# *Alternative Techniques to Obtain Monoclonal Antibodies at a Small Scale: Current State and Future Goals*

REVISIÓN

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***ABSTRACT***

The ascites method has been one of the most popular means for producing large quantities of highly concentrated monoclonal antibodies since its inception in 1972. Here we summarize some alternative methods to produce monoclonal antibodies and we compare them with the traditional method (ascites). The advantages and disadvantages of these techniques will be considered in order to investigate the practicality of using *in vitro* techniques to generate large quantities of MAbs. We also present the regulatory restrictions for MAbs production in different countries. Finally, a brief overview of the emerging technologies is described.

*Key words: in vitro, in vivo,* monoclonal antibodies

*Biotecnología Aplicada 2002;19:119-131*

***RESUMEN***

**Técnicas alternativas para la obtención de anticuerpos monoclonales a pequeña escala: Estado actual y perspectivas.** Desde su introducción en 1972 el método de producción de ascitis ha sido uno de los más populares para producir grandes cantidades de anticuerpos monoclonales (AcMs) altamente concentrados. En el presente trabajo resumimos los métodos alternativos de producción de AcMs y los comparamos con el método tradicional de producción. Para investigar la factibilidad práctica del empleo de técnicas de producción *in vitro* en la obtención de grandes cantidades de AcMs consideramos las ventajas y desventajas de estas técnicas. También evaluamos las restricciones regulatorias para producir AcMs en diferentes países. Finalmente se describe una breve revisión de las tecnologías emergentes para producir AcMs.

*Palabras claves:* anticuerpos monoclonales, *in vitro, in vivo*

## *I*ntroduction

The continuous production of monoclonal antibodies (MAbs) from mouse splenic lymphocytes after immortalization by fusion with a plasmocytoma cell line was first reported by Köhler and Milstein in 1975 [1]. At that time, the technique was just another tool for their research, but the impact was so enormous, that a Nobel prize was awarded to Köhler and Milstein, along with Jerne, in 1984.

This gave research a valuable, powerful and versatile tool, since the specificity of antibodies is exquisite and extremely sensitive. Since then, the production of monoclonal antibodies has reached multibillion-dollar markets in diagnostics, therapeutics and for purification of proteins and chemicals [2].

The challenge for many laboratories growing hybridoma cell lines is to produce adequate amounts of MAbs. The main limitating aspects are space, time and money. Media amounts of MAbs typically require an investment in expensive bioreactors, space allocated to many of tissue culture flasks stacked in incubators, or a very large number of mice.

The applications of MAbs are numerous and diverse. At present four user groups can be identified according to the amount of antibody required (Table 1). Currently there are more than 100 000 different MAbs available. Most of them are produced at a small scale (<0.1 g) solely for bench-related purposes. Larger amounts are often required for diagnostic kits and reagents (0.1–0.5 g), for routine diagnostic procedures and in pre-clinical evaluation studies (0.5–10 g). Finally, larger amounts are required for prophylactic, therapeutic purposes and to immunopurify antigens (>10 g). This outline clearly shows that the application of MAbs is almost exclusively limited to research and human medicine. Although the application of MAbs for other purposes is easily envisaged, this has not come materialized because the production costs MAbs on a large scale are extremely high, regardless of the production system used.

Table 1. Monoclonal antibodies: user groups.

| Group | MAb required | Proportion | Most widespread method | Applications |
|---|---|---|---|---|
| A | <0.1 g | ~60% | *In vivo* | Fundamental and applied research<br>Commercial production of diagnostic kits for research and analytical purposes |
| B | 0.1-0.5 g | ~30% | *In vivo* | Development and production of diagnostic kits<br>For evaluating the usefulness of novel therapeutic MAbs in animal experiments |
| C | 0.5-10 g | ~10% | *In vitro* | Routine diagnostic procedures<br>In pre-clinical evaluation studies |
| D | >10 g | <1% | *In vitro* | Prophylactic, therapeutic and for purification purposes |

Here we compare the classical way of producing MAbs (ascites) with the *in vitro* methods and finally we present some emerging technologies.

## *A*scites Production of MAbs

Antibodies can be obtained at high concentrations by injecting the hybridoma cells into the peritoneal cavity of genetically compatible mice or rats and obtaining the antibody in the ascitic fluid (Figure 1).

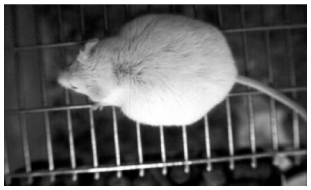

Figure 1. Production of monoclonal antibodies by the ascites method.

While culture supernatants yield on average 1–10 µg/mL, the ascitic fluid contains 1–20 mg/mL.

Several mechanisms have been reported [3, 4] to account for the production of ascites. To produce MAbs as ascites fluid, mice are primed with an intraperitoneal injection of pristane (2,6,10,14-tetramethyl pentadecane). The effect of the primer is two-fold: it suppresses the immune system so that the growth of the hybridoma cells in the abdomen is not strongly impaired, and it causes toxic irritation which leads to peritonitis and the secretion of serous fluid. After 7–21 days, the abdomen of the inoculated animals swells, indicating ascites production and tumor growth. The cells multiply and produce antibodies, whereas the animal responds with the production of fluid in the peritoneal cavity The antibody-rich ascitic fluid (about 5 mL from a mouse and 10–40 mL from a rat) is harvested by paracentesis, processed and animals are sometimes re-tapped after two days to increase the total volume of ascites obtained per animal. Various parameters affect the yield of ascites from inoculated mice or the concentration of MAbs [5].

Numerous parameters have been identified that may affect MAb production and/ or produce abnormalities, pain distress, or death in the animals as a result of the procedures used. These parameters include the hybridoma cell line used [6]; the stock or strain [7, 8]; sex and age [9] of the mouse selected; volume of pristane [10–12] or their ascitogenic priming agent administered [13–15] and the timing of pristane, or any other ascitogenic priming agent, administration in relation to hybridoma cell inoculation [6, 9, 13–16]; the hybridoma cell inoculum used [6, 9, 17]; frequency and total number of abdominal taps [6, 18–20]; the methods used for the abdominal taps [6, 7, 13]; and the frequency of clinical observations and criteria for euthanizing animals [20].

The ascites method to obtain MAbs can be analyzed from two different points of view: Manufacturing feasibility and regulatory issues.

## Manufacturing feasibility

Murine ascites production has been the time-honored technique for producing small-scale, research laboratory quantities of MAbs [6, 21–24].

Since the introduction of the hybridoma technology, MAbs produced in mice, the so-called ascites induction method, has been the method of choice. This method was preferred for a number of reasons: 1) Simplicity of the procedure; 2) No specific skills are required; 3) No capital investments are needed to set up the facilities for laboratory animals since they are generally available; 4) High concentration of MAbs are found in the ascites; 5) Ascites can be obtained in a relatively short period of time; 6) In the peritoneum the cell densities are similar to those of solid tissues, about $10^9$ to $10^{10}$ cells/mL; 7) Purity expressed as the specific antibody content is high; 8) Low cost; 9) No intensive use of labor or equipment is needed unless special (i.e., SCID, nude) animals are used; and 10) Many clones can be grown at the same time.

However, one of the main manufacturing advantages of the ascites method is the extremely high yield and productivity of MAb, which generally lies in the range of 1–20 mg/mL [25].

These points are particularly relevant to the small research laboratory with limited funds and time, yet having the ability to develop many MAbs of interest. These are, therefore, advantages which cannot be easily ignored.

The weak points are the low concentrations obtained in some of the wasted tissue culture supernatants (100–1000-fold lower than ascites); the fact that the use of serum can create problems similar to those of ascites in regard to contamination and downstream processing; the high volume of the medium and serum used in certain *in vitro* systems requires downstream concentration and purification; some *in vitro* systems have high initial equipment start-up costs; the high degree of contamination with animal serum proteins that is essential for the *in vitro* culture of hybridoma cells and the fact that sometimes (3–5%), cell lines do not adapt to *in vitro* conditions, limit the use of MAbs prepared by the conventional *in vitro* technique and have made the ascites procedure favorable for a long time, specially for small-scale production as needed for bench-related activities.

## Regulatory remarks

Although the original research of Köhler and Milstein was principally an *in vitro* technique, it was also apparent that MAbs could be produced by injecting the hybridoma cells into the abdominal cavities of different species of rodents. This was the initial use of the ascites method. Since these *in vivo* MAbs were easily made in any laboratory and the ascites process, widely viewed as both simple and inexpensive, had long been introduced, its use rapidly expanded. In the decades following the original discovery, tens of millions of animals suffered and died. Milstein noted, "in later years, both on practical and humane grounds, I became concerned with the use of ascitic fluids". The wide advantages of the ascites method are outweighed by a number of disadvantages. It is accepted that ascites production is extremely painful and causes suffering by peritonitis, abdominal distention and invasive malignancy and survival is of approximately 14 days [7, 26, 27]. An overview of clinical and pathological effects was published in 1999 [20]. Analysing the overall picture of clinical, pathophysiological and pathological changes shown in Table 2, it can be postulated that the production of ascites and the growth of tumors in rodents also lead to a substantial impairment of animal welfare.

Table 2. Clinical, pathophysiological and pathological effects of ascites production.

| Clinical | Pathophysiological | Pathological |
|---|---|---|
| Abdominal distension | Anorexia | Peritonitis |
| Decreased activity and body mass | Anaemia | Infiltrative tumor growth |
| Shrunken eyes | Dehydration | Adhesions in the abdomen |
| Difficulty in walking | Tachypnea and Dyspnea | Enlarged abdominal organs |
| Hunched posture | Circulatory shock | Blood in the abdominal cavity |
| Respiratory distress | Decreased venous, arterial, and renal blood flow | |
| Apathy | Ascites production | |
| Death | Immunosuppression | |

These limitations generate disadvantages, which makes the *in vivo* method a less attractive method. Some of these disadvantages are: it requires animal facilities, support services, trained personnel and daily monitoring to minimize pain and distress; it requires verification and approval of the Institutional Animal Use and Care Committee (IACUC); the use of pristane may produce residual contamination, reactivate endogenous murine retroviruses and promote production of IgG autoantigens; it requires manipulative skills during repeated tapping to locate remaining ascitic fluid; ascitic fluid is a more complex mixture than blood plasma, having the same difficulties associated with the use of serum for *in vitro* cultures and mice may not produce ascites due to premature death, development of solid tumors or failure to establish hybridoma growth.

There are other non-regulatory disadvantages that also limit the use of ascites: a greater variability (within animals) in some cases; the reduced MAb immunoreactivity generally observed, and the higher contamination levels obtained (biochemically identical immunoglobulins, growth factors, rodent plasma proteins, with bioreactive cytokines, bacteria and viruses).

"Ascites" means "bag" in Greek, and many laboratories have continued to use animals as if they were bags to fill with antibody-containing fluid. At the same time that the ascites method became widespread, the appropriateness of its use was increasingly questioned in Europe, particularly in certain countries of the European Union.

Thus the replacement, reduction and refinement (3R), the 3R concept had been used first 46 years before by Charles Hume to provide a framework for improving the conduct and ethical acceptability of experimental techniques on animals. Given that animals used in research may experience pain, suffering or lasting harm, the first step must be to consider whether less harmful or harmless alternatives could be used (replacement). Where this is not possible, care should be taken to minimize pain [28, 29], either before and during the experiment or after the experiment. Refinement is often achieved, for example, by providing the animals with an environment in which they can feel secure and comfortable, ensuring that they are free from infectious diseases, and by using appropriate anesthetics and analgesics if surgical techniques are to be used [30]. Finally, the number of animals used in a given project needs to be minimized (reduction), while ensuring that the objectives of the study are still achieved; typically, this will also reduce the total number of animal suffering. The 50's were described as the age of renaissance, the 60's, as the age of darkness, the 70's, the age of reason, the 80's the age of reformation and the 90's have been classified by some researchers as the age of revolution.

There are two general laws in Europe to protect laboratory animals: 1) The Council Directive 86/609/EU [31] and 2) The European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes, ETS 123 [32]. The directive came into force in 1986 and the convention in 1991. The convention has been signed and ratified by Belgium, Cyprus, Finland, Germany, Greece, The Netherlands, Norway, Spain, Sweden and Switzerland.

However, specific national policies have established guidelines or regulations which restrict or prohibit ascites production in rodents in Switzerland, Sweden, The Netherlands [33], the UK [34] and in German speaking countries (Table 3).

The Center for the Validation of Alternative Methods (ECVAM) has played one of the main roles in these regulatory steps. In 1993 the Scientific Advisory Committee of the ECVAM defined as its main goal, the promotion of scientific and regulatory acceptance of alternative methods which are of importance to the biosciences and which reduce, refine or replace the use of laboratory animals.

By 1996, *in vitro* production of MAbs was the method of choice in Europe for commercial concerns and other needs of individual researchers. As a result, a group of experts in immunology and the *in vitro* science representatives from many member states of the European Union met at the ECVAM to discuss the current status of *in vivo* and *in vitro* methods of MAb production [35]. After careful considerations of all uses for MAbs and all available *in vivo* and *in vitro* production options, the panel concluded that "for all levels of MAb production there are one or more *in vitro* methods which are not only scientifically acceptable, but are also reasonably and practically available; and as a consequence *in vivo* production can no longer be justified and should

Table 3. Some examples of national and institutional regulations and guidelines on the production of MAbs.

| Country/ Organization | Year | Regulation/Guidelines |
|---|---|---|
| CCAC | 1989 | Guidelines on acceptable immunological methods. |
| | | Guidelines regarding the collection of ascites and the use of humane endpoints. |
| Germany | 1989 | *In vivo* production of MAbs is only permitted: |
| | | • If the MAbs concerned are to be used for diagnostic and therapeutic purposes in case of emergencies; |
| | | • To reserve single hybridomas that are infected or do n ot grow *in vitro;* |
| | | • If the MAbs concerned are needed to investigate new scientific problems. |
| Netherlands | 1989 | Code of practice for the production of MAbs: |
| | | • *In vivo* production only permitted when < 10 animals are needed; |
| | | • Specific justification of the protocol ; |
| | | • Consultation of the animal welfare officer with regard to moment and frequency of collecting ascites, moment of euthanasia. |
| | 1996 | *In vivo* production of MAbs is prohibited. Requests for exemptions are granted or rejected by the Inspectorate after a grou p of experts is consulted. |
| SCAW | 1989 | Guidelines with regard to collection of ascites and the use of humane endpoints. |
| Sweden | 1990 | Recommendation of the National Board of Agriculture. |
| | | Existing alternatives should normally be used. |
| | | *In vivo* production needs specific justification. |
| Switzerland | 1989 | Rules for the judgment of approval requests for animal experiments for the production of MAbs. |
| | 1994 | *In vivo* production of MAbs is prohibited. Exceptions are only permitted: |
| | | • When the MAbs concerned are to be use d for diagnostic and therapeutic purposes in case of emergencies; |
| | | • To rescue single hybridomas that are infected or do not grow *in vitro*. |
| | | In case of exceptions: only 1 tap; increase in body weight may not exceed 20%. |
| UK | 1987 | United Kingdom Coordinating Com mittee on Cancer Research (UKCCCR) guidelines: 1 tap only, the amount of ascites may not exceed 20% of the body weight. |
| | 1991 | Home Office: antibody production -advice on protocols for minimal severity. |
| | | Only < 20 animals per hybridoma. Repeated tapping is n ot allowed. |
| | | Humane endpoints according to UKCCCR guidelines. |
| | 1999 | Project licenses for the ascites method are not granted unless under exceptional circumstances |

CCAC, Canadian Council on Animal Care; SCAW, Scientists Center for Animal Welfare. Source: R esearch in Immunology, 74th Forum in Immunology. Vol. 149(6):529–620.

cease". The conclusion continues to state that there was "no fundamental reason why hybridoma cells, which have been generated and cultivated *in vitro*, should suddenly be able to produce antibodies only in an animal's peritoneal cavity" [35].

The attention from animal-welfare organizations, the scientific community, and regulatory agencies has increased in the United States as well [36, 37]. Thus based on the results of the meeting and considering the similarities in the arguments used by both European and United States researchers to support the continued reliance on the ascites methods, the Alternative Research and Development Foundation (ARDF) and the American Anti-Vivisection Society (AAVS) decided it was time for an ascites prohibition to be enacted in the United States. Thus after a series of contradictions on August 29, 1999 [38] the ARDF sponsored an international panel of experts to participate in a workshop on the production of monoclonal antibodies in Bologna, Italy, and the panel concluded that "The National Institutes of Health and the National Center for Research Resources should aggressively promote and encourage the use of its (and other) Institutional Monoclonal Antibody Core Production Facilities. The NIH should also place greater emphasis on providing more opportunities for *in vitro* MAb production training for individual researchers/technicians at Core Monoclonal Antibody Production Facilities"; "*In vitro* production should be the accepted, required choice for MAbs; with ascites considered the alternative"; and "If the ascites methods are approved, every effort should be made to reduce and refine the procedures (e.g., limiting the number of taps or increasing body weight) to minimize the pain and distress experienced by the animals".

However, *in vitro* replacement alternatives for MAb production *in vivo* seem unlikely to be optimal elsewhere in Europe, since, under most circumstances, the production of MAbs can be undertaken *in vitro* [39] by methods which are reasonably and practicably available.

## *In Vitro* MAbs Production

### Should we use the *in vitro* MAbs production systems?

As expressed above, the advantages of the ascites method are widely minimized by the animal welfare concerns and also by certain production limitations. Hence, in recent years the *in vivo* method has been eventually substituted by *in vitro* production. Improvements in cell culture techniques and cell culture equipment have led to *in vitro* methods becoming available which are able to compete with the *in vivo* method, both in capacity as well as in cost-effectiveness.

The *in vitro* method offers the following advantages: 1) It does not use animals for MAb production; 2) No animal care issues or the IACUC approval are required; 3) It avoids or significantly decreases the need for experienced laboratory personnel in animal rearing and use; 4) A wide variety of production options is available; 5) Large scale production is possible at a lower cost than *in vivo* method; 6) Many *in vitro* systems produce MAbs with equal or better concentration and quality than those of ascites; 7) No host contributed immunoglobulin or typical ascitic fluid contaminants are found; 8) Immunoreactivity is sometimes higher than with ascites (90–95%); 9) The use of serum or a protein-free medium make downstream purification easier; 10) Most hybridomas can be adapted to serum/protein-free conditions; and 11) Under most circumstances it eliminates the need for Mouse Antibody Production (MAP) testing.

The demand for animal cell derived products and the needs to eliminate the *in vivo* methods have stimulated the development of bioreactors in the last decade. Most of those developments allow for a higher cell concentration in the bioreactor than that found in the past, primarily through the continuous flow of culture medium.

Much effort has been required to reproduce the biological conditions in terms of the medium and equipment. Therefore the question is not whether hybridomas can be grown *in vitro*, but whether they can be grown in a culture medium for long enough to yield the amount of MAbs needed, at a reasonable cost. Hybridomas are anchorage-independent cells, and can therefore be grown and maintained in either stationary or suspension culture. The amount of antibody produced depends on the concentration of cells attained and the time they remain viable and secrete MAbs. The viability and productivity of hybridomas depend on if they meet their nutritional requirements, removing metabolic waste products, and providing a stable pH, temperature, and dissolved oxygen for metabolism.

### Culture media

There are various basal media formulations suitable for growing hybridomas. These formulations frequently have a mixture of carbohydrates, amino acids, salts, vitamins, hormones and growth factors [40–42] and they are usually supplemented with glutamine as a supplementary energy; they are also buffered with sodium bicarbonate or organic buffers such as HEPES. Foetal calf serum (FCS) is frequently added to the media to provide growth factors and hormones. However, as a result of the disadvantages of the serum (it is expensive, undefined, there are batch to batch variations, the purity of the MAb preparation decreases, there is a potential contamination with germs, as well as animal welfare concerns) a variety of serum-free and more recently protein-free media formulations [43–45] and supplements are available for hybridoma growth. In contrast, FCS introduces a moral problem, because it is obtained by cardiac puncture from a bovine foetus on the slaughter line. Time between the slaughter of the cow and the death of the fetus by exsanguinations can be of 5 to 30 min. It cannot be excluded that fetuses used for FCS harvest experience pain or suffer from the bleeding procedure.

### Culture methods

Research should consider a number of factors when selecting an *in vitro* method or methods for MAb production to ensure the quality of the final product:

1. How many and how much MAb(s) do you need?
2. How concentrated do you need the sample?
3. What level of purity do you need?
4. How much space do you have in your facilities?
5. How much time and money do you have?

6. Is your personnel well trained?
7. Can you make in-house MAb production or a contract?

The amount of MAb produced depends on the concentration of the cells attained and the time they remain viable and secrete MAbs. The viability of hybridomas depends on whether they meet their nutritional requirements, removing metabolic waste products, and providing a stable pH, temperature, and dissolved oxygen for metabolism.

The *in vitro* systems can be classified in low and high cell density culture systems. The differences between them seem to be caused by the supply of gaseous ($O_2$) and of water-soluble non-gaseous nutrients (glucose, amino acids etc.) and the elimination of gaseous ($CO_2$) and non-gaseous water soluble products (lactic acid, ammonium ions). The degree to which both needs are covered determines the limits and effectiveness of the culture method:

- Nutrients consumed can be replaced by adding them to the culture;
- The demand oxygen can be met by agitating the cultures or by aerating them through gassing;
- The gaseous metabolite $CO_2$ can be removed in the same way.

Thus, in low-density systems the water-soluble non-gaseous metabolites can only be removed by changing the whole culture medium. Therefore the cell and MAb concentrations are low. If the culture vessel incorporates a continuous removal of the water-soluble non-gaseous metabolic products, higher densities and MAb concentrations can be obtained.

Once you know details on your true *status* you can start producing MAbs using one of the following production methods:

### *I. Low cell density culture systems*

#### I.I. Static suspension cultures

It is the simplest cell culture system. The cell requirements for nutrients and $O_2$ as well as the need to eliminate the gaseous and non-gaseous metabolic products from the direct environment of the cells are achieved by diffusion.

*Tissue culture flasks*. This system is used for stationary culture (Figure 2). Here, cells and media are placed in the flask, kept in a $CO_2$ incubator, and handled as a batch culture. The initial seeding density required for the reproducible, rapid proliferation of cells varies among cell lines, and the time between the end of proliferation, the start of cell viability decline, and the peak antibody level is also hybridoma-dependent [46]. Incubation periods are typically 7–10 days before harvesting. Little or no monitoring is required, and the flasks can be harvested when the medium turns yellow (become acid) and cell viability drops to approximately 5–10% [47]. The surface area for $CO_2$ and oxygen diffusion is low in these systems. The main disadvantage is that MAb concentrations are very low therefore flasks take up most of the incubator space and large volumes of media must be processed.

#### I.II. Agitated suspension culture (Figures 3 and 4)

Agitation solves the limitations of the static products, because the cells receive a supply of nutrients and $CO_2$ is removed more efficiently. As a consequence, cell and MAb concentration are higher than in the static suspension culture. Cell growth is however limited by the accumulation of water-soluble non-gaseous metabolic products, such as lactic acid. However, this system is superior to the stationary culture, only to a certain, but not very impressive, degree.

The low purity of the MAbs, the expenses in cell culture media as well as the costs of processing large volumes of the harvest and the tedious purification procedures, result in high costs and limit the applicability of these *in vitro* culture procedures.

*Roller bottles and stirrer flasks.* Among the major drawbacks of the roller bottle system is its labor-intensive nature and the lack of a pH and dissolved oxygen control. On the other hand, the roller bottles are a very flexible system [48]. For products whose market size may fluctuate drastically in a very short time period, the use of a roller bottles system allows the rapid expansion or reduction of production capacity. This flexibility in capacity with a minimum capital investment probably contributed to the slow adoption of the new technology by the vaccine industry. MAb concentration ranges from 10 to 220 µg/mL and the average culture period is of approximately 12 days.

Advantages and disadvantages are similar to those described for T-Flasks, but roller and spinner flasks require more incubator space and are more expensive than T-Flasks.

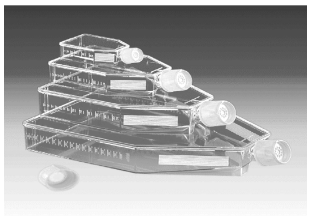

Figure 2. *In vitro* production of monoclonal antibodies in stationary systems. T-Flasks.

A B C

Figure 3. *In vitro* production of monoclonal antibodies in suspension cultures. A, spinner flasks; B, stirrer tanks; C, roller bottles.

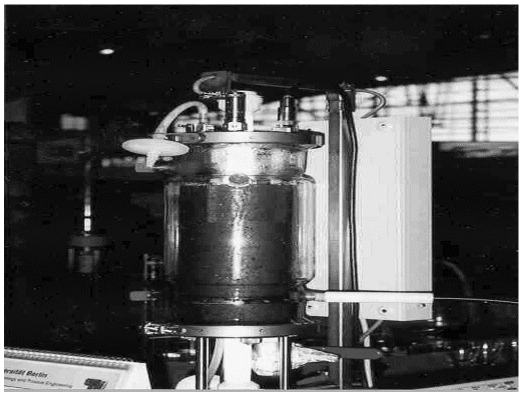

Figure 4. *In vitro* production of monoclonal antibodies in fluidized bed bioreactors.

Among various bioreactors, the stirred tank has found a wider acceptance in cell culture processing, especially for suspension cells. Microcarrier cultivation was first introduced by van Wezel [49] in 1967; it then matured and found wider acceptance in the late 70's and early 80's [50, 51] The original microcarriers were based on cross-linked dextran. Subsequent developments have concentrated on other materials such as polystyrene [52, 53], cellulose [54], gelatin [55, 56], and glass [57]. The microcarrier culture technique has become a good way of increasing the efficiency of stirred systems.

The trend in the development of the stirred bioreactor in the 80's was to look for non mechanical agitation. Thus, the bubble column or airlift bioreactors [58, 59] were designed to avoid an excess damage of the cells caused by direct sparging [60, 61]. Stirred tanks, airlift [62] or bubble column fermentors have been successfully used for antibody production at a large scale [63]. A microcarrier culture is typically initiated by inoculating with cells that have been trypsinized and detached from a surface. Three competing processes occur after inoculation: the cell attachment to the microcarrier surface, the clustering of cells to form clumps, or it is kept in suspension over an extended period, with a loss of viability. A relatively fast attachment rate is thus essential for a successful microcarrier culture.

### I.III. Membrane-based and matrix-based culture systems (Figure 5)

*Wave bioreactors™.* The wave bioreactor™, which was recently introduced, is a pre-sterilized and flexible bag. The bag is partially filled with the culture medium and the cells and the system remain fixed and in a $CO_2$ atmosphere. The waves facilitate the gas diffusion to the culture. The 2 L bags and 20 L bags are suitable for small-scale production; the scaling up to a 500 L culture volume has been demonstrated using this technology. Cell densities reach $\geq 7 \times 10^6$ cells/mL.

*Gas-permeable cell culture bags.* This system features attached ports, and tubing with roller clamps, which are used for the inoculation of cells and media, sampling during production and bags. The bag is inoculated with cells and media, placed in a $CO_2$ incubator, and generally handled as a batch culture. The medium is harvested when MAb concentration reaches a plateau or cell viability drops to approximately 10%.

Compared to standard T-Flasks, bags have more surface area for $CO_2$ and oxygen diffusion, thus improving cell oxygenation. This has the following advantages: the MAb concentration per cell is higher than in T-Flasks, bags are a completely closed system that reduces microbial contamination, the space required in the incubator is reduced, it is technically simple, and it may require less media than T-Flasks.

*miniPERM® systems.* Falkenberg *et al*. developed it in 1993 [64]. This modular system consists of a disposable 40 mL production module and a nutrient reservoir, which holds up to 550 mL of the medium, a semi-permeable membrane separates both modules. The cells and the secreted MAbs are retained in the production module. Nutrients, metabolic wastes, and dissolved gases are exchanged across the dialysis membrane between the production and nutrient modules. By rolling the vessel at high speed (5 rpm), the cells are kept in a stable suspension. The reported advantages of this system include: the growth of cells at high cell densities ($\geq 10^7$ cells/mL), high MAb concentration (10–30-fold higher than in stationary cultures) high product purity related to serum reduction or removal from the nutrient compartment, it is relatively easy to use, it is able to maintain the cultures for a relatively long period of time, and some components may be re-used.


Figure 5. *In vitro* production of monoclonal antibodies in membrane-based and matrix-based culture systems. A, wave biorreactor; B, gas-permeable bags; C, CELLine culture system.

*CELLine culture systems.* They are based on a membrane compartmentalization technology. Cells and secreted MAbs are retained in a small-volume cell compartment, which is separated by a semi-permeable membrane from a larger volume of the basal medium contained in the nutrient medium compartment.

It is a very special flask, that has the following advantages over other classical stationary systems: it requires 70% less time, the reduction in total cost is of 40%, less material is needed, 50–100X more MAb concentration is obtained. The system has other additional advantages: a good optical quality, reduced costs and concentration steps, a self-contained cell cultivation-without the need for pumps and gassing systems, the long term use in the incubator is possible, cost is substantially lower for storage and logistics, considerably less waste is produced, etc [65].

### *II. High cell density culture systems (Figure 6)*

These systems make it difficult to maintain the oxygen and nutrient levels. They are, however, more advantageous than the low cell density culture systems: they require less serum, they give higher MAb concentration and the culture may be maintained for a longer period of time.

*Hollow fiber bioreactors (HFB).* These bioreactors share the advantages of the perfusion systems: they are good for both stable and unstable products, they offer an optimal culture control, there is no product degradation, there is a low variability, they also offer high cell viability and the system is scalable (from g to kg).

The first work on the hollow fiber bioreactor systems was reported twenty nine years ago by Knazek *et al.* [66], who had the intention of reproducing the *in vivo* capillary system to provide a more physiological environment for cells with regard to nutrient supply, metabolic waste removal, and pH, while providing a stable pericellular microenvironment [67]. Many researches have used HFB [68–71].

The supply of water-soluble non-gaseous nutrients (glucose, amino acids etc.) and the elimination of gaseous ($CO_2$) and non-gaseous water soluble products (lactic acid, ammonium ions) can only be achieved by two chamber culture devices, which are more complex to manufacture and more complicated to operate than the low cell culture density described above. In such systems, the cells are grown in a small cell culture chamber, which is connected to a significantly larger medium supply chamber by a semi-permeable membrane. Agitation of both the cell culture and the supply media is essential for optimal membrane exchange.

The hollow fibers have inner diameters of 100 microns or less. The membranes are semi-permeable and have defined pores with known molecular weight thresholds, in most cases between 10 and 50 kD.

The medium passes through the lumen of the fiber at a high flow rate, but only a small fraction permeates through the fiber membrane. The membrane can be made of several materials including polysulfone and cellulose esters [72]. By recirculating the medium through the HFB, the cells are supplied with nutrients and dissolved oxygen, and water-soluble non-gaseous metabolites. Both the gaseous metabolite and the $CO_2$ are removed in the same way. Considering the low solubility of oxygen in the tissue culture media, high volumes of aerated nutrient media have to be pumped through the HF. The high cell density (close to that of the solid tissue) and the high antibody concentration (close to 10 mg/mL) make this system attractive for small-scale production and even at the industrial scale.

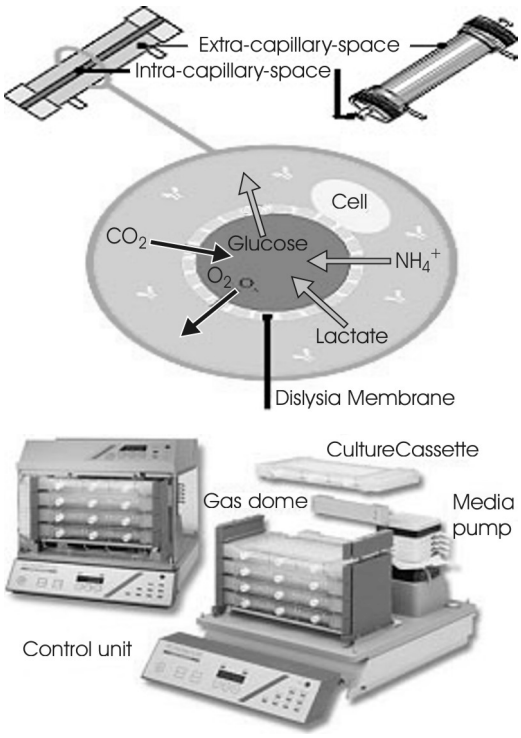


Figure 6. *In vitro* production of monoclonal antibodies in hollow fiber biorreactors.

Hollow fiber systems are more highly recommended for suspension cells like hybridoma cells and they have the following advantages: 1) High antibody concentrations that minimize and simplify purification steps; 2) There is no need to develop serum free lines/optimal medium combinations; 3) Minimum serum usage and purity is facilitated [73, 74]; 4) They are simple to keep sterile and endotoxin free; 5) Low capital and consumable costs are needed; 6) They are relatively compact and convenient; 7) Cells are protected from shearing; and cell viability and production are maintained for long periods [75–77], ranging from weeks to months.

The disadvantages of these systems include their potential for mechanical failure, large initial investment, large amounts of media expanded and the need for technical expertise, cells are vulnerable to rapid changes in lactic acid and familiarity with the system is needed.

The scaling up of hollow fiber bioreactors is hindered, however, by the diffusion barrier across the fiber membrane which causes nutrient limitations and waste buildup as well as the formation of gradients within the cartridge [78]. In an HFB, due to the positive trans-membrane pressure difference between the lumen and the shell side, the medium permeates through the membrane onto the shell side at the bioreactor inlet. As this pressure difference decreases along the length of the bioreactor to become increasingly negative towards the outlet, it causes the medium to flow back into the fiber lumen. This phenomenon is called starling flow [79, 80]. It is also difficult to enumerate the cell population directly, which is a drawback for online monitoring and control.

In the Technomouse a layer of HF is spread between two thin sheets of gas-permeable silicone rubber membranes. Unlike conventional HFB containing a cylindrical bundle of fibers within a hard plastic shell, the fibers in this HFB are arranged in single parallel rows and encased in a silicone membrane, within a flat rectangular plastic cartridge. It can accommodate five clones at one time, it is easier to handle and suffers less frequently from leakage.

*Ceramic systems.* Both in the ceramic [81] and the HFB systems, oxygen is supplied by recirculating the medium continuously through the reactor.

The ceramic system is a cylinder of porous ceramic with square channels passing through the cylinder. Cells are inoculated into the channels and they either adhere to the surface or they are entrapped in the pores of the ceramic. The medium passes through the channels to provide nutrients and to remove the metabolites. The cells are directly bathed in the recirculating medium.

## Which System to Select?

The fermentation process has to be designed in such a way that the biological potential of the cell is optimally exploited. Besides the optimization of the technique and medium formulation it is necessary to know how a certain cell line is growing and producing under well-defined conditions. This provides the information necessary for the design of the process.

According to results reported by Merten in 1988 [82] different hybridoma cell lines express different production kinetics and these production patterns can be classified into three groups. The first group (pattern type I) shows the best release of monoclonal antibodies at the beginning of a batch culture during the lag phase and the onset of the exponential growth phase and with no increase during the death phase [83–85]. Production pattern II is characterized by a relatively high initial specific production, which decreases during maximum growth, and, after having reached the stationary and death phases it increases again [86–88]. Production pattern number III is characterized by a relatively stable production during the growth of the cells, and almost no production when the cells enter the stationary and death phases [89].

The three specific production kinetic patterns may have implications on the construction of a production process. If the product released is more or less growth associated (pattern III), a continuous culture system might be the best choice because it provides for the permanent growth of cells. Cells, showing production patterns II or I, might preferentially be cultivated in a discontinuous mode, because growth and specific production are more or less inversely related. Pattern II implies that the culture system has to provide a growth phase independently from a production phase. This can be achieved by the use of the following process modes: batch, fed-batch, repeated batch with or without a second stage, or a continuous culture system with a second stage. Cells showing production pattern I should have the highest production in repeated batch systems.

Assuming that the differences between high and low density cell cultures are not too great, the above mentioned implications of batch production kinetics might be extended for high cell density systems, also. In any case, the cells have to be held in a physiological state, which is optimal for overall production.

The growth and production kinetics of cell lines are within the most important parameters for MAb production on an industrial scale.

For researchers working in the academic field, the use of the available *in vitro* techniques indicated above is often problematic.

For *in vitro* production the following points should be considered: high yields obtained at high concentrations, the reproduction of the *in vivo* condition, and the cells need to be grown at high cell densities. There are many types of equipment on the market. In roller bottles, which are suitable for adherent cells and stirred bottles, the cells are grown until the medium is exhausted. The concentration of the MAb is not much greater than that grown in normal tissue culture flasks, with the additional problem that proteases and toxins are released by the dying cells. Also, there are numerous types of bioreactor of all sizes, but they are aimed at commercial production. To obtain tissue-like densities, the dialysis tubing technology, miniPERM, and the hollow fiber technology can be used. There is a natural reluctance for researchers to invest so much and to face the high costs of consumable products and maintenance. The miniPERM system seems to be very promising, it takes up little space, it may be run at once and the initial and maintenance costs are low.

The production requirements and the appropriate production technologies based on the quantities required are shown on Table 4.

The optimum system for large scale cell culture must meet the following requirements: 1) Cell concentrations must be higher than $10^8$ cells/mL; 2) The rate of nutrients fed must be at least similar to the consumption rate, and waste removal should be enough to avoid toxic effects; 3) Cells must be viable; 4) Sophisticated process control methods are needed that minimize medium and serum requirements and that allow for an optimum process control; 5) Downstream processing must be facilitated; 6) It must be an adequate automatic process; and 7) Scaling-up using efficient equipment must be cost-effective. No system exists with all of these properties. In recent years some bioreactors have been designed with most of these characteristics; however, there is a certain resistance to use them, mainly due to the absence of adequate scaling-up and because of the variability of the results according to the cell line used [89].

Figure 7 and Table 5 show some of our results using different culture systems. The cell line evaluated was hybridoma CB.Hep-1 (anti-rHBsAg) under protein-free and serum containing medium conditions respectively (PFM, SCM). In our work a relatively drastic hybridoma weaning procedure was successfully used for mouse hybridomas using TurboDoma™ HP-I.

The first *in vitro* system evaluated was T-Flasks, where the highest cell densities started 7–10 days after seeding and the antibody production ranged from 10–100 µg/mL. In this case the time needed for the highest cell density ranged from 6 to 9 days in both media.

Hybridoma CB.Hep-1 showed a type I production kinetic pattern in T-Flasks. For these experimental conditions the PFM did not change the production kinetic pattern compared with SCM. Nevertheless, the saturation cell density was affected in PFM, which could be caused by a stoichiometric limitation under PFM conditions.

The maximum antibody production in T-Flasks was between 15.45 and 55.56 µg/mL in SCM and 18.78 and 29.16 µg/mL in PFM. It can not be attributed to the low cell density reached by this hybridoma because IgG production per cell was also 1.5-fold lower in PFM, expressing medium limiting factors for IgG production or that the weaning protocol should be optimised for this cell line.

For roller bottles the maximum exponential growth rate was almost similar in both media. These results

Table 4. *In vitro* MAb production technology suggested according to the amount of MAb needed.

| | Production scale | | | | |
|---|---|---|---|---|---|
| | ≤ 1 g | 1–10 g | 10–100 g | 100 g–2 Kg | > 2 Kg |
| Applicable *in vitro* technology | T-Flasks, roller bottles, spinner cultures, perfusion and hollow fiber bioreactors | Roller bottles, spinner cultures, perfusion and hollow fiber bioreactors | Roller bottles, hollow fiber bioreactors, stirred tank bioreactor systems | Roller bottles, hollow fiber bioreactors, stirred tank and airlift bioreactors | Roller bottles, stirred tank, and airlift bioreactors |
| Number of mice required assuming 20 mg/mouse | ≤ 50 mice | 500–500 mice | 500–5 000 mice | 5 000–100 000 mice | > 100 000 mice |

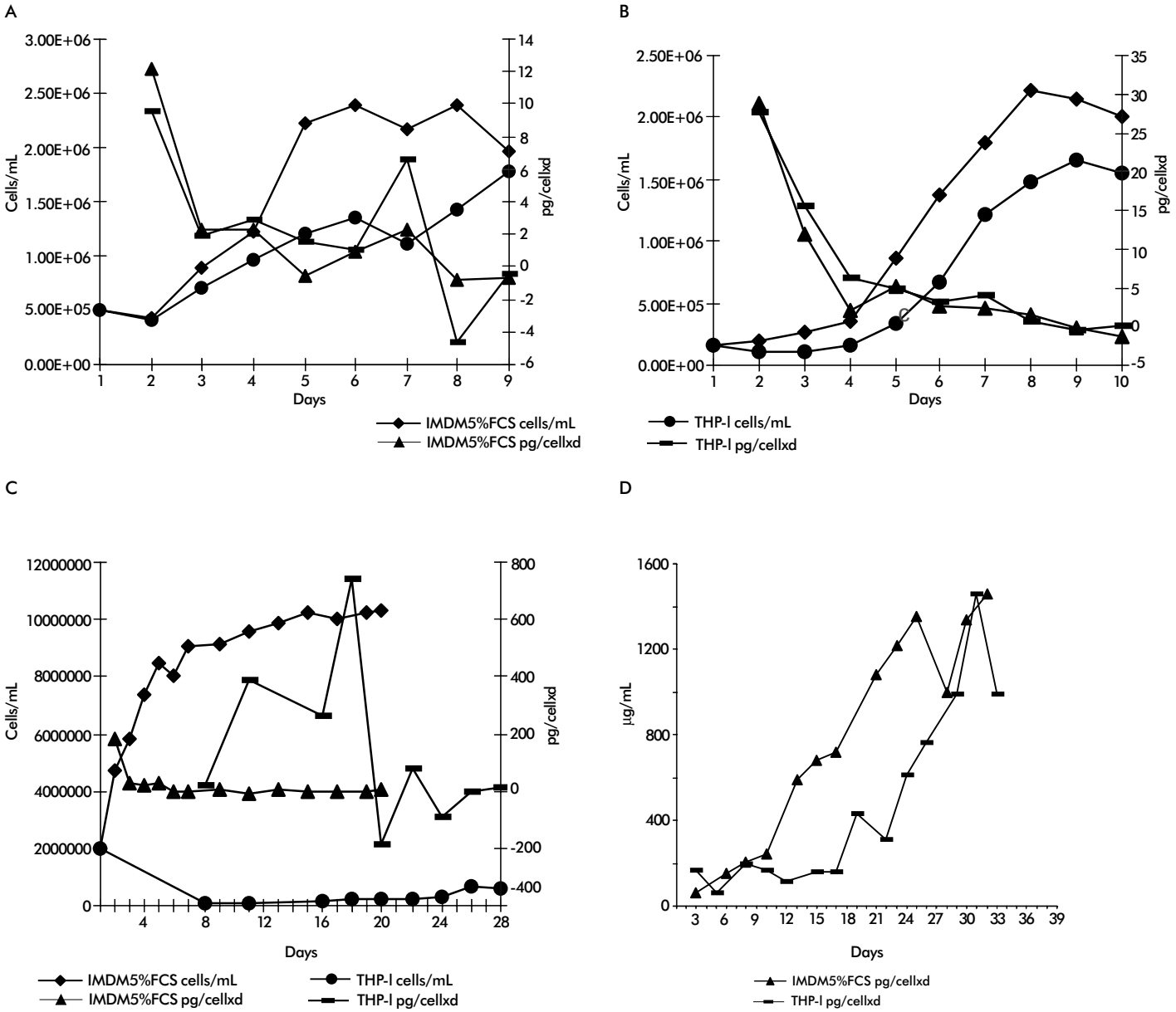


Figure 7. Cell proliferation and MAb CB.Hep-1 production using different *in vitro* systems. A, T-Flasks; B, roller bottles; C, miniPERM; D, hollow fiber biorreactors.

Table 5. CB.Hep-1 hybridoma performance in serum containing a protein-free medium.

| Parameters | T-Flasks | | Roller bottles | | miniPERM bioreactors | | Hollow fiber bioreactor | |
|---|---|---|---|---|---|---|---|---|
| | SCM | PFM | SCM | PFM | SCM | PFM | SCM | PFM |
| Production time (days) | 9 | 9 | 10 | 10 | 20 | 28 | 32 | 37 |
| Exponential growth rate ($h^{-1}$) | -0.008–0.032 | -0.0012–0.024 | 0.005–0.024 | -0.0008–0.034 | 0.063–0.044 | 0.007–0.035 | - | - |
| Specific production rate (pg/cell/day) | 12.2–2.22 | 9.598–1.04 | 28.78–1.40 | 27.69–0.59 | 184.45–0.84 | 384–0.84 | - | - |
| Total IgG (mg/run) | 2.31 | 2.33 | 20.09 | 13.17 | 51.33 | 54.29 | 100.72 | 90.28 |
| Scale factor | 1 | 1 | 8.69 | 5.62 | 2.5 | 4.12 | 1.96 | 1.66 |
| Medium consumed (L/run) | 0.25 | | 1.02 | | 1.65 | 1.39 | 7.28 | 10.16 |
| Production kinetic pattern | I | I | I | I | I | I | - | - |

express differences compared with T-Flasks, demonstrating the influence of the culture system on this parameter. Similar to T-Flasks, the maximum cell density obtained under SCM conditions was 1.3-fold higher than in PFM, confirming that PFM allows hybridoma proliferation but with a lower efficiency than SCM. The production kinetic patterns obtained in this culture system were type I in both media.

The MAb produced by these hybridoma cells gradually increased in the culture. The PFM TurboDoma HP-I did not modify the IgG production pattern for this hybridoma.

As in T-Flasks the stationary phase was maintained for several days in all cases and the total cell density was almost the same as that of the viable cell density during the experiments (data not shown).

The third system selected was the miniPERM® bioreactor. The exponential growth rate was also similar for the hybridoma CB.Hep-1 in SCM and in PFM, but under PFM conditions the cell population showed a higher death rate. The time required to reach maximum cell density was almost 2-fold higher in THP-1 than in SCM for Hep-1. The specific production rate expressed as pg/cell/day showed a notable increment compared to T-Flasks and roller bottles, ranging from 184.45 to 0.84 in SCM and 384 to 0.84 in PFM for these three cell lines, respectively. The production kinetic patterns did not differ from T-Flasks and roller bottles in both media.

Finally, we selected a hollow fiber bioreactor as a model for perfusion systems. The average difference between both media to reach a maximum IgG concentration was of only two days but the average antibody production was reduced to 5.47 mg/day in PFM, which represented 80% of the result obtained in SCM. In contrast with other culture systems the maximum IgG concentration was higher. If type III and I MAb production kinetic patterns are obtained in T-Flasks and roller bottles, good results should not be expected for MAb production in a continuous culture system. The best condition to predict good performance in continuous systems is the type II pattern because MAb production is initially relatively high, then decreases during maximum growth and increase again after the stationary and death phases. In this sense the kinetic of the production profile was seriously affected when PFM was used. Only an average of 8 days was needed to show an evident increase in MAb production in SCM while 15 days was the time required for PFM. The perfused condition seems to be responsible for this incongruence.

## Emerging Technologies

With their commercialization, high amounts of highly purified MAbs are required [90–94], with their production being carried out in different ways. However, since the large scale production of MAbs by hybridoma cells is extremely expensive for applications other than for diagnostic use and human medicine, the need for their applications in other fields could not be fulfilled. To meet the increasing demand, laboratory scale operations have been developed into full scale production processes to handle the larger amounts of material required. Thus, the most important consideration for the application of MAbs is an inexpensive production on a large scale.

As the need for more antibodies increases, the development of novel recombinant DNA-based technologies provides a cost-effective and viable alternative to cell culture. Developments in the field of the recombinant DNA technology in the past 10 years resulted in the establishment of many heterologous expression systems for recombinant antibodies. This was the result of demands from the scientific community and the pharmaceutical industry.

It is now possible to express recombinant antibodies and antibody fragments using a variety of expression systems such as bacterial, insect, mammalian and yeast systems.

Each system has its own advantages and disadvantages. The technology for expressing recombinant proteins is well established and relatively inexpensive. Bacterial expression systems are by far the most commonly used methods for producing recombinant antibody fragments because of the obvious ease of genetic manipulation [95, 96]. The technology for expressing recombinant proteins in bacteria is well established and relatively inexpensive.

Yeasts are rapidly becoming a preferred host for the efficient expression of heterologous antibodies [97, 98]. The main advantage of yeasts over other expression systems is that they are both microorganisms and eukaryotes and their genetics are more advanced than *E. coli*, but they are easier to manipulate than mammalian cells.

Mammalian cells appear to offer all the requirements for the production of monoclonal antibodies. This has the advantage of a more sophisticated refolding machinery. Insect cells share some advantages with mammalian cells and this system is particularly attractive for the large-scale production of proteins due to the fact that it is cost-effective and has a high expression efficiency [99]. The choice of the expression system depends on many factors. These include the nature of the molecule, the experience of the laboratory in using different expression systems and the quantity and quality needed.

The use of a transgenic technology is particularly attractive for pharmacologically active MAbs, which are required in large amounts because of their annual market demand, high therapeutic dose or long-term application. Transgenic production offers the advantage that it does not require a large initial capital for production facilities, particularly at the early stages of product development and the production of the clinical trial material. The transgenic herd can be expanded when and according to needs, minimizing both capital and running costs. Postlaunch expansion can be adapted to market requirements without a significant cost increase.

Although for the large scale bulk manufacturing of proteins there is already much experience with transgenic animals [100–103], in the future, transgenic plants [104–113] might be favorable, due to the lower risk of contamination with adventitious agents. Thus, higher organisms can be exploited for the large-scale production of soluble antibodies.

The application of these systems will allow that in the near future the *in vitro* systems may be com-

pleted with all of these alternative methods and at that moment the *in vivo* method to produce MAbs will only be a filed image.

## Conclusions

At present, we believe that there are sufficient *in vitro* methodologies and technologies available which can supplant the use of mice in the routine production of MAbs from hybridomas for most applications. The best choice depends on the line, media and purposes. The substitution by *in vitro* methods must come about by persuasion by those who have had "hands on" experience and are familiar with the problems and can therefore recommend suitable alternatives without being beholden to any particular commercial organization. Although the total exclusion of mice for the generation or production of MAbs will not likely occur soon, there are many forces at work, now and in the future, that will greatly decrease the need for *in vivo* production.

It is clear that there are now many ways to express high quantities of soluble and functional recombinant antibodies in both research and industrial settings. The obvious key is to find the system that best suits your needs.


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*Received in April, 2001. Accepted for publication in October, 2001.*