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INVITRO PRODUCTION OF MONOCLONAL ANTIBIOTICS AS AN ALTERNATIVE OF INVIVO PRODUCTION IN RODENT

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ABSTRACT

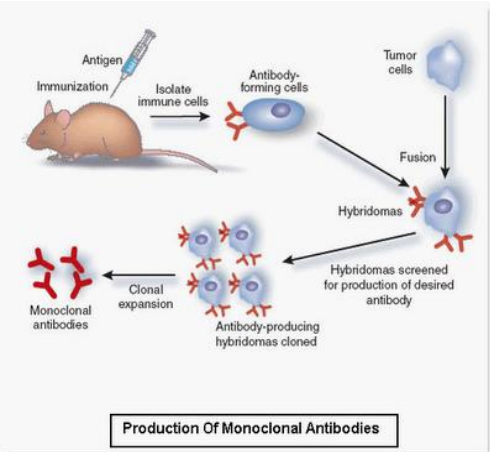
Many areas of biomedical research focus on the study of human-specific diseases and medical concerns for which induced animal models are seldom, if ever, appropriate or scientifically relevant. This largely reflects obvious species-specific differences in anatomy, biochemistry, physiology, pharmacokinetics, and toxic responses. Use of replacement methods, especially incorporating human cells and tissues, avoids such confounding variables. A specific example of a basic research alternative method, and one that potentially has saved up to one million animals, is the in vitro production of monoclonal antibodies (mAbs), which are used in nearly every field of biomedical research and critical areas of clinical practice. In brief, monoclonal antibodies are antibodies which have a single, selected specificity and which are continuously secreted by immortalised hybridoma cells. The widely-used ascites method of producing mAbs, involves injecting cells into rodent abdominal cavities and is extremely painful and unnecessary. Due to this, a need for developing invitro model was felt, as invitro models are of moderate cost, and can be shown to be either better than, or equal to, the ascites production method in terms of antibody quality. We hereby discuss that the in vivo production of mAbs is no longer necessary, except in rare cases where it is already approved for clinical applications.

Keywords: Monoclonal antibodies, Rodents, Gene, Production

INTRODUCTION

Monoclonal antibodies (mAb) are important reagents used in biomedical research, in diagnosis of diseases, and in treatment of diseases and infections. Monoclonal antibodies are antibodies which have a single, selected specificity and which are continuously secreted by immortalised hybridoma cells. A hybridoma is a biologically constructed hybrid of a mortal, antibody-producing, lymphoid cell, and a malignant, or immortal, myeloma cell. Monoclonal antibody therapy is the use of monoclonal antibodies (or mAb) to specifically target cells[1]. The production of monoclonal antibodies was pioneered by georges kohler and cesar milstein in 1975.

The applications of mAb are numerous and diverse. They are extensively used in fundamental research, medicine and biotechnology [5]. The global therapeutic proteins market will grow at a moderate pace, with monoclonal antibodies considered to be the highest revenue generating market in 2010. This will also be the leading market in the coming years. The global therapeutic proteins market was worth $93 billion in 2010 and is forecast to grow to $141.5 billion by 2017, which represents a growth rate of 6.2% between 2010 and 2017. With the advancements in the bio generics and gene therapy markets, the therapeutic proteins market is expected to show a moderate growth rate in the coming years. On the other hand, the introduction of new oral therapeutic proteins will increase the patient pool due to better drug compliance. Even though the competition from gene therapy and the



Demand and growth of Monoclonal Antibodies

biogenerics market will influence the market, the market is still very attractive for the pharmaceutical companies.

Hybridoma Technology

Essential two stages in the production of mAbs:

a) The induction of antibodyproducing lymphoid cells *in vivo* and the selection of antibody-producing hybridoma cells *in vitro.*

b) *In vitro/in vivo* propagation of selected hybridoma clones [2].

The first stage, the formation and selection of the hybridoma clone, involves the use of one or

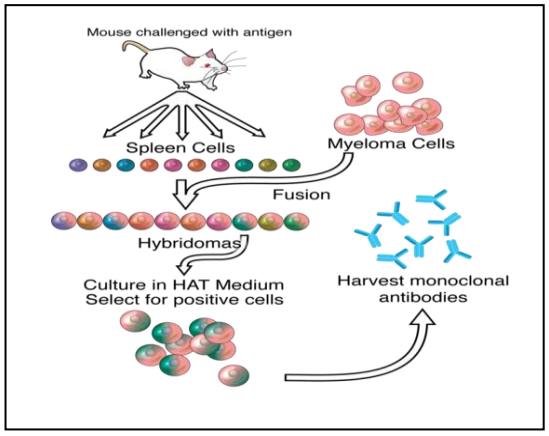
More animals (except in rare cases when a human mAb is being developed), and is carried

Out in the following way:

1. The antigen is injected into mice (or rats). The antigen is often injected in combination with an adjuvant, to enhance the immune response, even though the use of adjuvant generally leads to severe side-effects.
2. After an appropriate interval (5-21 days), the immunised animals are killed andlymphoid cells (including progenitor antibody-producing cells) are isolated from the spleen[14].
3. The lymphoid cells are fused with myeloma cells which have been grown *in vitro.*
4. The two original cell types and the newly formed hybrids are cultured in a selective medium, such as HAT (hypoxanthine/ aminopterin/thymine) medium, which only allows the hybridoma cells to grow.
5. The supernatant media from the numerous *in vitro* microcultures exhibiting a recognisable growth of hybridomas are screened for secretion of the desired antibody, by using various immunoassay procedures.
6. The selected cells are subcultured *in vitro*, using special cloning procedures toensure that each *in vitro* culture consists of hybridomas with a single antibodyspecificity only[17].
7. Hybridoma cells can be cryopreserved at this stage.

Monoclonal Antibodies Production *in Vivo*

The *in vivo* procedures entail the use of mice or rats. Initially, the immune systems of the Experimental animals are suppressed weeks before the intraperitoneal [i.p] injection of hybridoma cells)a primer, such as pristane (2,6,10,14 tetramethylpentadecane) . The hybridoma cells then multiply in the peritoneal cavity, and the ascitic fluidwhich forms is a very rich source of the secreted antibodies [12].



When an adequate amount of ascites has formed, the animal is killed and the ascetic fluid is collected. Sometimes, the ascitic fluid is first tapped or drained from the peritoneal cavity while the animal is under anaesthetic, with a second and final harvest being taken once the ascites has reformed[3,6].

The mAb product can be harvested 5-21 days after the injection of hybridoma cells. Approximately 5ml of ascites can be obtained from a mouse, and 10-40ml from a rat. Thus, for the production of a mAb with a given specificity, it may be necessary to use one or more mice, depending on the amount of antibody required [9, 11].

Advantages of Mouse Ascites Method [7]

- The high concentration of the desired mAb in mouse ascites fluid avoids the effects of contaminants in in vitro batch-culture fluid when comparable quantities of mAb are used.
- The mouse ascites method avoids the need to teach the antibody producer tissue-culture methods.

Disadvantages of Mouse Ascites Methods [8]

1. It is extremely painful for the animals used, due to the following:

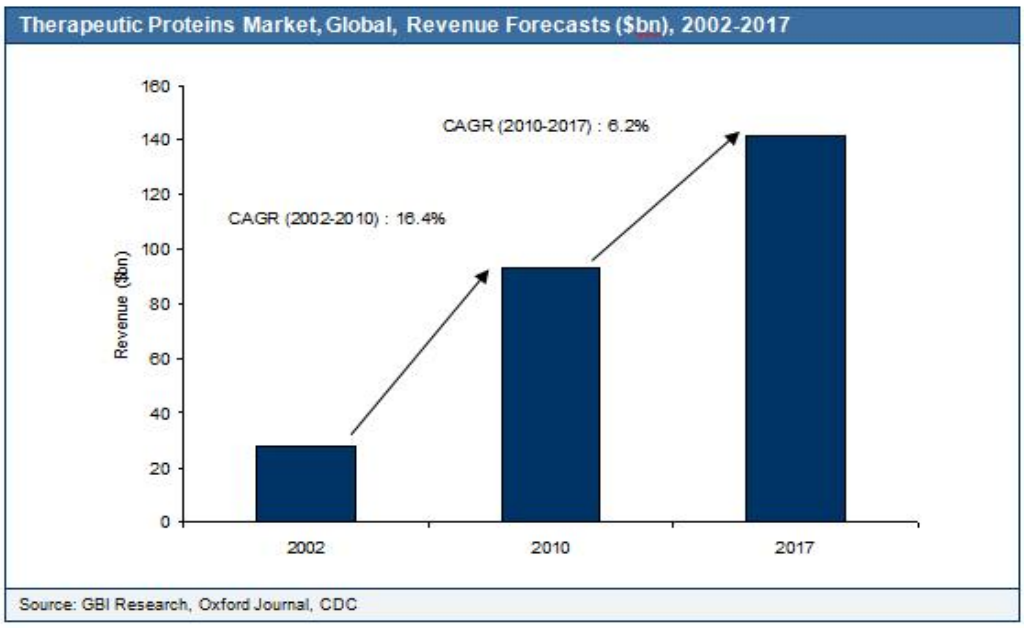
   a. the injection of primer

   b. the resulting peritonitis caused by the primer

   c. abdominal tension

   d. The invasive tumour.

2. The mAbs produced generally show a reduced immunoreactivity of 60-70%, as opposed to an



immunoreactivity of 90-95% for antibodies produced *in vitro*, due to contamination by biochemically identical immunoglobulin.

3. There is also a potential risk of product contamination by viruses which are pathogenic to humans.
4. The individual batches of harvested ascitic mAb are of variable quality, a nd they are contaminated with bioreactive cytokines.

In Vitro Production Procedures [4, 10, 11, 15, 16]

In vitro production systems,during the last 20 years, a wide range of*in vitro* production systems have been developed for different purposes. While mostof them are useful for the *in vitro* productionof mAbs, they differ in terms of:

a) The ease with which they are handled

b) The antibody yield per culture or bioreactor runs

c) The maximum antibody titre achievable.

The antibodies produced generally express an immunoreactivity of 90-95%, irrespective of the system used. Three categories of *in vitro* production system can be identified according to the principle underlying the culture system:

a. **static and agitated suspension cultures;**
b. **membrane- based and matrix-based culture systems;**
c. **High cell density bioreactors.**

Advantages of In Vitro Methods [20]

- In vitro methods reduce the use of mice at the antibody-production stage.
- In vitro methods are usually the methods of choice for large-scale production of mAbby the pharmaceutical industry because of the ease of culture for production, compared with use of animals and of economic considerations.
- In vitro methods avoid the need to submit animal protocols to animal ethical committee.
- In vitro methods avoid or decrease the need for laboratory personnel experienced in animal handling.
- In vitro methods using semipermeable-membrane-based systems produce mAb in concentrations often as high as those found in ascitic fluid and are free of mouse ascitic fluid contaminants.

Disadvantages of In Vitro Methods [20]

- Some hybridomas do not grow well in culture or are lost in culture.
- In vitro methods generally require the use of FCS, which limits some antibody uses. The use of in vitro methods for mAb production generally requires the use of FCS, which is a concern from the animal-welfare perspective.
- The loss of proper glycosylation of the antibody (in contrast with in vivo production) might make the antibody product unsuitable for in vivo experiments because of increased immunogenicity, reduced binding affinity, changes in biologic functions, or accelerated clearance in vivo.
- In general, batch-culture supernatants contain less mAb (typically 0.002-0.01) per milliliter of

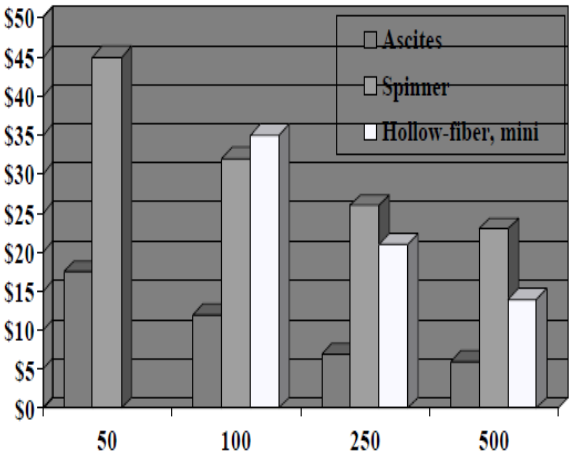
medium than the mouse ascites method. Semipermeable-membrane-based systems have been developed that can produce concentrations of mAb comparable with concentrations observed in mouse ascites fluid.

- In batch tissue-culture methods, mAb concentration tends to be low in the supernatant; this necessitates concentrating steps that can change antibody affinity, denature the antibody and add time and expense. Adequate concentrations of mAb might be obtained in semipermeable-membrane-based systems.
- Most batches of mAb produced by membrane-based in vitro methods are contaminated with dead hybridoma cells and dead hybridoma-cell products, thus requiring early and expensive purification before study.
- mAb produced in vitro might yield poorer binding affinity than those obtained by the ascites method.
- In vitro culture methods are generally more expensive than the ascites method for small-scale or medium-scale production of mAb.
- The number of mAb produced by in vitro methods is limited by the amount of equipment that it is practical to have available.
- The Food and Drug Administration (FDA) estimates that proving the equivalence of a mAb produced by in vitro methods to a mAb previously produced by the mouse ascites method would cost the sponsor $2-10 million.

In Vivo and In Vitro Methods for Commercial Production of mAb[13,17]

Commercial mAb production uses both the mouse ascites method and in vitro methods. Cost is usually the major consideration in determining the method except for marketed therapeutic products.

When all fully-loaded production and pre-production and post-production costs are considered for a commercially viable line, economics usually favour in vivo production. However, as the amount of mAb increases, existing in vitro production technology can become more economical because high, fixed optimization costs associated with in vitro production are spread over a larger production amount, making cost per gram competitive with in vivo production, which has a higher and more variable cost structure. When production costs are compared with small-scale production, in vitro methods are ½ to 6 times higher, depending on the cell line. However, these costs might not include all factors, such as animal housing costs and technician time. In large-scale production runs, in vitro systems are economically competitive and are usually selected because they reduce animal use and decrease the presence of contaminating foreign antigens if serum-free media can be used. When the time of mAb production is critical and small amounts are required, in vivo production is selected because it takes only 6 weeks. For in vitro systems, time requirements vary considerably. Production time depends on the amount of time required to optimize the hybridoma to the system being used and on the quantity of mAb needed. Commercial-quantity in vitro production of mAb requires more time than in vivo production because of the lengthy optimization process and the increased time for producing a given quantity of mAb. The human immune system tends to reject mouse-derived antibodies, which can lead to allergies or decreased effectiveness of injected mAb. Therefore, techniques that replace most of the mouse's antibody genes with human DNA have been developed.



Advanced technologies and future developments[21, 22, 23, 24]

With novel recombinant DNA-based technologies, such as phage display libraries and direct cloning into plasmids, either experimental animals are used solely for the immunisation stage, or the need to use animalsis obviated altogether. The realisation that antibody fragments can be expressed on the surface of bacteriophage particles has revolutionised our ability to mimic B-cell immune systems *in vitro*. Very large collections of antibody molecules

(libraries) can be expressed on the surface of filamentous bacteriophage particles so that antibodies with desired specificities and high affinities can be obtained from these libraries by affinity selection, by using a wide variety of target antigens such as recombinant proteins and intact prokaryotic and eukaryotic cells. Phage display libraries can be constructed from immunoglobulin genes of any species, including humans, and often incorporate synthetic nucleotide sequences. In many cases, sufficiently large repertoires enable the selection of antibodies without prior immunisation of B-cell donors, and this therefore avoids the need to use living animals. Selected antibody fragments can be recloned into a variety of vectors to produce molecules with tailor-made properties such as whole immunoglobulin's of any isotype as well as bivalent or bispecific antibodies. The incorporation of affinity tags enables these recombinant proteins to be rapidly purified after their expression in prokaryotic and eukaryotic expression systems. Importantly, phage antibody display libraries allow the selection of novel specificities against nonimmunogenic or unknown target antigens. Similarly, large libraries of linear or conformationally constrained small peptides expressed on phage particles enable the selection of even smaller .binding. molecules with desired specificities and affinities. It can be envisaged that, in the near future, binding molecules could be selected from an array of peptide and antibody phage display libraries, and relevant molecules could be produced in *in vitro* expression systems or by peptide synthesis.

FUTURE PROSPECT

- There is a need for the scientific community to avoid and minimize pain & suffering oflaboratory/experimental animals. Therefore, over the next several years, as tissue-culture systems are further developed, tissue-culture methods for the production of monoclonal antibodies should be adopted as the routine method unless there is a clear reason.
- Also, the mouse ascites method of producing monoclonal antibodies should not be banned, because scientific necessity for this method.
- When the mouse ascites method for producing mAb is used, every reasonable effort should be made to minimize pain or distress, including frequent observation, limiting the numbers of taps, and prompt euthanasia if signs of distress appear.
- mAb now being commercially produced by the mouse ascites method should continue to be so produced, but industry should continue to move toward the use of tissue-culture methods.

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