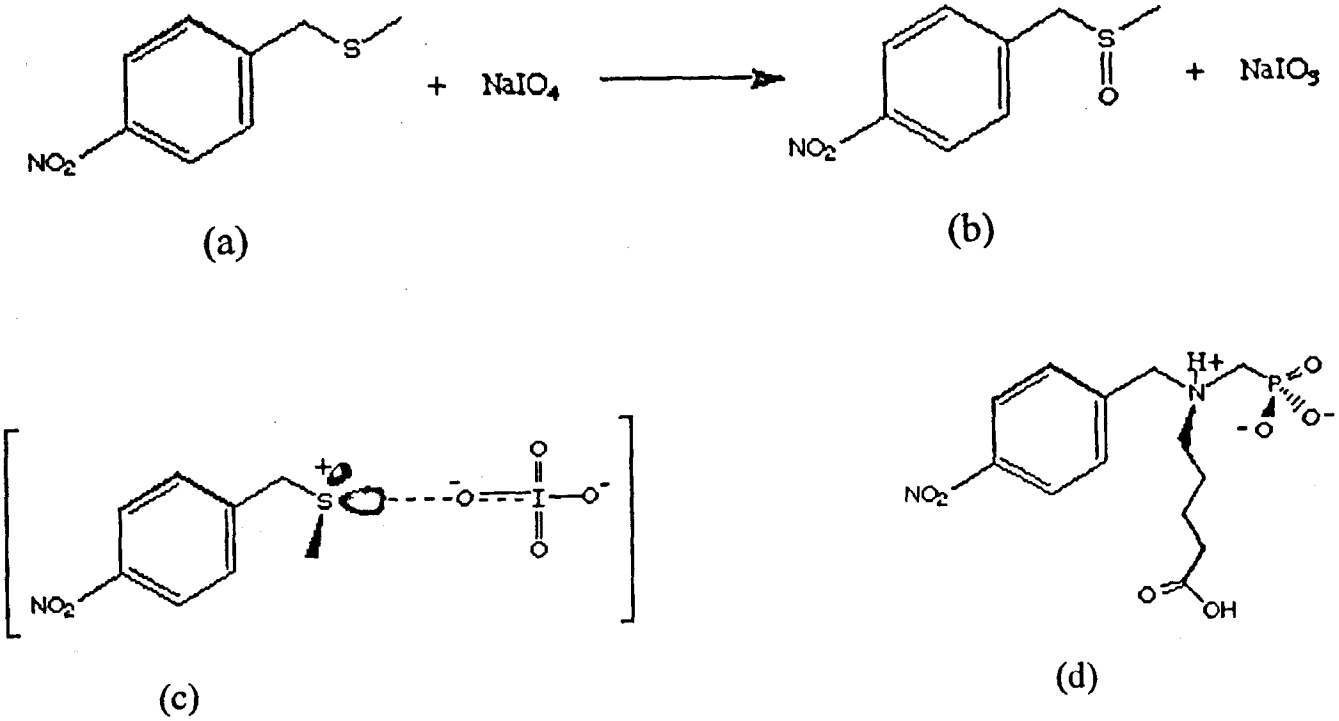

Fig. 7: "28B4 – abzymes" catalyses periodate oxidation of *p*-nitrotoluene methyl sulfide to sulfoxide.

a) It can occur via two possible transition states to give sulfoxide product. b) Sulfoxide product. c) Transition state.

d) Hapten, mimics the stereoelectronic features of both transition states and therefore used to raise antibody 28B4.

6. Plantibodies: Not only can genes for monoclonal antibody be expressed in bulk in the milk of lactating animals but plants can also be exploited for this purpose. So called 'plantibodies' have been expressed in bananas, potatoes and tobacco plants[88,89]. One can imagine a high tech farmer drawing the attention of a bemused visitor to one field growing anti-tetanus toxoid, another anti-mennigococcal polysaccharide, and so on. Multifunctional plants mighty be quite profitable with, say, the root being harvested as a food crop and the leaves expressing some desirable gene product. At this rate there may not be much left for science fiction authors to write about!

CONCLUSIONS

The monoclonal antibody production technology has revolutionised the world of immunology. An outstanding advantages of monoclonal antibody as a reagent is that it provides a single standard material for all laboratories throughout the world to use in an unending supply if the immortality and purity of cell line is nurtured; antisera raised in different animals on the other hand may be as different from each other as chalk and cheese.

The monoclonal antibody approach again shows a clean pair of heels relative to conventional strategies in production of antibodies specific for individual components of complex mixture of antigens. It must be clear that we now have in our hands a really powerful technique whose applications are truly legion, like diagnosis of lymphoid and myeloid malignancies, tissue typing, ELISA, RIA, serotyping of microorganisms, immunological intervention with passive antibody, antiidiotype inhibition, or magic bullet therapy with cytotoxic agents coupled to antimouse specific antibody. These and many other areas are being transformed by monoclonal antibody production technology.

ACKNOWLEDGEMENTS

Authors are grateful to Dr. K.G. Bothara, Principal, AISSMS College of Pharmacy for his constant encouragement and support without which this article could not have been completed.

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