Production of monoclonal antibodies

Five steps are important in the production of hybridomas to be used for the secretion of mAbs of a determined specificity:
(1) immunization of mice;
(2) fusion and selection of secreting hybridomas;
(3) cloning of hybridomas;
(4) definition of the isotype of mAbs obtained;
(5) further development (Figure 17.3).

Step 1: Immunization
Most myeloma lines used in cell fusion originate from BALB/c mice. These mice can be immunized with exogenous proteins (50–100 _g/ml), with cells (10^7 cells) or with peptides conjugated with carrier proteins, such as the keyhole limpet hemocyanin (KLH).

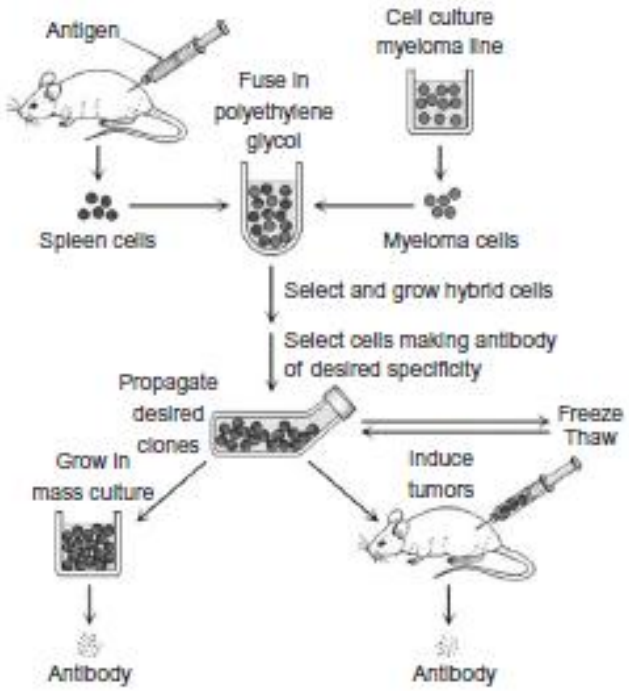


Figure 17.3
Scheme for hybridoma production.

The proteins and peptides are generally mixed with adjuvants (complete or incomplete Freund adjuvant, potassium alum (K2Al24SO4 + 24H2O) or commercial adjuvants such as Titer Max) and introduced **subcutaneously** or **intraperitoneally.** Cells are generally administered intraperitoneally in the absence of adjuvants. It is usually necessary to repeat the administration of the antigen once or twice to obtain an immune response with a high level of antibodies. **The level of specific antibodies in the serum** is assessed by immunoenzymatic assays of the ELISA type, using serum separated from a small sample of blood collected from the tail or the ocular plexus veins. The immunization of laboratory animals such as mice or rats is designed to increase the number of B-lymphocyte clones specific for the antigen, thus increasing the chances of obtaining hybridomas that will secrete the antibodies of interest in fusion experiments. **The booster doses promote the switch of immunoglobulin class and the maturation of antibody affinity due to somatic hypermutation** of the variable genes for the immunoglobulins that arise after repeated exposure of the animal to the antigen.

Step 2: Fusion and selection of secreting hybridomas

Myeloma cells are generally of the SP2Ag14/0 line; they are cultivated in RPMI 1640 medium containing 10% fetal calf serum until semiconfluence and then collected from the culture flasks by centrifugation. Animal spleens that present the highest antibody levels are collected aseptically, disrupted in the culture medium, and then the spleen cell suspensions are transferred to the centrifuge tube containing the myelomas. The mixture contains 2-3x10^7 myeloma cells for each 10^8 spleen cells. These cells are allowed to sediment and are then washed twice with a serum medium and centrifuged. The cell mixture is then resuspended in 1 ml of a 10% DMSO and **50% PEG solution**. This solution is added slowly to the cells over a period of 2.5 minutes. The first 60 seconds are at room temperature, after which the temperature is increased to 37°C (for the final 90 seconds). The volume of the cell suspension is then slowly increased to a total of 50 ml with culture medium or physiological saline solution. After 5 minutes the cells are allowed to sediment and washed twice by centrifugation. The final sediment is **resuspended in HAT medium** containing 20% fetal bovine serum. In the standard procedure, the cells are now plated at a density of 10^5 per well in 96-well plates containing a feeder layer of macrophages, although in some protocols, subsequent selection is simplified by plating 24 wells with a density of 106 cells per well. The feeder layer is prepared by seeding the wells with macrophages collected from the peritoneal cavity of normal mice some 48 hours prior to the fusion procedure. In addition to providing growth and differentiation factors, such as interleukin (IL)-6, this feeder layer provides the cell density necessary for the growth of the hybridomas that manage to survive the process. Some 10–15 days after seeding, the hybridomas are already sufficiently established on the surface of the plate that the antibodies of interest can be detected in the supernatants of the cultures. Their identification involves the use of specific assays, which are defined by the nature of the antigen and the properties of the antibody being produced. For cell surface antigens, the most frequently used assays are immunofluorescence or immunoenzymatic, with their numerous variations. For soluble protein or peptide antigens, the most common assays are immunoenzymatic, enzyme-linked immunosorbent assays (ELISA) or Western blot tests.

Step 3: Hybridoma cloning

Once the antibody-secreting hybridomas are obtained, they are **cloned by limiting dilution**, a procedure which consists of seeding a 96-well plate with equal aliquots of a 100-cell suspension of the clones, which **will hypothetically result in cultures of one cell per well.** Some 10 days after the cloning, the culture plate is investigated to determine whether or not clones are actually growing. These clones are similar in appearance to a bacterial colony in culture. The wells are then tested for the presence of the antibody, and each clone with the proper characteristics is expanded in culture flasks. **The culture will now secrete a single type of antibody, known as an mAb**.

Once hybridoma clones are established, they can be propagated in vitro indefinitely. An alternative means of propagation involves in vivo intraperitoneal inoculation in histocompatible animals previously inoculated with mineral oil (Pristane or Nujol), since these animals will develop **ascitic tumors.** These tumors accumulate a liquid that will contain large quantities of the mAbs. The hybridomas can also be frozen in liquid nitrogen for future use, thus providing an unlimited source of specific antibodies.

Step 4: Definition of the isotype of monoclonal antibodies obtained

Depending on the isotype (i.e. class/subclass and kind of light chain), immunoglobulin molecules will display a particular biological property and will require an appropriate method for purification. The identification of mAb isotypes generally employs the culture supernatants of hybridomas and commercially available kits for the specific immunoenzymatic assays. This knowledge about the specific isotype facilitates the selection of the purification process in the next step.

Step 5: Follow-up/later developments

Depending on the use of the mAbs, certain adaptations may be required for their preparation. When large quantities of mAbs are required, in vivo production of the antibody in ascitic fluid is not practical, because it will require the use of a large number of animals. Thus, it is often easier to cultivate the antibodies in an appropriate in vitro culture medium. However, given the strict nutritional requirements of the hybridomas and their fragility in the face of osmolality, pH variations, and the

accumulation of metabolites, the production of large quantities of antibodies in vitro will necessitate special care. The purification of mAbs of the IgG class is usually carried out by affinity chromatography on a resin coated with protein A or protein G from Staphylococcus sp., ligands that have affinity for this kind of immunoglobulin. More recently, other affinity ligands have been introduced, such as protein L, which has a special affinity for kappa light chains. Other Ig molecule classes, especially those with lambda light chains, can be purified by other methods, such as pseudo-bioaffinity. In this case, the chromatography uses metallic ions or certain dyes as ligands. These will interact with the antibody molecules due either to electric charge or to hydrophobic reactions. After purification, mAbs can be covalently linked with other reagents for use in specific assays. These reagents include radioisotopes (for radioimmunoassay or the in vivo tagging of antigens), enzymes (for immunoenzymatic assays), or fluorochromes (for immunofluorescence assays).
All of the procedures require time and exhaustive work in reagent standardization, but the most complex are those produced for therapeutic use in humans. Some of the developments in obtaining reagents for human applications will be discussed here, especially the use of recombinant DNA technology.

Production of recombinant antibodies

The development of the technique for the construction of hybridomas has made possible the rapid dissemination of mAbs as an analytic tool, and these products have had a profound impact on the procedures of diagnosis and the purification of other proteins. Although numerous applications of mAbs have been developed, the greatest interest has always been in medicinal uses. However, the mAbs obtained from hybridomas have – at least initially – proved to be less efficient as therapeutic agents. Problems include:
i**) insufficient activation of effector functions** in humans and
ii) **the stimulation of an immune response to the rodent proteins**. This latter phenomenon, known as the **HAMA response** (for human anti-murine antibody), results from the fact that murine antibodies are recognized as antigens by the human immunological system, and they will be rapidly eliminated from circulation by antimurine antibodies, thus reducing the effects of the treatment. To overcome this difficulty, larger doses of the medicine are required, although this will increase the risk of undesirable effects. These problems explain why so few products of murine origin have been launched on the market (Table 17.1). The only therapeutic product of murine origin that is well established is OKT3 mAb. This antibody is used in the reversal of acute transplant rejection. It involves an anti-CD3 (cluster of differentiation 3) antibody, which is part of the receptor of the membrane of T lymphocytes, enabling the temporary elimination of the T lymphocytes involved in the rejection process from circulation, thus facilitating the acceptance of the transplanted organ. Since the treatment required is of **short duration**, the immune response of the patient against the murine antigen, if it occurs, can be kept to acceptable levels.

Ideally, mAbs for therapeutic use should be **completely human**. However, the construction of hybridomas capable of producing such proteins presents both technical and ethical problems. The immunization of humans is unacceptable, especially since this would entail a biopsy for the collection of secondary lymphoid organs. One alternative would be the in vitro production of human mAbs using B lymphocytes collected from the peripheral blood of naive or naturally immunized human donors. In both of these procedures, however, the frequency of B lymphocytes specific for a given antigen is quite low. Moreover, **B lymphocytes producing antibodies against self-antigen, frequent targets of mAbs, are generally extremely rare**, since such B lymphocytes will normally be eliminated before maturation by a process known as ‘‘tolerance induction.’’ If they could be isolated, these lymphocytes could be perpetuated by techniques such as immortalization by **Epstein–Barr virus (EBV).** However, the cells would be difficult to clone and have a low productivity of the antibody, as well as facing an inherent risk of contamination of the final product by viral particles. Since it is impossible to obtain human mAbs from technologies such as those outlined above, other alternatives have been sought to enable the production of molecules with the appropriate variable domains of heavy and light chains, as well as its correct alignment, to guarantee the same affinity and specificity of the murine antibody, but with the human characteristics of the constant regions.

Table 17.1 Approved human monoclonal antibodies

Company	Commercial names (generic names)	Type	Category	Approval date
Johnson & Johnson	Orthoclone® OKT3® (Muromonab-CD3)	Murine	Immunologic*	1986
Centocor	ReoPro® (Abciximab)	Chimeric	Heart ischemic disease	1994
Centocor/Glaxo	Panorex® (Edrecolomab)	Murine	Antineoplastic	1995
Biogen IDEC	Rituxan® (Rituximab)	Chimeric	Antineoplastic	1997
Hoffmann La Roche		Humanized	Immunologic*	1997
Zenapax® (Daclizumab)				
Novartis	Simulect® (Basiliximab)	Chimeric	Immunologic*	1998
MedImmune	Synagis® (Palivizumab)	Humanized	Anti-infection	1998
Centocor	Remicade® (Ifiximab)	Chimeric	Immunologic*	1998
Genentech	Herceptin® (Trastuzumab)	Humanized	Antineoplasic	1998
Wyeth	Mylotarg® (Gemtuzumab ozogamicin)	Humanized	Antineoplastic	2000
Millenium/ILEX	Campath® (Alemtuzumab)	Humanized	Antineoplastic	2001
Biogen IDEC	Zevalin® (Ibritumomab tiuxetan)	Murine	Antineoplastic	2002
Abbott	Humira® (Adalimumab)	Human	Immunologic*	2002
Genentech	Xolair® (Omalizumab)	Humanized	Immunologic*	2003
Corixa	Bexxar® (Tositumomab – I131)	Murine	Antineoplastic	2003
Genentech	Raptiva® (Efalizumab)	Humanized	Immunologic*	2003
Imclone System	Erbitux® (Cetumimab)	Chimeric	Antineoplastic	2004
Genentech	Avastin® (Bevacizumab)	Humanized	Antineoplastic	2004

Adapted from Reichert and Pavlou (2004).
*Including arthritis, immunological and inflammatory diseases, and prevention of transplantation rejection.

Various strategies have been tried, including recombinant DNA technology for the expression of mAbs on phages or in transgenic animals. A result of the success of these strategies is the ever growing number of drugs for human use that have recently appeared on the market (Table 17.1). Moreover, some new products can be found in various phases of clinical trials, and some are expected to enter the market in the coming years. It seems as if the potential of mAbs as therapeutic drugs has finally been realized. Various procedures are used for obtaining humanized or human antibodies. Some of these using microbial or eukaryotic systems of cell expression will be presented further in this chapter. Other means of synthesis of these molecules, such as expression in plants, or even in the milk of transgenic animals, will not be discussed further.

Humanized antibodies

The technique of recombinant DNA provides a tool for the manipulation of the genes encoding antibodies present in murine hybridomas and those of human B lymphocytes that determine the expression of human antibodies, independent of specificity. It is thus possible to construct a genetic sequence that will express a molecule containing **both the variable fragments (Fv) of the light and heavy murine chains of interest and the human functional fragments (Fc)**. Such a combination is called a **chimeric antibody** (Figure 17.4). These chimeric constructs can be inserted into cell lines, which will express antibodies that are 70% human, with half-lives and effector characteristics similar to those of the human molecule, although they preserve the specificity of the murine antibody. These chimeric antibodies still present a **human antichimeric antibody** (HACA) immune response, but this is less pronounced than that observed with the use of completely murine antibodies. Moreover, these humanized antibodies have been successful in numerous clinical treatments. There are currently five products on the market using this technology; they account for 70% of the sales of therapeutic antibody treatment. Moreover, a large number of chimeric antibodies can be found in various phases of clinical trials, and new ones are expected to be approved in the next few years. Humanized antibodies can also be made by genetic recombinant grafting so that **specific regions of the chain that determine complementarity in the human molecule** (complementarity-determining regions, CDRs) **replace the murine ones,** thus guaranteeing the specificity of the antibody produced by the hybridoma, but obtaining 90% of the properties of human antibodies. Each domain (VH and VL) has three of these CDR regions (Figure 17.1). These regions vary greatly and determine the specificity and affinity of binding sites of antibodies. Like chimeric antibodies, these genetic constructs can be inserted into animal cells, which will then express the protein of interest in a suitable culture system.

Unlike the antibodies obtained by hybridoma technology, this type of antibody generally has a reduced affinity, but adverse reactions are of lesser intensity.

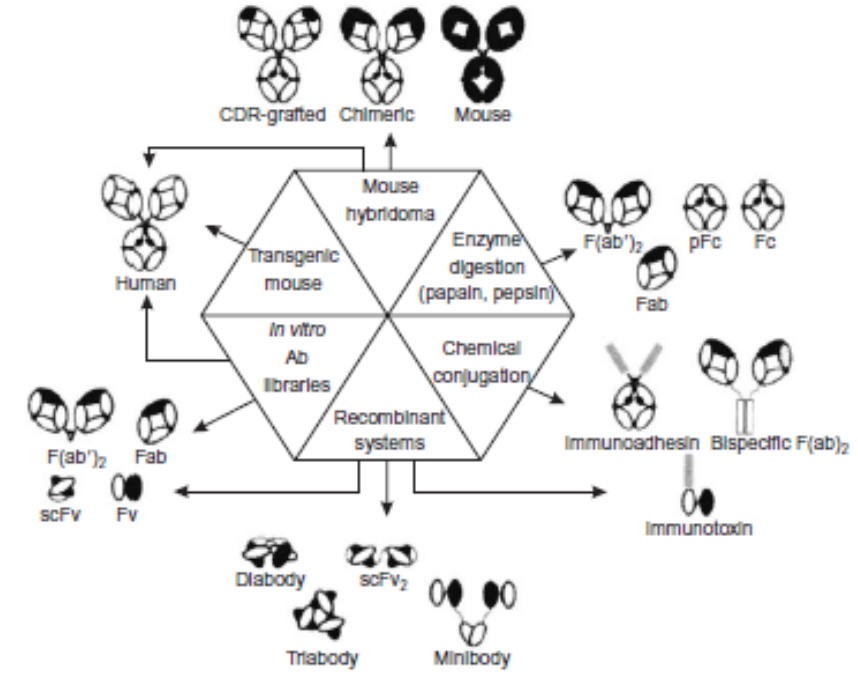


Figure 17.4

Recombinant antibodies: examples of possible types of constructions and technology employed (Reprint from Roque *et al.*, 2004. Copyright 2004 American Chemical Society).

At present, there are eight therapeutic products on the market that make use of this technology (Table 17.1), and the prospects for more are high, since some 42% of the mAbs in clinical trials involve molecules humanized by CDR grafting.

Human antibodies

The same technologies used for obtaining humanized antibodies can be used for the production of fragments of completely human antibodies, which have tremendous advantages for clinical application. The most successful methods used to obtain human mAbs involve the construction of transgenic mice or the synthesis of human antibody fragments, based on "DNA libraries" of human cells, by using viral vectors to deliver the genetic material into cells inside a living organism or cultured in vitro.

Transgenic antibodies

In producing transgenic antibodies, it is the live mouse that is subjected to genetic modification, rather than the cells that will produce the humanized antibodies. Initially, the genes codifying the light and heavy chains are inactivated in an embryonic cell. Then large segments of DNA containing the genes for the light and heavy chains of human immunoglobulin are introduced into this cell. The cell will then grow into a transgenic mouse, which will be able to produce completely human antibody molecules. With this technology it should be possible to allow for isotype switching and affinity maturation.

These transgenic mice can be immunized with any target molecule to obtain lymphocytes that synthesize human antibodies, with hybridomas being produced from these cells.

Another alternative is the insertion of small sequences of human chromosomes into embryonic animal cells, thus generating **trans-chromosomic mice**. These "mini" chromosomes are isolated from human chromosomes 2 and 14, which contain the genes for the light and heavy chains, respectively. This means that all of the V, D, and J segments of the variable N-terminal portion, as well as those of the constant regions, will become part of the mouse genome.

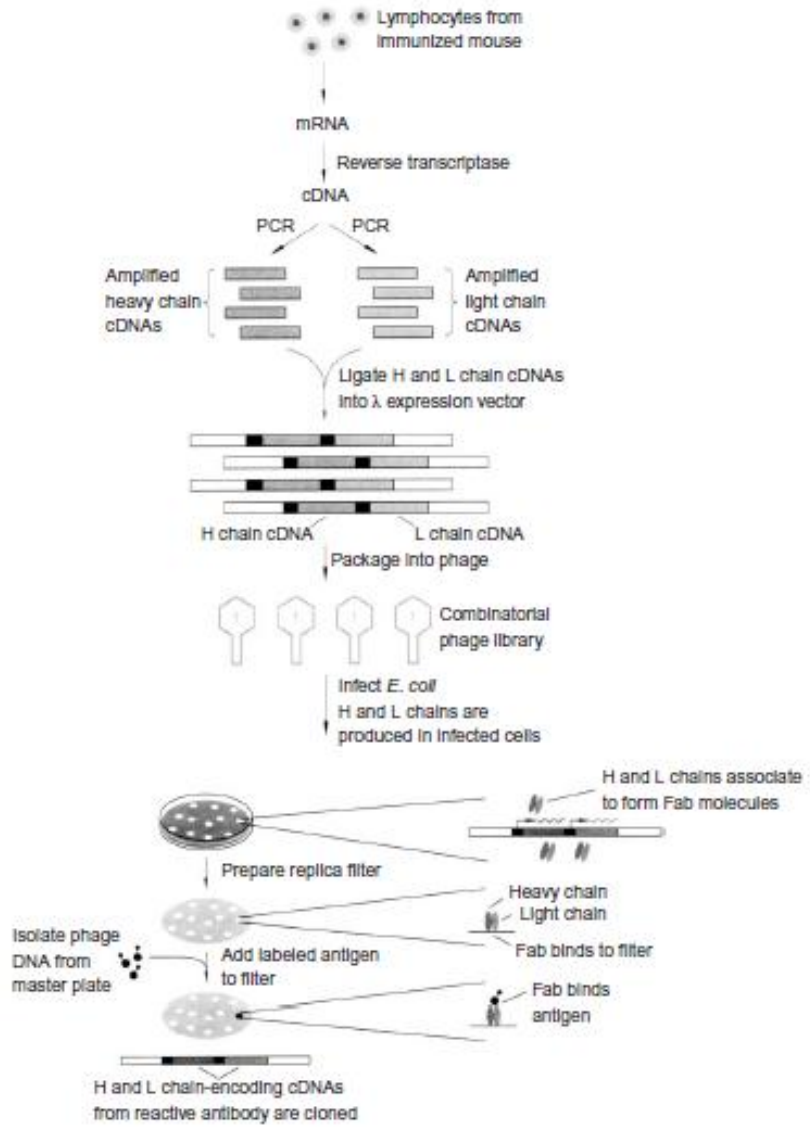
A third option uses ‘‘trimera’’ mice, which are mice that have been subjected to lethal irradiation, but are then prevented from suffering the effects of radiation by transplanting bone marrow cells from severe combined immunodeficient (SCID) mice, which have no B or T lymphocytes. To become trimera mice, the animals are repopulated with lymphocyte precursors from healthy human donors, and are then immunized with the antigen of interest. The immune system of the trimeras will then produce B lymphocytes that express specific human antibodies for the antigen, and their spleen cells can be used to produce hybridomas producing human immunoglobulins.

Antibody fragments

Various strategies have been used to combine the variable region of antibodies, which bind to the antigen determinants, with small functional proteins. Such constructs can be produced on a large scale in various expression systems; bacterial expression systems are relatively simple and less expensive than the alternatives, but eukaryote expression systems (yeast, mammalian, and insect cells) are also being used for this purpose. Most genetic constructs for obtaining antibody fragments express the **Fv portions** (Figure 17.4), which are the smallest antibody fragments that still retain the binding affinity of the parental antigen binding site. These Fv fragments can also be expressed as **single chain Fv molecules (scFv),** or **minibodies**, in which variable domains of heavy and light chains are permanently linked by flexible peptide bridges (Figure 17.4). This makes it possible to align the CDR regions of the chains in the same way that they were on the natural antibody. The peptide bridges, with 10–25 amino acids, should preferentially be of a hydrophilic nature to prevent association with the hydrophobic V domains (I) and can incorporate tags that will be useful in the purification of the fragment after propagation in an expression system. One frequently used ligand is a combination of residues of glycine and serine (GLY4Ser).

The immunoglobulin genes can be obtained from either animals or humans, both **naı̈ve** and **immunized**, although human sources are severely limited due to the ethical issues mentioned above. The repertoire of genes from immunized sources is smaller but they normally generate antibodies of high affinity. These genes are usually **stored in DNA libraries, which are cloned on phages**. These phages serve as vectors for expression in eukaryotic cells, or in ribosomes, thus leading to the development of totally in vitro systems. The technique of phage presentation is widely utilized for the construction of these libraries. In this procedure, the Ab fragments are expressed as fusion proteins linked to the N-terminal of proteins on a viral surface, with various copies of the fusion proteins being expressed in the virus envelope. Figure 17.5 illustrates the steps necessary for the construction of such a combinatory library of human DNA. Sequences of mRNA are isolated from lymphocytes of naive or immunized sources and utilized to synthesize the complementary DNA (cDNA), using the enzyme reverse transcriptase. The polymerase chain reaction (PCR) then makes it possible to increase the number of gene sequences of both light and heavy chains of the immunoglobulin of interest. The stability and half-life of complete antibodies is greater than that of fragments. These characteristics are crucial in certain therapeutic applications. However, fragments are especially useful for diagnostic processes involving images, as well in the treatment of solid tumors, where good penetration of the tissues and rapid elimination from the bloodstream are desirable characteristics. They are also useful for inactivation of cytokines, neutralization of viruses, and blocking of receptors. The fragments which retain the F(ab) portion (Figure 17.4) normally last longer in circulation than do smaller fragments (Fab, Fv, or scFv). If necessary, however, the half-life of the smaller fragments can be increased by mixing them with a PEG, or by chemical conjugation to form dimers, trimers, and tetramers (‘‘diabodies, ‘‘triabodies’’, and ‘‘tetrabodies,’’ respectively (Figure 17.4).

These polymeric structures are obtained from the modulation of the length of the flexible peptide bridges of the scFv modules. It is also possible to produce bi-specific antibodies (Figure 17.4), which have two variable regions, each with a distinct specificity, but which bind, for example, to two adjacent epitopes of a given antigen, thus increasing the avidity of the connection of the antigen to the antibody. Bi-specific antibodies can also be generated so that they bind simultaneously to a tumor antigen and a cytotoxic T lymphocyte, thus facilitating the elimination of the tumor. These bi-specific antibodies can be produced by hybrid hybridomas (‘‘quadromas’’), by either chemical or genetic conjugation, as well as by the fusion of adhesive heterodimer domains of two or more Fab modules. Bi-functional antibodies can also be obtained from the fusion of natural antibodies or recombinant antibodies with compounds that might carry out auxiliary functions after antibody binding to its specific target .



.

These compounds can be radioactive conjugates, cytotoxic drugs, proteins, toxins, enzymes, and viruses, any of which can have diagnostic or therapeutic uses.

Production systems

The development of a specific process for the production of an mAb requires the selection of
(a) a system of expression;
(b) a bioprocess for obtaining the product,
(c) a purification technique, and
(d) analytical methods for determining purity and product quality.

It is clear that all the choices must be compatible. So, for example, the selection of a process must be compatible with the expression system. Moreover, the process chosen must consider the specific conditions of the product in relation to the competitive market, the quality required, and the total volume to be produced. Thus, for diagnostic or biogeneric industries, the production cost dominates the choices, and the optimization of the productive process is extremely important. This is not true for the therapeutic industry, or for products protected by patents, which are more dependent on the regulatory requirements. Unlike the production of other proteins from animal cells, mAbs can also be produced in vivo by inducing ascitic tumors in laboratory animals, as described above. This is an

option when using hybridomas. However, there is a drive worldwide to find suitable in vitro production methods, to avoid the suffering of laboratory animals, as well as minimizing the risks of contamination of the final product by adventitious substances. However, in certain specific situations, the use of an in vivo system of production is unavoidable.
For antibodies produced in vitro, as is the case for many other proteins obtained from animal cell cultures, the main question involves **the low level of expression of these products in the culture medium**. This necessitates the use of large culture volumes for production, thus involving higher costs, especially for purification. In general, the optimization of these in vitro processes attempts to increase the concentration of the product in the medium. This is often possible by using high cell densities. Typical values for traditional processes are in the range of 20–100 mg/L, whereas for optimized systems, this can rise to 4.6 g/L. It is unusual to find an optimized generic process for obtaining mAbs since each producer cell has a unique pattern of response to stress, consumption of nutrients and synthesis of products and byproducts. However, it is clear that the systems utilized for the production of mAb do not differ in any significant way from those using animal cells for the synthesis of other products.

Cell lines Although various systems have been developed for the production of mAbs, only those involving cultures of animal cells will be presented in here since this is the system used to produce most of the antibodies on the market today.
Those of murine origin are produced directly from hybridomas, whereas the humanized or completely human ones come from the **culture of animal cells transfected with specific genetic sequences**, which are capable of following the patterns of glycosylation and the desired structural conformation, required for adequate drug performance.
Hybridomas constitute the most widely used cell lines for the production of mAbs, on both small and large scales. however, these cells produce antibodies that have limited therapeutic application, due to the immunological responses to murine antibodies that often arise in patients. As discussed above, one solution for this problem is the genetic manipulation of cell lines to enable them to synthesize humanized antibodies. In this situation, the hybridomas serve as an important source of gene sequences that codify antibody molecules (or their fragments), although these are later transfected into other animal cells. In general, the cell lines used are **more robust than hybridomas**, due either to their greater stability or even a reduced tendency for apoptosis. The **Chinese hamster ovary (CHO)** cell line is now being used as a standard host cell for transfection with genes of interest for later use in the production of recombinant antibodies. Other cells with equivalent performance are the murine myelomas NS0 and Sp2/0, as well as baby hamster kidney (BHK), human embryonic kidney (HEK-93), and a derivative of the human retina (PER.C61). The selection of the best cell line should consider **antibody productivity**, as well as **cell growth rate**, although these parameters frequently follow opposite trends. For the production of large amounts of mAb, it is fundamental that the productivity of the selected cell line is high. Otherwise, larger reaction volumes will be required, and the cost of purification will be increased. A reference value for specific protein productivity is 20 pg/cell per day. Another important aspect involved in the selection of transfected lines is **the capacity to grow without physical support**, since the scale-up of such processes is much simpler than those designed for growth of anchorage dependent cells

Various combinations of bioreactors and operation mode have been used for the production of mAbs in several systems of expression. All cells utilized for the production of mAbs grow in suspension. Those that did not initially have this capacity have been adapted (as is the case for CHO and BHK). This results in a large number of options for production systems. Cells with this characteristic are easily cultivated in stirred-tank reactors, which have been scaled up to a volume of 10 000 L. This kind of bioreactor provides excellent homogeneity, facility for the implementation of control techniques, and the principles of scaling up are relatively well known. Other kinds of bioreactors for the production of mAbs are also available, such as air-lift, with volumes up to 1000 L, and also fixed-bed bioreactors. Products with less demand, such as those used in diagnosis, are developed in small-scale systems such as T-flasks, rollers, and hollow-fiber bioreactors.