Monoclonal Antibody Production

The Report and Recommendations of ECVAM Workshop 23\textsuperscript{1,2}

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Preface

This is the report of the twenty-third of a series of workshops organised by the European Centre for the Validation of Alternative Methods (ECVAM). ECVAM’s main goal, as defined in 1993 by its Scientific Advisory Committee, is to promote the 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. One of the first priorities set by ECVAM was the implementation of procedures which would enable it to become well-informed about the state-of-the-art of non-animal test development and validation, and the potential for the possible incorporation of alternative tests into regulatory procedures. It was decided that this would be best achieved by the organisation of ECVAM workshops on specific topics, at which small groups of invited experts would review the current status of various types of \textit{in vitro} tests and their potential uses, and make recommendations about the best ways forward (1).

The workshop on Monoclonal Antibody Production was held in Angera, Italy, on 19–22 November 1996, under the chairmanship of Uwe Marx (University of Leipzig,
Germany). The aim of the workshop was to evaluate the present status of in vitro methods for monoclonal antibody (mAb) production, and to compare the advantages and disadvantages of the in vitro methods with those of the traditional in vivo (malignant ascites) method. The workshop participants assessed various in vitro culture systems for the propagation of hybridoma cells in terms of: a) the antibody production capacity; b) the concentration, yield and quality of the mAbs produced; and c) the capital and running costs of operation. The participants felt that there are already several scientifically satisfactory in vitro methods which are both reasonably and practicably available. As these 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, they concluded that the in vivo production of mAbs is no longer necessary, except in rare cases where it is already approved for clinical applications. In this respect a guideline on mAb production was discussed at the workshop, and a proposed guideline is included as an Appendix to this report.

Introduction

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. Following the discovery of hybridoma technology in 1975 (2), developments in mAb production and in their application have had profound implications not only on medical research, diagnosis and therapy, but also on biology in general. Hybridoma technology represents a significant advance because, in principle, it provides a means for obtaining unlimited supplies of highly specific antibodies.

In the production of mAbs, animals (generally rats or mice) first have to be immunised with the target antigen to obtain mortal antibody-producing cells. The biological construction of hybrids, and the selection of hybridomas which produce antibodies with the desired specificities, are carried out in vitro. In the early days of hybridoma technology (the late 1970s), the hybridomas developed in vitro were injected into the peritoneal cavity of an animal so that useful amounts of the desired mAb could be harvested from the ascitic fluid. This procedure was considered necessary at the time, since no efficient large-scale in vitro methods were available. By the mid-1980s, there were already serious doubts regarding the necessity of such a painful animal procedure. Nevertheless, as a result of its early introduction as part of the hybridoma technology, ascites production of mAbs is now employed worldwide, in spite of the ongoing development of in vitro technologies and the growing public pressure to replace or reduce animal experiments. The urgent need for experts to disseminate information and make recommendations about antibody production, taking animal welfare issues into consideration, was recognised by ECVAM in holding a workshop on avian antibodies in March 1996 (3) and, subsequently, in organising this workshop on mAb production.

Hybridoma Technology

There are essentially two stages in the production of mAbs: a) the induction of antibody-producing lymphoid cells in vivo and the selection of antibody-producing hybridoma cells in vitro; and b) the in vitro/in vivo propagation of selected hybridoma clones. 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 and
lymphoid cells (including progenitor antibody-producing cells) are isolated from
the spleen.

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 anti-
body, by using various immunoassay procedures.

6. The selected cells are subcultured in vitro, using special cloning procedures to
ensure that each in vitro culture consists of hybridomas with a single antibody
specificity only.

7. Hybridoma cells can be cryopreserved at this stage.

The second stage, the propagation of cloned hybridoma cells, can be accomplished either
by continuing to grow the cells in vitro, or by propagating them in vivo in the form of
ascites tumours.

Current Demand for Monoclonal Antibodies

The applications of mAbs are numerous and diverse. They are extensively used in funda-
mental research, medicine and biotechnology. At present, four user groups can be
identified, according to the amount of antibody required. These are summarised in Fig-
ure 1.

User group A: < 0.1g
Approximately 60% of the mAb users in Europe fall within this group, as do many of
the current users of the in vivo (ascites) method. Small amounts of antibodies are
produced for use in fundamental and applied research, the commercial production of spe-
cial diagnostic kits for research, and for ana-
lytical purposes.

Figure 1: Monoclonal antibody user groups
User group B: 0.1–0.5g

This group accounts for approximately 30% of mAb users and encompasses a significant number of people still using the \textit{in vivo} method. Antibodies in these amounts are required for the development and production of a wide range of \textit{in vitro} diagnostic kits and reagents, as well as for evaluating the usefulness of novel therapeutic mAbs in animal experiments.

User group C: 0.5–10g

In this group, which accounts for approximately 10% of mAb users, adoption of the \textit{in vivo} method is comparatively rare. The mAbs produced are used in routine diagnostic procedures and in preclinical evaluation studies. They are usually produced by large biotechnology companies but, during the last few years, the production of these mAbs has increasingly been contracted out to smaller facilities.

User group D: > 10g

Users in this group, who require mAbs for prophylactic and therapeutic purposes \textit{in vivo}, make up less than 1% of all mAb users in Europe. The mAb production processes they use are first developed and validated by the pharmaceutical industry, and are then submitted to a regulatory body for approval.

The extensive use of the ascites method by groups A and B can be attributed to its supposed economic advantage as well as to a lack of inclination to adopt the new techniques. Most of the mAbs produced by these groups are not used in clinical studies and therefore do not have to comply with the standard requirements for pharmaceutical products. This has led to a lack of awareness in these user groups of the disadvantages of ascites production, such as the potential for infection by animal viruses, and the reduced immunoreactivity of the mAb due to contamination with non-specific animal immunoglobulins.

\textbf{Monoclonal Antibody Production \textit{In Vivo}}

The \textit{in vivo} procedures entail the use of mice or rats. Initially, the immune systems of the experimental animals are suppressed (1–2 weeks before the intraperitoneal [i.p.] injection of hybridoma cells) by injection (i.p.) of a primer, such as pristane (2,6,10,14-tetramethylpentadecane) or Freund’s incomplete adjuvant. The hybridoma cells then multiply in the peritoneal cavity, and the ascitic fluid which forms is a very rich source of the secreted antibody.

When an adequate amount of ascites has formed, the animal is killed and the ascitic 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. 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.

The main advantage of the ascites method is the extremely high yield of antibody, which generally lies in the range 1–20mg/ml. In addition, the method is not excessively labour-intensive.

However, these advantages are outweighed by a number of disadvantages. The main disadvantage of the ascites method is that 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; and d) the invasive tumours which result (4–6). Proper animal husbandry facilities are mandatory. The mAbs produced generally show a reduced immunoreactivity of 60–70%, as opposed to an immunoreactivity of 90–95% for antibodies produced \textit{in vitro}, due to contamination by biochemically identical immunoglobulins. There is also a potential risk of product contamination by viruses which are pathogenic to humans. A further disadvantage is that the individual batches of harvested ascitic mAb are of variable quality, and they are contaminated with bioreactive cytokines.

\textbf{In Vitro Production Procedures}

\textit{In vitro} production systems

During the last 20 years, a wide range of \textit{in vitro} production systems have been
developed for different purposes. While most of them are useful for the in vitro production of mAbs, they differ in terms of: a) the ease with which they are handled; b) the antibody yield per culture or bioreactor run; and 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; and c) high cell density bioreactors. Some of these systems have been reviewed recently (7, 8).

Static and agitated suspension cultures
Systems in this category, which include the widely used T-flasks, roller cultures and spinner cultures, allow the growth of a maximum of two litres of supernatant per culture unit, and a maximum antibody yield of 100–200mg. They are easy to handle in cell culture laboratories, enable various hybridoma cell lines to be propagated simultaneously, and are useful for most of the users in group A.

Investment costs are low because disposable plasticware is readily available, particularly when using T-flasks. The use of serum-free media, or low-cost additives permitting a reduction in the serum concentration, can greatly reduce costs, while efficiently supporting hybridoma growth (9–13). For example, two serum-free media use a combination of transferrin and insulin (9, 10), whereas two low-serum media use a combination of 1% fetal calf serum (FCS) and 0.1% Primatone®, a peptic digest of animal tissues. This supports hybridoma growth in all culture methods tested at least as efficiently as 5% FCS, at approximately 25% of the cost (M.J. Embleton, personal observation).

For the production of mAbs in amounts greater than 100mg, conventional stirred tank bioreactors of different sizes are available. These bioreactors need to be used by specially trained staff and are relevant for user groups B, C and D.

The concentration of hybridoma cells in suspension cultures hardly ever exceeds $5 \times 10^6$ cells/ml and, in general, the maximum antibody concentration achievable is below 100µg/ml. As a result of the low antibody concentration, the supernatant usually has to be concentrated by ultrafiltration if any further purification steps are to be carried out.

Feeding of cultures may be carried out periodically if required but, in practice, antibody concentration is increased by 2–4 times if the cultures are allowed to grow to exhaustion over 2–3 weeks without feeding.

Membrane-based and matrix-based culture systems
This category includes membrane-based and matrix-based static cultures as well as suspension bioreactors. These systems are suitable for user groups A, B and C, which require up to 10g of mAb.

In membrane-based systems, the cells are cultured in compartments separated from the nutrient supply by perfusion membranes; special gassing membranes enhance the oxygen transfer into these systems. They produce yields of up to 100mg per culture (user group A), and generate intermediate antibody concentrations of up to 500µg/ml. In addition, they are easy to handle and enable various different cultures to be run simultaneously in routinely equipped cell culture laboratories.

In matrix-based systems, such as fluidised bed or ceramic bioreactors, the immobilisation of cells on matrices enables them to be perfused actively and continuously with fresh medium. Irrespective of the size and running time of the bioreactors, 0.1–10g of mAbs (user groups B and C) can be produced, corresponding to a maximum concentration of 500µg/ml. In most cases, the supernatant produced has to be concentrated by precipitation or ultrafiltration before special purification procedures can be carried out. Special training is required for the proper handling of these systems.

High cell density bioreactors
This category includes all culture systems which are capable of generating cell densities greater than $10^8$ cells/ml and which, in certain cases, can maintain viable tissue-like cultures. The bioreactors meet the needs of user groups B and C, as they are capable of generating 0.1–10g mAb. The corresponding concentrations lie in the range 0.5–5mg/ml, due to the high cell densities in these systems. They can be run in conventional cell
culture laboratories and models are available for the simultaneous propagation of different cell lines. The product can be used directly or purified without prior concentration. Training is recommended for these systems and is usually provided by the manufacturer.

In the most common system within this category, the hollow fibre bioreactor, the culture medium is passed through bundles of hollow fibres, enabling the cell growth compartment to be perfused continuously and effectively. Due to the high antibody concentration, the maximum amount of 500mg of antibody needed by user group B can be produced in a bulk of only 500ml of supernatant, which is easy to handle and process in a conventional cell culture laboratory. Even for user group C, which requires up to 10g of mAb, the total product can be produced in only 10 litres.

The different categories of culture system are listed according to their usefulness to the different user groups in Table I. Instead of the maximum achievable mAb concentration, the concentration which is normally achievable is given. The types of systems recommended for the different user groups, on the basis of their ease of handling, production costs, and advantages with respect to antibody purification, are highlighted.

In vitro process development

Several problems are associated with the use of serum-containing media for the in vitro production of mAbs, the most important being the high protein content which makes antibody purification either difficult or impossible. Other problems are animal welfare concerns relating to the production of fetal serum, its cost, its uncontrollable variability in quality from one batch to another, and the risk of its contamination by viruses, mycoplasma and unsuspected prions (14). All commercial companies with a long experience of cell culture, and many small new biotechnology groups, now offer various serum replacements from bovine plasma and serum substitutes, and ready-to-use serum-free media which may contain many serum-derived proteins (~3mg/l) or reduced amounts of essential proteins (~30µg/ml), or which may be devoid of proteins and peptides. Potentially important supplements are also supplied separately to fortify and optimise basal versions of the classical media currently used (15, 16).

Hybridoma growth and mAb production in serum-free media are variable processes which depend on the physical and nutritional requirements of: a) the specific hybridoma cell line; b) the complexity of the serum-free formulation; and c) the culture conditions of the bioreactors (17). Therefore, during the weaning process by which a subpopulation of cells is adapted to growth in a new environment, one needs to optimise criteria such as the cell growth rate, the maximum cell concentration, the final mAb concentration, and the quality of the mAb and its production rate. It is also necessary to ensure that the selected subpopulation exhibits the same immunoreactivity as the population which was cultured in the presence of serum (16).

In most cases, the use of an optimised serum-free formulation rather than a serum-containing medium offers two advantages: a) the mAb is produced in greater yield and with less expense (16, 18); and b) subsequent downstream processing is facilitated.

Monoclonal antibody quality

Both monoclonal and polyclonal immunoglobulin G (IgG) antibodies are N-glycosylated at amino acid 297, a conserved asparagine (Asn) residue in the second constant domain of the heavy chain (CH2). Human serum IgG might be associated with at least 30 different biantennary complex oligosaccharides (19), but these represent only 2–5% of the antibody's molecular weight.

Under physiological conditions, N-glycosylation at Asn 297 plays an important role in several biochemical processes: a) the fixation of complement C1q (17, 20; G. Winter, A.R. Duncan & D. Burton, patent number PCT/GB88/002111); b) the binding of Fc-γ receptors; and c) the resistance of the antibody to proteolysis. In addition, biologically important processes, such as phagocytosis, antigen-dependent cellular cytotoxicity, and the clearance and placentation of mAbs, can be influenced by the type, sequence and structure of their glycosylation.

In addition to glycosylation at Asn 297, glycosylation also occurs, in very rare cases, in the variable region of mAbs (21). If such an additional glycosylation is present on a mAb, it may influence its antigen binding capacity, with the result that the respective hybridoma clone is unlikely to be picked out.
Table I: Appropriate culture systems for the four monoclonal antibody user groups

<table>
<thead>
<tr>
<th>Categories of culture system</th>
<th>User group</th>
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<tbody>
<tr>
<td></td>
<td>A</td>
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<tr>
<td>Static and agitated suspension cultures</td>
<td>for example, T-flasks, rollers, spinners</td>
</tr>
<tr>
<td>Usual mAb concentration 50µg/ml</td>
<td>Vmax = 2 litres</td>
</tr>
<tr>
<td>Membrane-based and matrix-based static cultures</td>
<td>for example, membrane-based static disposable cell culture flasks and rotatable devices, spinner flasks with an incorporated harvest with cell retention</td>
</tr>
<tr>
<td>Usual mAb concentration 0.2mg/ml</td>
<td>Vmax = 0.5 litres</td>
</tr>
<tr>
<td>High cell density bioreactors</td>
<td>for example, miniaturised hollow fibre bioreactors with several culture modules</td>
</tr>
<tr>
<td>Usual mAb concentration 1mg/ml</td>
<td>Vmax = 100ml</td>
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Vmax = maximum volume of supernatant to be processed during purification, assuming the usual antibody concentration for that category. The most suitable culture systems for the four user groups are shown in bold.
by the initial antigen-specific selection procedures.

Glycosylation is a complex post-translational event which can be influenced by a variety of factors, such as the culture conditions, the protein and carbohydrate supplements in the medium, and the purification procedures. Thus, the in vitro methods enable the desired glycosylation structure to be obtained by making an appropriate choice of these factors. What is often needed, for example, are mAbs with a glycosylation pattern of the biantennary complex oligosaccharide type, with terminal sialic acid residues, and this can be generated in hollow fibre bioreactors. In contrast, when antibodies are produced by the ascites method, it is impossible to influence their glycosylation pattern, which may vary from mouse to mouse.

Generally, the glycosylation issue is only relevant to users who want to use the antibodies in vivo, either in humans or in animal experiments (user groups B, C and D), and to users who need to perform experiments on the binding of mAbs to complement proteins or Fc-receptors. In summary, there are no reasonable arguments based on antibody glycosylation which support the use of in vivo methods.

Economic aspects

The relative costs of mAb production by in vitro methods as opposed to the in vivo ascites method has been addressed by several authors (22–25). Although many have concluded that in vitro alternatives are comparable in cost to the in vivo method, individual calculations have been based on different assumptions. As a consequence of the “outsourcing” policy which is currently widely adopted by industry and universities, “full cost analyses” have to be made for given technologies. Such analyses reveal a trend in which the costs of mAb production by the ascites method are continually increasing, whereas the costs associated with the various in vitro methods are decreasing. The increasing costs of in vivo production are largely a result of the increasing costs of laboratory animals.

In contrast, the disposable materials needed for in vitro mAb production are decreasing in cost as production technology improves. The increasing demand for bioreactors is reinforcing this trend by allowing manufacturers to produce them on a larger scale, leading to a reduction in their production costs.

These two cost development curves indicate that there is no driving force which will eventually favour the in vivo production of mAbs. The adoption of in vitro methods by user groups C and D has led to moderate increases in costs which, at present, are no more than 1.5–3 times higher than those associated with the in vivo production procedure.

It is desirable that centres of excellence become available for an intermediate period, to help the different user groups adapt their own facilities for mAb production in vitro. Such centres of excellence would also be of enormous educational value, by providing training in in vitro cell culture technologies.

Advanced technologies and future developments

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 animals is 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 (26, 27). 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 (26–28). 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 immunoglobulins 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 non-immunogenic or unknown target antigens (26). 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 (29).

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.

**Regulatory Aspects**

**General remarks**

Two important laws exist in Europe for the protection of laboratory animals: a) *Council Directive 86/609/EEC* (30); and b) the *European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, ETS 123* (31). Both the Directive and the Convention require alternatives to be used when “reasonably and practicably available”, but each country is free to adopt stricter measures.

The Directive came into force in 1986; the Convention was opened for signature by the Member Countries of the Council of Europe on 18 March 1986, and came into force in 1991. The 15 Member States of the European Union (EU) are required to incorporate the Directive into their national laws, but the 43 Member Countries of the Council of Europe are not legally obliged to sign the Convention. However, once a Member Country has voluntarily signed and ratified the Convention, it is required under international law to implement the provisions of the Convention within its territory. So far, the Convention has been both signed and ratified by ten countries, namely, Belgium, Cyprus, Finland, Germany, Greece, The Netherlands, Norway, Spain, Sweden and Switzerland.

**National policies and their impact on reducing the use of the ascites method**

**United Kingdom**

The *Animals (Scientific Procedures) Act 1986* (32), which came into force in 1987, effectively implements *Directive 86/609/EEC* in the UK. A project licence issued under the terms of this Act is required for all *in vivo* production of mAbs by the ascites method. Applicants for project licences are required to justify their proposals in writing, and the Home Secretary (acting on the advice of an expert Inspectorate) then decides whether, and on what terms, to grant the licence.

In December 1991, the UK Home Office issued advice on protocols for minimal severity for raising antibodies using live animals (33). According to this advice, “The malignant ascites method may be justified where less than 20 mice are needed on a one-off basis for a particular mAb. If appropriate facilities for the production of the mAb *in vitro* are available, it is expected that these will be used in preference to the ascites method in mice.” The Home Office advice also included recommendations for the use of pristane, for tapping ascites, and on the humane endpoints to be observed when using the malignant ascites method.

The use of animals with hybridomas for mAb production *in vivo* was identified for the first time in the statistics for 1990. From 1990 to 1994, the number of animals with hybridomas (mainly mice) fell by 51.5%, from 46,188 to 22,391, at a time when total animal use decreased from 3,100,553 to 2,772,758. Thus, hybridoma use decreased not only in absolute terms, but also as a percentage of the total number of animals used (including those used for breeding strains with harmful defects) from 1.49% to 0.83%. Assuming that the total production and use of mAbs did not decrease in the UK over this period, the statistics are fully compatible with an increasing use of *in vitro* production methods in preference to *in vivo* ones. Indeed, it is known that, by using *in vitro* methods, some large mAb producers have reduced the number of mice used for *in vivo* production by a factor of ten.

It is not yet known whether the Home Office has conducted a review with the following objectives: a) to confirm wherever possible that all project licence holders in the UK are following the formal advice referred to previously; b) to determine the nature, rationale and geographic location for all current use of the ascites method and, in particular, to establish whether such use is routine or exceptional; and c) to discover whether an alleged lack of equipment or expertise for
MAB production in vitro are acceptable reasons for allowing the continued use of the ascites method.

**Germany**

In 1989, a national hearing was held at ZEBET (National Centre for the Documentation and Evaluation of Alternatives to Animal Experiments) to evaluate the current in vitro methods for the production of mAbs as replacement alternatives to the ascites mouse procedure (34). The consensus of opinion among national experts was that the production of mAbs in vivo should only be permitted in the following exceptional cases: a) when the Mabs are intended for diagnostic and therapeutic purposes in humans, provided that no other options are available; b) when hybridoma cells need to be rescued because they have either failed to grow in vitro or they have become infected; and c) when the Mabs are needed to investigate new scientific problems.

Several legal technicalities in connection with these exemptions are noteworthy. Exemption 1 does not breach Article 7.1 of the German Animal Protection Act (Tierschutzgesetz Article 7.1), since the production of Mabs in this case is not considered to be part of an experimental procedure, and is therefore not considered to be an animal experiment according to this Act. On the other hand, Exemptions 2 and 3 do relate to animal experiments according to Article 7.1 of the German Animal Protection Act, and therefore have to be authorised in accordance with Article 8.1. Furthermore, Exemption 2 will only be granted if the Mabs are produced for a specific research project and not for distribution to third parties.

**The Netherlands**

The Netherlands Code of Practice for the Production of Monoclonal Antibodies (4) was issued in 1989 by the Netherlands Veterinary Public Health Inspectorate, which is empowered to supervise compliance with the provisions of the Experiments on Animals Act (1977). The Code consists of a small set of guidelines and general information concerning technical matters, pathology, clinical signs and distress in relation to MAb production. Among other things, the guidelines concern: a) the maximum number of mice to be used (5–10) per hybridoma; b) the skill and authorisation of the persons concerned; c) the justification for the protocol; and d) the responsibilities of the day-to-day caretaker, the researcher and the animal welfare officer. The Code was drawn up by a working group established by the Inspectorate. The working group consisted of representatives from five scientific societies: the Netherlands Society for Immunology, the Netherlands Society for Microbiology, the Netherlands Society for Pathology, the Netherlands Society for Infectious Diseases, and the Netherlands Society for Laboratory Animal Science. The Code is not mandatory, but is intended to serve as a tool for researchers, animal welfare officers, biotechnicians and local ethical review committees.

Three years after the Code was issued, an evaluation of its effect led to the following conclusions: a) many institutes were holding discussions on the subject of MAb production, as a result of the Code; b) a number of institutes had changed their institutional policies; c) in several institutes, facilities for in vitro production had been established; d) in some institutes, in vivo production had been completely replaced by in vitro production; e) the total number of animals used for the in vivo production of Mabs had been significantly reduced (from more than 10,000 in 1990 to less than 1000 in 1995); f) some institutes were contracting out the in vitro production to other institutes; and g) in some institutes, the adoption of in vitro production was being hampered by the relative ease of in vivo production.

In 1995, a symposium was organised entitled The Production of Monoclonal Antibodies: Are Animals Still Needed? (25). There were about 120 participants, who were mainly researchers and animal welfare officers. Several researchers presented their experiences of the in vitro production of a large number of Mabs. The Inspectorate used the symposium to investigate whether there was consensus of opinion among the experts concerned. This played a key role in the legislation which followed; Article 10 of the Netherlands Experiments on Animals Act, which is the equivalent of Article 7.2 of Directive 86/609/EEC (30), states:

“No animal experiment shall be conducted for a purpose which, according to the consensus of opinion among experts, may also be achieved by means other than an experiment on animals, or by means of an experiment...
using fewer animals or entailing less distress than the experiment in question”.

Taking into consideration the discussions and information presented, the Inspectorate decided that Article 10 was fully applicable to the *in vivo* production of mAbs. One month later, on 1 January 1996, a ban on *in vivo* production came into force. Exemptions could only be granted on the basis of a good scientific justification. By the end of 1996, the Inspectorate had received five requests for exemption.

These results make it clear that the Code of Practice had a substantial effect and created a climate in which a ban could eventually be established. The involvement of researchers and animal welfare officers at all stages of the process appears to have been essential in achieving this ban.

**Sweden**

Sweden is bound by three different regulations concerning the use of alternative methods: the *European Convention* (31; Article 6); the *Swedish Animal Protection Act* (35; Section 49:2); and *Directive 86/609/EEC* (30; Article 7). The Swedish law is stricter than the Convention in that it states that existing alternative methods must be used and instructs the animal ethics committees to “advise against the use of animals for such purposes where it is possible to acquire comparable information by other means”. This wording does not allow for exemptions, such as for economic reasons, lack of equipment, and/or lack of familiarity with alternative methods on the part of the scientist.

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**Switzerland**

In 1989, the Swiss Federal Veterinary Office (BVET) informed all scientists that the production of mAbs by the ascites method would become a fundamental breach of Swiss animal welfare legislation from May 1994, and that they had 5 years in which to change their methods. The general ban on ascites production was implemented in 1994 by Animal Welfare Guideline 5.01 (BVET, 20 May 1994), which stated that, in principle, mAbs could be obtained *in vitro*, and that, as a rule, applications for ascites production were to be refused. However, two exceptions were envisaged: a) the development of mAbs for diagnostic and therapeutic purposes in cases of medical emergency; and b) the development of mAbs to rescue single hybridomas when it can be documented that they are not growing satisfactorily *in vitro* or are contaminated.

If exemptions are granted, each animal has to be documented and checked at least once a day. Animals with a weight gain of over 20% have to be killed immediately to harvest the ascites. Although this should usually be drained from dead animals, living animals may also be used, but the authorities have to be notified in every case. In 1996, there were no reported instances of exceptional mAb production in ascites mice. However, some scientific groups ordered custom-made mAbs from commercial suppliers outside Switzerland.

In 1993, the Swiss Foundation Research 3R started a validation study on the *in vitro* production of mAbs and provided hollow fibre reactors free of charge to 31 research centres throughout Switzerland. The preliminary
results show that 24 groups are still working with mAbs; eight of them have changed to other in vitro mAb production systems, mostly with a lower yield (Foundation Research 3R, Switzerland, unpublished data). Four groups indicated that the yield obtained with the hollow fibre reactor was insufficient; on average, six mAbs were produced per year by each group, with a concentration range of 20–200mg/ml. Of the 24 groups still working with mAbs, 17 thought that universities should provide central mAb production units, and 13 of them felt that this should be done on a non-profit basis. Twenty-two groups bought custom-made mAbs in 1995; 16 of these bought imported mAbs. About 80% of the mAbs purchased were produced in vitro. Twenty-one of the 24 groups welcomed the labelling of commercially available mAbs as either “in vitro produced” or “in vivo produced”. The expected demand per group was 18 mAbs per year, with amounts ranging from 110–1150mg.

Conclusions and Recommendations

The workshop participants noted a number of difficulties which are preventing a complete assessment of the impact and usefulness of in vitro methods. There is a lack of information on the extent of in vivo production in most EU Member States, due to incomplete statistics on laboratory animal use. Several countries within the EU do not have an effective system for project review or for the justification of animal use, nor do they require explanations of why in vitro methods cannot be used. The workshop participants felt that all Member States should collect such information, albeit in summary form, and make it available. They also suggested that mAb manufacturers supply information on how their antibodies are produced, for example, by listing this in their catalogues.

Difficulties also arise from mAbs produced in vivo being imported into countries where such in vivo production is either prohibited or is only permitted in exceptional cases. Without any restrictions being placed on the importation of such mAbs, it is possible for scientists in countries where guidelines are strictly applied, to export hybridoma cell lines to countries with lax policies, so that they can later re-import mAbs which have been produced in vivo. In Switzerland, for example, one third of the mAbs which are imported have been produced in vivo (René Fischer, unpublished observation). The workshop participants felt that the importation of products obtained by methods which breach existing guidelines, such as Directive 86/609/EEC (30) and the European Convention (31), cannot be justified.

Many mAb users merely require the antibodies as a tool. Such users may not have the knowledge or experience of relevant in vitro methods, so their opinions on the usefulness of in vitro methods cannot be objective and should therefore be treated with caution. It is desirable that such scientists, and those reviewing their applications, take advice from those with experience in in vitro methods and in the supply of products manufactured by such methods.

Article 7.2 of Directive 86/609/EEC (30) states that:

“An experiment shall not be performed if another scientifically satisfactory method of obtaining the result sought, not entailing the use of an animal, is reasonably and practicably available”.

This is comparable to Article 6.1 of the Council of Europe Convention (31). In the light of the above requirement and current knowledge, it was concluded that for all levels of mAb production: a) 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, b) in vivo mAb production can no longer be justified and should cease. However, to enable users time to acquire and implement the new techniques, and for administrative reasons, a transitional period of no more than 2 years should be allowed, before a complete ban on in vivo production is implemented.

Where there is an exceptional need for an emergency therapeutic application, the in vivo production of mAbs should continue to be permitted. In those cases where there is an existing regulatory approval for a diagnostic or therapeutic mAb produced by the ascites method, such an in vivo method has to be accepted until the approval expires. In addition, the ascites method may be needed in other very exceptional circumstances, where verifiable efforts have failed to produce the mAb in vitro. In this situation, each
animal experiment should be scientifically justified on a case-by-case basis, and mAb production should be limited in terms of time and the number of animals to be used. It is also expected that continued efforts be made to produce the mAb in vitro.

The main conclusions and recommendations from this ECVAM workshop on mAb production are summarised below:

1. Various in vitro mAb production systems have been developed to meet the needs of a diverse range of users making the ascites method of mAb production redundant.

2. New recombinant DNA technologies are emerging which enable the expression of designer peptides and proteins, thus permitting the rapid selection of a range of previously unidentified mAbs and their subsequent very specific opsonisation.

3. There are differences in the regulations between different European countries, as well as differences in the extent to which they are implemented.

4. The in vivo production of mAbs should be prohibited in those countries which are members of the EU and/or have ratified the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes.

5. Before a ban on in vivo production comes into force, centres of excellence offering advice and, if appropriate, assistance should be established, to help laboratories adapt to the use of in vitro methods. A transitional period of no more than 2 years should be allowed to enable users time to acquire and implement the new techniques, and for administrative reasons, before such a ban is implemented.

6. Commercially available mAbs should be unambiguously labelled to show whether they were produced in vivo or in vitro.

7. Ascites-produced mAbs imported into the EU should be labelled to indicate their country of origin.

8. To ensure that in vivo mAb production is not performed unnecessarily, there is an urgent need for effective inspection systems, as well as for the resources to implement these, at the level of individual user establishments.

9. Project reviews and inspection systems should be subject to approval. In countries where there is no project review system, one should be introduced. In countries where there is a project review system, it should be considered whether this system meets the necessary approval criteria, especially with respect to the requirement to justify any use of in vivo methods. During the review of applications, advice should be sought from those with experience in in vitro methods and the supply of products manufactured by such methods.

10. The collection of statistics must be improved in all Member States of the EU, and these should include the numbers and species of animals used for mAb production by the ascites method.

11. In scientific reports, it should be mentioned how the mAbs were produced. Editorial Boards of scientific journals should include this requirement in their Instructions to Authors.

The text of a proposed European guideline on mAb production embodying these conclusions and recommendations was discussed at the workshop (Appendix 1). This should provide the basis for controlling mAb production and for national practice where an appropriate guideline is currently lacking.

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References


Appendix 1

Proposed European Guideline on Monoclonal Antibody Production

**Directive 86/609/EEC and Convention ETS 123**

The purpose of this Guideline is to advise Member States on the application to monoclonal antibody (mAb) production of the Three Rs principles enshrined in Article 7 of Directive 86/609/EEC (1) and Articles 6, 7 and 8 of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, ETS 123 (2), whilst having regard to the right of the Member States to apply stricter measures (Article 24 of the Directive and Article 4 of the Convention). In particular, this Guideline aims to provide specific advice to scientists and project reviewers on what is currently regarded as best practice by experts in the field.

Article 7.2 of the Directive requires that “an experiment shall not be performed if another scientifically satisfactory method of obtaining the result sought, not entailing the use of an animal, is reasonably and practicably available”. Also, Article 7.3 of the Directive states that “in a choice between experiments, those which use the minimum number of animals . . . cause the least pain, suffering, distress, and lasting harm and which are most likely to provide satisfactory results shall be selected”, while “as a general principle”, Article 7.4 of the Directive requires that “all experiments shall be designed to avoid distress and unnecessary pain and suffering to experimental animals”. These requirements are also documented in Articles 6.1, 7 and 8a, respectively, of the Convention (2).

Article 2 of the Directive and Article 1 of the Convention cover any use of an animal for experimental or other scientific purposes which may cause it pain, etc., while Article 3 of the Directive and Article 2 of the Convention apply to the use of experimental animals for purposes including the manufacture of drugs, and other substances or products. Thus, Directive 86/609/EEC and Convention ETS 123 apply unequivocally to all use of live animals in the production of mAbs, whether the antibodies are intended for use as research tools, for assays, or for therapeutic or diagnostic purposes.

**Monoclonal Antibody Production**

After an initial immunisation *in vivo*, immunocompetent cells are fused with myeloma cells *in vitro* to produce single hybridoma cells secreting the specific antibody. Consequently, all existing hybridoma cell lines are initially grown up in a static *in vitro* culture.

In the light of present knowledge, it can be concluded that, for all levels of mAb production, one or more *in vitro* methods are scientifically acceptable and reasonably and practicably available. Such *in vitro* methods have the additional advantage of producing antibodies with very high immunoreactivities. A previous objection to the *in vitro* methodology was that significant practical effort was needed to concentrate spent culture fluid and produce useful amounts of mAbs. However, modern technology provides a variety of economically acceptable *in vitro* systems which enable the generation of both high concentrations and/or high yields of mAbs. Thus, most production facilities and up-to-date research institutes are now producing all of their mAbs *in vitro*.

The use of the traditional method, which causes a considerable amount of pain and distress to the animals involved (3), is a matter of great concern. In this method, selected antibody-producing hybridoma cells are injected into the peritoneal cavity of compatible laboratory animals under aseptic conditions to produce rapidly progressive local tumours secreting mAbs in high titre in the ascitic fluid. Substantial pain and discomfort result from the following: a) the initial priming with the irritant pristane; b) the subsequent rapidly growing tumour (which may disseminate); c) the rate and volume of ascites production; and d) the procedures for, and frequency of, harvesting. Clearly, the use of this method
in the majority of circumstances where it is not necessary and cannot be justified breaches the provisions of Directive 86/609/EEC and the European Convention and, as a consequence, such in vivo production should cease.

Where there is an exceptional need for an emergency therapeutic application, the in vivo production of mAbs should be allowed. In those cases where there is an existing regulatory approval for a therapeutic or diagnostic use, the ascites method can only be accepted until the end of the approval period. In addition, the ascites method should be allowed in other very exceptional circumstances, where verifiable efforts have failed to produce the mAb in vitro. In this situation, each animal experiment should be scientifically justified, and limited in terms of time and the number of animals to be used. Continuing efforts to produce the mAb in vitro would be expected. In themselves, convenience, "custom and practice", lack of equipment, and/or lack of familiarity with cell culture methods are not justifications for new or continued use of the ascites method.

Pristane continues to be used to encourage consistent ascitic, rather than solid, tumours. In such cases, it is usually satisfactory to give a single priming injection of 0.2ml pristane intraperitoneally 7–10 days before injecting 10⁶–10⁷ hybridoma cells. However, before resorting to the use of pristane, it must be borne in mind that this causes painful peritonitis (3–5) and other malignant effects (6, 7). The Dutch Code of Practice suggests that pristane should not be used (4), and Freund’s complete and incomplete adjuvants have been suggested as possible alternatives (8, 9).

Animals should be inspected frequently by suitably trained personnel so that their clinical conditions can be assessed. Initially, the animals should be handled and inspected by such personnel twice a day and, if necessary, more frequently later on. Animals must be killed without delay when they show more than mild distress, overt tumour deposits or spread, or significant dehydration or cachexia. The volume of ascites should not normally exceed 20% of the host body weight in mice and rats, in the absence of overt cachexia. A 20% increase in body weight is indicative of a very small, almost imperceptible, swelling of the abdomen. Ascites fluid must be harvested once only, either under terminal anaesthesia or post-mortem.

References


