A Call for a European Prohibition of Monoclonal Antibody Production by the Ascites Procedure in Laboratory Animals

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Summary — Monoclonal antibodies (mAbs) are particularly valuable in therapeutics and research. Unfortunately, one of the most familiar methods of producing mAbs, the ascites induction method, causes pain and distress to the animals used. In most cases, non-animal or *in vitro* alternatives can be employed to reduce or eliminate the use of animals for mAb production. Prohibition of the use of animals in the production of mAbs is recommended, except when the replacement *in vitro* methods prove to be insufficient, and in a limited number of other well-documented cases, such as an exceptional need for an emergency therapeutic application. A total ban on the use of animals for mAb production is impractical and it is imperative that an appeals process should accompany the prohibition. The need for the establishment of core facilities for *in vitro* mAb production is emphasised.

Key words: ascites method, in vitro production, in vivo production, monoclonal antibodies, replacement alternatives.

History and Applications

Monoclonal antibodies (mAbs) are antibodies produced by a single B-cell clone when an antigen epitope interacts with the immune system. They differ from their easily reproducible counterpart, polyclonal antibodies, in that mAbs are more specific and more homogeneic.

The development of hybridoma technology marks a major breakthrough in mAb production. By fusing B-cells with myeloma cells, Köhler & Milstein (1) developed a way to immortalise the B-cells and produce virtually unlimited quantities of mAbs. Since Köhler & Milsteins Nobel Prize-winning work, mAbs have become important tools in the laboratory and in the clinic (2). These products are now widely used as therapeutic agents, components of kits for immunoassays and affinity chromatography, as carriers in drug targeted therapy, and in basic research experiments (for example, as immunomarkers).

The introduction of mAb technology also created possibilities for replacing animal tests with *in vitro* tests. For example, the mAb-based *in vitro* "potency" test for the rabies vaccine (an ELISA test with the monoclonal antibody against one of the glycoproteins and nucleoproteins of the rabies virus as coat) serves as an alternative to the lethal challenge tests performed in mice (3).

At present, more than 100,000 different mAbs are available (4). Most are produced in small quantities (< 0.1g) solely for bench-related purposes. Larger amounts are often required for use in diagnostic kits and reagents (0.1–0.5g), for routine diagnostic procedures and in preclinical evaluation studies (0.5–10g), and for prophylactic and therapeutic purposes (> 10g; 5).

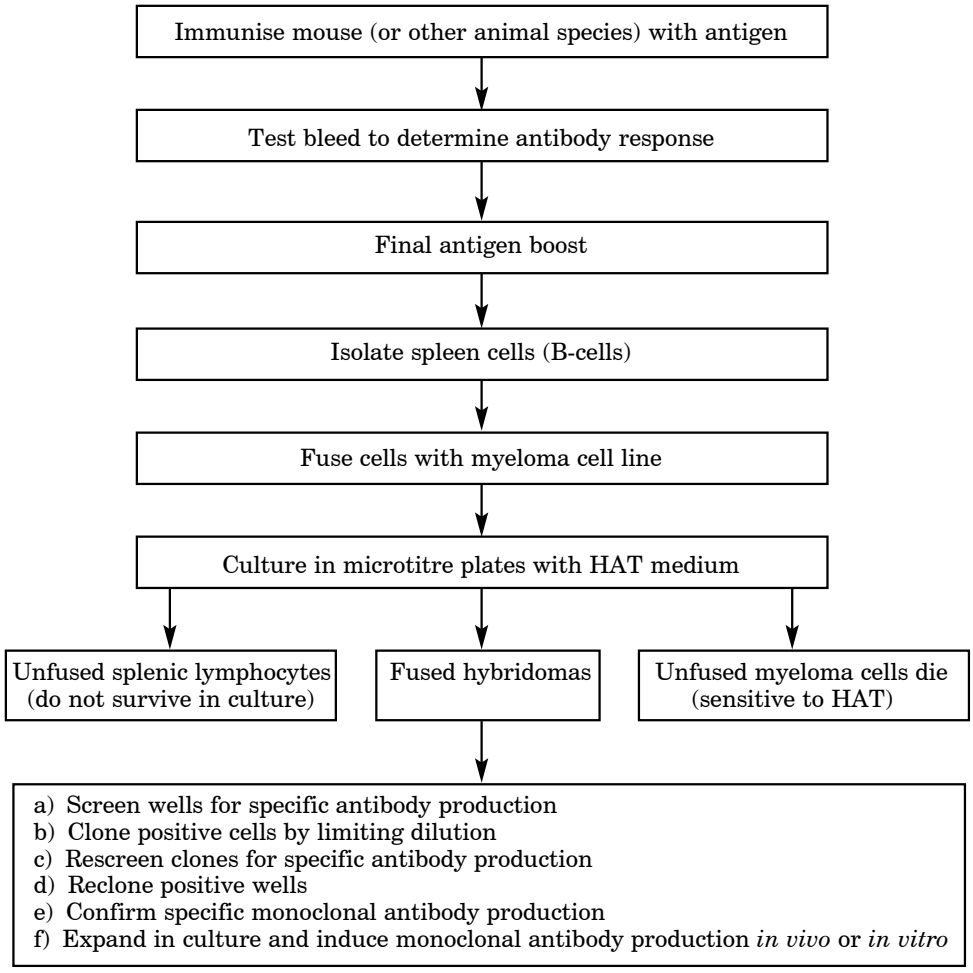
Development and Production

The mAb production process includes a number of steps (Figure 1). In order to obtain the

necessary B-cell clones, a donor animal is immunised. The schedule in laboratory animals consists of a primary immunisation followed by one or more boosts. The use of a purified antigen increases the probability of obtaining a highly specific B-cell response. Also, the antigen is often mixed with an adjuvant to enhance the immune response.

Balb/c mice are the most common hosts used in immunisation, since many of the myeloma cell lines available for fusion are of Balb/c origin (6). When Balb/c mice are unable to produce the B-cell clone of interest or for specific purposes, mAbs are obtained from other species such as the rat, hamster and human (4). At the end of the immunisation period, the animal is sacrificed and its spleen is used as the source of cells for fusion. The B-lymphocytes can also be obtained from gut-associated lymphoid tissue and mesenteric or peripheral intestinal lymph nodes. Peripheral B-lymphocytes are

Figure 1: Schematic outline of the production of monoclonal antibodies in mice



From reference 6.

HAT = hypoxanthine, aminopterin, thymidine.

generally required for human mAbs (7). Although the primary *in vitro* stimulation of B-lymphocytes has been reported, the cells have been almost entirely of the IgM type, and it is the IgG type that is preferred (8, 9). Usually, the IgG type of B-cell clones can only be obtained by the restimulation of memory cells derived from *in vivo* primary immunisation (10).

Having been obtained, the antibody-producing cells are fused with non-secretory myeloma cells. Fusion procedures include electrofusion, Epstein-Barr transformation and chemical fusion (the most frequently used method) by polyethylene glycol. All fusion procedures are based on *in vitro* techniques. The lymphocyte–myeloma hybridomas are then cultured *in vitro* on microtitre plates containing HAT (hypoxanthine, aminopterin, thymidine) medium. Selection in HAT medium allows only hybridoma cells to survive. Hybridomas positive for the specific antigen epitope are further selected and subcloned *in vitro*. Finally, the desired mAbs are produced by injecting the clones into mice or by growing them in culture.

Aspects of *In Vivo* Immunisation and *In Vivo* Production

The classical process of mAb production includes two separate procedures in laboratory animals: immunisation to generate B-cell clones; and production of mAbs by ascites induction. Both procedures raise animal welfare concerns and have prompted the pursuit of *in vitro* alternatives. Since the IgG-type of B-cell clones are usually only obtained after *in vitro* restimulation of memory cells derived from *in vivo* primary immunisation, refinement is, for the time being, the only possible Three Rs approach to minimise the side-effects of immunisation. Possible refinements include the selection of the adjuvant inducing the least severe side-effects, and adjusting the volume and selection of the route of immunisation (11).

In contrast, hybridoma clones can be grown in tissue culture, or as a tumour line implanted in mice (or in rats for rat hybridoma cells), a technique referred to as ascites production (12). Both procedures were mentioned in the original article on mAb production by Köhler & Milstein (1). However, for various reasons, such as production capacity and the ease of the procedure, the main production method is ascites production.

According to a 1991 report by the Business Communications Company, an estimated 2.6 million mice were used worldwide for the production of mAbs each year, at that time, 40% of them in the USA (13). Other reports estimate the use in the USA to be at least 0.5 million (9) to 1 million (Kimbrell, A. Petition requesting the National Institutes of Health to prohibit the use of animals and implement non-animal alternatives in the production and use of monoclonal antibodies. 23 April, 1997). In the UK, the use of animals for ascites production fell from 46,188 to 2,391 in the period 1990 to 1994 (14).

With *in vivo* ascites production, the mice are primed, preferably by intraperitoneal (i.p.) inoculation with pristane (6). The effect of the primer is two-fold: it suppresses the immune system so that the growth of the hybridoma cells in the abdominal cavity is not (strongly) impaired; and it causes toxic irritation, which leads to peritonitis and the secretion of serous fluid (15). A hybridoma cell suspension is injected (i.p.) 7–10 days later. After 7–21 days, the abdomen of the inoculated animal becomes swollen, indicating ascites production and tumour growth. The ascites fluid is removed and the animals can be re-tapped two days later.

Some modifications of the procedure have been reported to optimise or modify ascites production. These include the use of Freunds incomplete adjuvant (FIA) as an alternative to pristane (16), the use of nude and severely compromised immunodeficient (SCID) mice (17), or the use of Balb/c-derived cross-bred F_1 hybrids (18).

In vivo mAb production has a number of advantages and disadvantages, which are summarised in Table I. The characteristics of this production method are shown in Table II.

Traditionally, *in vivo* production is favoured, amongst others due to an inherit resistance to, or lack of familiarity with, *in vitro* methods (for example, 24–26). This cannot be entirely valid, since an essential part of the production of all mAbs is the cloning and subcloning of hybridoma cell lines of potential interest, by using *in vitro* culture methods.

Generally, it is accepted that ascites production involves some (27) to substantial (28)

Table I: Advantages and disadvantages of *in vivo* monoclonal antibody production

Advantages	Disadvantages
High monoclonal antibody concentration per ml ascites (1–28mg/ml)	Contamination of preparation with endogenous mouse immunoglobulins
Ease of the procedure, not requiring specific skills	Variability in the capacity of hybridomas to elicit ascites (19, 20)
Not labour intensive	Generally lower immunoreactivity (60–70%) compared to monoclonal antibodies produced *in vitro* (90–95%; 5)
Rapid production	Potential for change of DNA in the hybridoma (21)
Relatively low costs of production	Reduced specificity due to serum contaminants (22, 23)
Most (but not all) hybridoma cell lines grow after intraperitoneal administration	Need for animal facilities
Animal facilities are often available in the laboratory	Inter-animal and inter-batch differences in quality of monoclonal antibodies
	Animal welfare aspects

animal pain and suffering, as caused by the various procedures: i.p. injection of primer (pristane or FIA), the effect of the primer after administration; the i.p. inoculation of hybridoma cells; the growth of the tumour cells in the abdominal cavity (primary effects) as solid peritoneal plaques that infiltrate the abdomen wall and/or abdominal organs; and the tapping of the animal. An overview of the clinical, pathophysiological and pathological effects is presented in Table III.

More specifically, i.p. injection, as classified according to the Dutch statistics, is believed to cause moderate distress. Ascites and tumour growth are known to cause suffering in human patients (15) and, in particular, the complex pathophysiological and pathological changes may cause severe fear, distress, pain and suffering in the animals involved. Although ascites tapping is believed to give relief, the immobilisation of the animal and the abdominal clamping create distress.

In Vitro Production

Although *in vitro* procedures for mAb production have been available for many years, the use of these procedures has been limited, due to their inadequacy and/or high costs. Traditionally, *in vitro* methods were expen-

Table II: Characteristics of monoclonal antibody production by induction of ascites

Characteristic	Specification
Scale	< 200mg/mouse
Volume	1–10ml/mouse
Concentration	1–28mg/ml
Production time	2–4 weeks
Cost	0.5–12 US$/mg

Table III: Clinical, pathophysiological and pathological effects of ascites production

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

From reference Kimbrell, A. Petition requesting the National Institutes of Health to prohibit the use of animals and implement non-animal alternatives in the production and use of monoclonal antibodies. 23 April, 1997.

sive to set up, produced low concentrations of antibodies, and were labour intensive. In recent years, however, *in vitro* methods have been substantially improved, even for small-scale production.

Ideally, an *in vitro* system developed to replace an *in vivo* method should be simple, should be equally or less expensive, should not require special culture conditions, should produce high concentrations of mAb/ml, should be free of contamination, should be re-usable, and should require a reasonable period of time to produce relatively pure mAbs in adequate quantities (29, 30).

Currently, there are no *in vitro* techniques that meet all these criteria, but, depending on the quality and quantity of mAbs needed, investigators can now select a simple, easy to handle, small-scale production system or a technically complex, large-scale production system.

Since the *in vitro* systems are still being optimised, any overview of their limitations reflects upon state-of-the-art technologies that are not yet widely used. The general advantages and disadvantages of the *in vitro* systems are summarised in Table IV. However, because of the wide range of *in vitro* methods, the comments made do not necessarily apply to all the systems.

These methods can be categorised according to the principles underlying their culture systems (5). There are static and suspension culture systems, membrane-based and matrix-based culture systems, and the high cell density bioreactor. Bioreactors include all culture systems which are capable of generating cell densities greater than 1×10^8 cells/ml. In the matrix/membrane systems, cells are cultured in compartments separated from the nutrient supply by perfusion membranes, or cells are immobilised on matrices and are perfused continuously with fresh medium. *In vitro* methods can also be categorised as small-scale (< 0.1g), medium-scale (0.1–0.5g) and large-scale (> 0.5g) production methods.

The quantities produced depend on different variables, for example, production capacity per cell, cell density in culture, production volume, and opportunities for replenishment of the medium. The pros and cons of employing *in vitro* methods vary from system to system.

The static and agitated suspension cultures (i.e. T-flasks, roller bottles, spinner flasks) are inexpensive and easy to handle. In these systems, medium and cells are in the same compartment and generally there is no (continuous) replenishment of the medium. In closed systems, there is no microbial contamination. The production time is relatively short. Even so, these systems produce mAb at low concentrations and the product is

Table IV: Advantages and disadvantages of *in vitro* methods

Advantages	Disadvantages
Low variation in quality between batches of monoclonal antibodies	Low level of monoclonal antibodies/ml requiring concentration[a]
No contamination with murine immunoglobulins	Introduction of *in vitro* systems might require a substantial capital outlay[b] and expertise
No contamination with murine pathogens	Use of fetal calf serum[c]
No animal suffering	Hybridoma cells might be damaged by shear forces[d]
High immunoreactivity (compared with *in vivo*-produced monoclonal antibodies)	

[a]*Monoclonal antibody concentration in hollow fibre systems is comparable to monoclonal antibody concentration in ascites.*

[b]*Depending on the type of in vitro systems. Small-scale production units are cheap, whereas hollow fibre systems require a substantial capital outlay.*

[c]*Medium containing fetal calf serum is being increasingly replaced by serum-free medium.*

[d]*Only where stir growth systems are being used. By cell encapsulation in polymers, cells can partly be protected (24).*

impure. The supernatant fluid has to be purified and concentrated before use. There is also a risk of product decomposition by enzymatic processes.

Recent modifications of the static and suspension cultures (i.e. the gas-permeable bags [24] and super-spinner flasks [31]) have increased mAb yields.

Membrane-based and matrix-based culture systems, such as the dialysis membrane technique (20), are also easy to handle and do not require expensive equipment. Low amounts of medium are required, so low costs for medium and serum supplements (for example, fetal calf serum [FCS]) are incurred. Unlike suspension cultures, these cultures show a high level of mAb purity, but the disadvantages are far more numerous. The potential for microbial contamination is high. No samples can be collected while the cells are being cultured, so optimisation of the technique is more complicated. The desired product can be decomposed by the enzymatic processes that are part of the system. The membrane-based and matrix-based culture systems produce relatively low mAb concentrations (27–400g/ml), although, with some modifications (for example, the Glass Mouse and Tecno-Mouse), they can yield higher cell densities. A combination of the dialysis technique and the roller bottle is the oscillating bubble chamber (32).

The high cell density (heterogeneous) bioreactors category includes all culture systems which are capable of generating cell densities greater than 1×10^8 cells/ml (5, 33). In these systems, cells, or in some cases mAbs, are physically retained in the system. An example of this system is the hollow fibre bioreactor. Medium passes through bundles of hollow fibres, enabling the cell growth compartment to be perfused continuously. Hollow fibre bioreactors are universally the first choice if large or medium quantities of mAbs are needed. The advantages of these systems include high yield, high purity and high concentration (0.6–20mg/ml) of mAbs. Purity can be improved by gradual serum reduction in

the culture media. Sustained production can be continued for a long time. Frequent harvesting decreases the potential for degradation resulting from prolonged exposure to cellular proteases in the culture media. There is a risk of microbial contamination and mechanistic failure. The system is relatively expensive because of the capital investment costs and the use of disposables, and a high level of expertise is also required. Other examples of a high cell density bioreactors include the (mini-)fermentor (30), ceramic matrix systems, and systems based on microencapsulation.

A detailed overview of the various mAb production methods will be published in late 1998/early 1999, as a special issue of *Research in Immunology*.

In Vivo Versus *In Vitro* Production

Various systems for the production of mAbs have been compared in a number of papers, according to a number of parameters, including the concentration of mAb/ml, the total production volume, the production time, and the costs per mg. Table V summarises this information. In Table V, concentrations of mAbs/ml are hybridoma-related and affected by medium composition, gassing, etc. In a single system, different mAb concentrations for one hybridoma can be found (34). The cost calculations are based on various assumptions, as some authors base ascites production costs on the cost price of the animals, while others include labour costs and the overheads

Table V: Comparison of *in vivo* and *in vitro* systems for the production of monoclonal antibodies

Production system	Process mode	Cell density	Volume (ml)	Concentration (μg/ml)	Scale (mg)	Production time (weeks)	Cost/mg (US$)
Ascites	Batch		1–10	1,000–28,000	< 250	2–4	0.5 –12
Roller bottle	Batch	Low	150–2,000	40–220	20–120	2–3	1–15
Dialysis tubing	Batch	Low	10–25	27–400	< 50	2–5	2.5–40
Spinner flask	Batch	Low	1,000	10–200	50	2–3	1–20
Ceramic matrix	Continuous	High	3,000	30–50	10–30	3–12	[a]
Hollow fibre	Continuous	High	25–1,000	600–20,000	100–500	3–12	0.75–20
Fermentor	Continuous	High	< 2,000	500–5,000	2–100	2–12	0.75–5

The figures are based on information included in references 15, 31 and 34–41.

[a]*No information available.*

of the animal facility. The same holds true for the *in vitro* systems.

The investments that need to be made for mAb production vary significantly, depending on the technique chosen, and on whether some of the equipment and other necessities are already available. Apart from the need for basic equipment for cell culture laboratories, such as a flow cabinet and an incubator (which must be available anyway for subcloning of hybridomas), other investments must be made to set up an *in vitro* system. These can vary between $25,000 and $40,000 for a central unit for *in vitro* production (36). Equipment for purification and concentration can result in additional costs.

The development and use of *in vitro* production methods has brought about some shifting trends with regard to methods, costs and animal welfare. In small-scale, easy to use systems such as T-flasks and roller bottles, the limiting factor for replacing *in vivo* methods is largely due to the fact that mAb concentrations are lower than when they are produced by the ascites method. The *in vitro* product therefore requires more concentration before use than do *in vivo* mAbs. This factor has driven the development of small-scale systems that optimise mAb concentrations (for example, gas-permeable bags, oscillating bubble chambers). These techniques have only recently become available and are not yet routinely used. On a larger scale, high cell density bioreactors (for example, hollow fibre systems) and mini-fermentors provide the same concentration levels as the ascites production method.

Previously, *in vivo* ascites production was clearly more cost-effective than *in vitro* production. As animal studies become more expensive because of animal welfare regulations and quality assurance policies, the gap is closing (Table VI). The cost of *in vitro* production methods is now becoming relatively low (for example, as costs for equipment decrease) in comparison.

Any *in vitro* versus *in vivo* policy must take into account the fact that some hybridoma cell lines do not adapt well to *in vitro* conditions, especially under serum-free culture conditions. Cell lines that grow poorly make *in vitro* mAb production impossible. Even so, poor growth of hybridoma cells in culture is becoming the exception rather than the rule. With the addition of serum or a serum-replacing additive, virtually all hybridomas can be prompted to grow well. About 8% (42) to 1% (W. de Leeuw, personal communication) of the hybridomas made are problematic, mostly due to techni-

Table VI: Summary of costs for monoclonal antibody production by ascites systems versus *in vitro* systems

Ascites production	***In vitro* production**
Animals	Equipment for culture and production
Housing (specific pathogen-free conditions)	Materials, including medium, fetal calf serum and disposables
Disposables	Labour
Labour (especially daily observations)	Downstream processing to concentrate and (in some systems) to purify monoclonal antibodies
Protocol review by the local Animal Care and Use Committee or external review committee	
Downstream processing to purify monoclonal antibodies	

cal problems (for example, contamination, the effects of shear forces). The initial selection and subcloning of hybridoma cells is by *in vitro* culture, so these cells must have some potential for growing *in vitro* (B. de Geus, personal communication).

Additionally, there are, albeit rarely, some hybridoma cell lines which fail to grow *in vivo* after i.p. inoculation.

In vitro culture requires the use of FCS and thereby introduces another moral problem. Obviously, the harvesting of FCS raises animal welfare concerns (43), but, in comparison, ascites production is among the most stressful procedures conducted in laboratory animals. The animal welfare issues are of a different order (both qualitatively and quantitatively), and involve inducing both tumour growth and ascites production. Furthermore, FCS is increasingly being replaced by FCS serum-replacing additives or the use of serum-free media, partly because one of the major costs of mAb production *in vitro* is that of FCS-containing media (44).

Some publications reported a difference in the quality of mAbs produced *in vitro* compared with those produced *in vivo* (45). In general, however, the quality of mAbs is primarily to do with the glycosylation structure of the mAb. Glycosylation can be influenced by a variety of factors, such as the culture conditions and supplements in the medium (46). These factors can be standardised in *in vitro* culture, but not in *in vivo* culture (5, 47). For this reason, differences can be found in mAbs produced in different animals, but not in a mAb batch produced after *in vitro* culture. Greater consistency is desirable, especially in the production of therapeutics.

New Developments

Several new approaches which escape from the traditional procedure of mAb production are being studied or are already in use. Ultimately, these methods will replace the use of laboratory animals altogether, including those used for immunisation purposes. A promising approach is phage-display technology. This technique permits the selection of antibody fragments, which are expressed on the surface of filamentous phage particles, from large libraries constructed from the B-cells of individuals or assembled *in vitro* from the genetic elements encoding antibodies (48, 49). The procedure is extremely rapid and is independent of the immunogenicity of the target antigen.

Other recombinant DNA-based technologies are based on mAb production by the introduction of genes encoding for the heavy and light antibody chains into, for example, plants (50), insect cells (51), bacteria (52) or yeast (53).

Legislative Framework

In several European countries, developments in *in vitro* technology have resulted in the establishment of guidelines on *in vivo* ascites production. A summary is given in Table VII. The guidelines differ from country to country. Recently, the UK government implemented a policy leading to the eventual prohibition of use of the ascites production method (54).

In the US, the National Institutes of Health issued a very clear directive to the Institutional Animal Care and Use Committees, requiring the use of *in vitro* methods (55). The agency stated that: "the validity and reliability of alternative methods for the production of mAbs are well-established", and that "these methods should be used whenever scientifically appropriate" (56).

In general, the European guidelines refer to the national laws on animal experimentation, as well as to the European laws on animal experimentation, namely *Directive 86/609/EEC* (57) of the European Union (EU) and the European Convention of the Council of Europe (58). The Directive is legally binding on the 15 Member States of the EU. Article 2 of the Directive covers any use of an animal for experimental or other scientific purposes which may cause pain, etc., while Article 3 applies to the use of animals for other purposes, including the manufacture of drugs and other substances or products. Thus, *Directive 86/609/EEC* applies unequivocally to all use of live animals in the production of mAbs, whether the antibodies are intended for use as research tools, for diagnostic assays, or for therapeutic purposes (D. Straughan, unpublished document).

Article 7 of the Directive is of direct relevance to the discussion on mAb production, since it includes the following measures.

Table VII: Guidelines in European countries on *in vivo* monoclonal antibody production

Country	Year	Type of guideline
Germany	1989	*In vivo* production only permitted for (59): — monoclonal antibodies for diagnostic and therapeutic purposes — passage of infected hybridoma cells — monoclonal antibodies for new scientific problems
The Netherlands	1989	*In vivo* production is only permitted with< 10 animals (60)
	1996	Prohibition of the use of animals Exemptions to be approved by a national committee of experts[a]
Sweden	1990	*In vivo* production can be justified in certain cases, to be decided by the local animal ethics committees (61)
Switzerland	1994	*In vivo* production only permitted for (28): — monoclonal antibodies for diagnostic and therapeutic purposes in case of emergencies — to rescue single hybridomas
UK	1991	*In vivo* production only permitted using < 20 animals per hybridoma (62)
	1998	Prohibition of the use of animals Exceptions only possible if *in vitro* production fails or for specific diagnostic or therapeutic purposes (54)

a Official letter of the Veterinary Public Health Inspectorate. VHI/D/U/-967/jw dated 5 January 1996.

— Article 7.2: "An experiment shall not be performed if another scientifically satisfactory method of obtaining the result sought, not entailing the use of animals, is reasonably and practically available."

— Article 7.3: "In a choice between experiments, those (should be selected) 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."

— Article 7.4: "All experiments shall be designed to avoid distress and unnecessary pain and suffering to experimental animals."

From the outcomes of the recent conferences, workshops (Appendix 1) and reports on *in vitro* and *in vivo* mAb production, it can be concluded that:

1. *in vitro* production systems produce mAb of the same quality as mAbs produced *in vivo* (i.e. they are scientifically satisfactory); and
2. *in vitro* production systems are now available, which produce mAbs as cost-effectively and labour-effectively as *in vivo* production (i.e. they are reasonably and practicably available).

Therefore, it is concluded that Article 7.2 of the Directive should now be applied to mAb production in laboratory animals.

Occasionally, hybridomas might not be produced *in vitro* or might not grow *in vitro* (just as, occasionally, hybridomas do not

grow *in vivo*). In such cases, and for other scientifically justified reasons, exemptions to the general prohibition may be granted. Experience in The Netherlands has shown that, since *in vivo* production was prohibited, on 1 January 1996, nine requests for ascites production have been made to the national committee of experts. No information is available on the total number of mAbs produced in The Netherlands, but this number must be substantial. Six applications were approved based on the following arguments: a therapeutic mAb with existing regulatory approval (one application); poor growth or production *in vitro* (three applications); differences in quality between *in vivo* and *in vitro* mAbs (one application); and an exceptional diagnostic emergency (one application). Three applications were rejected and the applicants were advised to optimise their culture and/or purification process (W. de Leeuw, personal communication).

The establishment in The Netherlands of institute-related or university-related core facilities on *in vitro* mAb production, which started early in the 1990s, has been very helpful in the shift from *in vivo* to *in vitro* production.

Statement

Since alternatives to the *in vivo* production of mAbs (both large-scale and small-scale) are available and experts agree on their scientific validity and practicality, the use of animals for this purpose should be prohibited, with reference to Article 7.2 of *Directive 86/609/EEC*. As the responsible authority, the European Commission should be requested to take action toward the prohibition of ascites production in the Member States of the EU. A transitional period of no more than two years should be allowed, to enable users time to acquire and implement *in vitro* techniques, and for administrative reasons, before such a ban is implemented. The establishment of core facilities should be supported.

As in rare circumstances there might be very good scientific reasons for *in vivo* production, exceptions for *in vivo* production should be permitted on a case-by-case basis, and criteria should be developed for the consideration of applications. It is recommended that an EU expert group should be established to develop such a set of criteria, to examine applications for *in vivo* production and to monitor the implementation of the prohibition in the EU. National expert groups should be established for the approval of *in vivo* ascites applications. To keep expertise in ascites production at a high level, it is recommended that *in vivo* production be centralised, and that only one or two laboratories in each Member State should be permitted to perform production by the ascites method.

The success of a prohibition partly depends on the efficient distribution and communication of information to individual scientists, ethics committees, and national and international organisations. European and national scientific associations, such as the Federation of European Laboratory Animal Science Associations, as well as international umbrella organisations from the pharmaceutical industry (for example, European Federation of Pharmaceutical Industries Associations), should be asked for their support. An important ramification of a ban is that a European scientist might by-pass the regulations by subcontracting mAb production to non-European countries. Hence, harmonisation of legislation on mAb production and of institutional policies should be sought worldwide.

Appendix 2 summarises some of the general objections to a prohibition of ascites production. The conclusion is that these objections are neither relevant nor scientifically justified.

Summary of Conclusions and Recommendations

1. Monoclonal antibodies are antibodies of defined specificity, produced by a single clone of B-lymphocytes.
2. Monoclonal antibodies are important tools both in laboratories and in clinics.
3. Most mAbs are produced in small quantities (< 0.1g) by research scientists. Larger amounts are often required for diagnostic kits and reagents and for therapeutic and prophylactic purposes.
4. The classical process of mAb production includes two separate procedures in laboratory animals: a) immunisation of donor animals to generate B-cell clones;

and b) production of mAbs by ascites induction.

5. Currently, no *in vitro* alternatives are available for immunisation. However, there are possibilities for refinement (adjuvant, route of immunisation, etc.).
6. Ascites induction is based on initial priming, followed by i.p. administration of hybridoma cells. Large numbers of animals are being used for this purpose worldwide.
7. Ascites production leads to complex clinical, pathophysiological and pathological changes, and is believed to cause substantial pain and suffering in the animals used.
8. EU *Directive 86/609/EEC* applies unequivocally (Article 3) to mAb production, irrespective of whether mAbs are used as research tools, for diagnostic assays, or for therapeutic or prophylactic purposes.
9. Nowadays, *in vitro* production systems are available, for both small-scale and large-scale production, which are equally or almost equally as cost-effective as ascites production. In addition, mAbs produced *in vitro* are of equal quality to mAbs produced *in vivo*.
10. Only a limited number of hybridoma cell lines fail to grow or produce *in vitro,* generally because of technical failures or cell culture problems.
11. Experience in some of the EU Member States that have banned ascites production shows that this does not impede the quality of research.
12. Based on points 9, 10 and 11, it is concluded that Article 7.2 of *Directive 86/609/EEC* is applicable to ascites production: "An experiment shall not be performed, if another scientifically satisfactory method of obtaining the result sought, not entailing the use of animals, is reasonably and practicably available." Therefore, a policy to prohibit *in vivo* mAb production in the EU Member States should be endorsed. A transitional period of two years should be allowed.
13. *In vivo* ascites production should only be permitted in exceptional cases and under the following conditions: full documentation and verification of efforts in cases where *in vitro* production is claimed to have failed; expertise in *in vitro* culture of the research group; approval by an expert committee, either at a national or an international level; if approval is given, it should be only for *in vivo* production in an assigned laboratory having experience with *in vivo* production; specification in the statistics on the use of animals.
14. A number of steps should be taken to try to improve expertise in *in vitro* mAb production, i.e. the establishment and financial support of core facilities to centralise expertise and the organisation of training courses in *in vitro* production techniques.
15. Activities should be undertaken (for example, with the US counterparts) to seek harmonisation in legislation and regulation with regard to mAb production, as a matter of urgency.

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References

1. Köhler, G. & Milstein, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature, London* **256**, 495–497.
2. Asai, D.J. & Wilder, J.K. (1993). Making monoclonal antibodies. *Methods in Cell Biology* **37**, 57–74.
3. Anon. (1998). Rabies vaccine inactivated for veterinary use, Monograph No. 0451. *European Pharmacopoeia*, Suppl., 445–447. Strasbourg: Council of Europe.
4. Lindl, T. (1995). Entwicklung humaner monoklonaler Antikörper. (Development of human monoclonal antibodies.) *Alternativen zu Tierexperimenten* **12**, 13–23.
5. Marx, U., Embleton, M.J., Fischer, R., Gruber, F.P., Hansson, U., Heuer, J., de Leeuw, W.A., Logtenberg, T., Merz, W., Portelle, D., Romette, J-L. & Straughan, D.W. (1997). Monoclonal anti-

body production. The report and recommendations of ECVAM workshop 23. *ATLA* **25**, 121–137.
6. Johnson, D.R. (1995). Murine monoclonal antibody development. In *Methods in Molecular Biology, Vol. 51, Antibody Engineering Protocols* (ed. S. Paul), pp. 123–137. Totowa, NJ, USA: Humana Press.
7. Lagace, J & Brodeur, B.R. (1985). Parameters affecting *in vitro* immunization of human lymphocytes. *Journal of Immunological Methods* **85**, 127–136.
8. Borrebaeck, C.A.K., Daniellson, L. & Moller, S.A. (1988). Human monoclonal antibodies produced by primary *in vitro* immunization of peripheral blood lymphocytes. *Proceedings of the National Academy of Sciences USA* **85**, 3995–3999.
9. Lewin, D.I. (1997). Animal Welfare Group seeks ban on mAbs from mouse ascites. *The Journal of NIH Research* **9**, 22–23.
10. Ho, M-K., Rand, N., Murray, J., Kato, K. & Rubin, H. (1985). *In vitro* immunization of human lymphocytes. I. Production of human monoclonal antibodies against Bombesin and Tetanus toxoid. *Journal of Immunological Methods* **135**, 3831–3838.
11. Leenaars, P.P.A.M., Koedam, M.A., Wester, P.W., Baumans, V., Claassen, E. & Hendriksen, C.F.M. (1998). Assessment of side-effects induced by injection of different adjuvant/antigen combinations in rabbits and mice. *Laboratory Animals*, *in press*.
12. Abrams, P.G., Ochs, J.J., Giardina, S.L., Morgan, A.C., Wilburn, S.B., Wilt, A.R., Oldham, R.K. & Foon, K.A. (1984). Production of large quantities of human immunoglobulin in the ascites of athymic mice: implications for the development of anti-human idiotype monoclonal antibodies. *Journal of Immunology* **132**, 1611–1613.
13. Goettel-Connolly, C. (1997). Alternatives in monoclonal antibody production workshop. *Animal Welfare Information Center Newsletter* **8**, 21.
14. Anon (1997). Antibody "cruelty" to be phased out. *New Scientist*, **2108**, 12.
15. Kuhlmann, I., Kurth, W. & Ruhdel, I. (1989). Monoclonal antibodies: *in vivo* and *in vitro* production on a laboratory scale, with consideration of the legal aspects of animal protection. *ATLA* **17**, 73–82.
16. Gillette, R.W. (1987). Alternatives to pristane priming for ascites fluid and monoclonal antibody production. *Journal of Immunological Methods* **99**, 21–23.
17. Pistillo, M.P., Sguerso, V. & Ferrara, G.B. (1992). High yields of anti-HLA human monoclonal antibodies can be provided by SCID mice. *Human Immunology* **35**, 256–259.
18. Stewart, F., Callander, A. & Garwes, D.J. (1989). Comparison of ascites production for monoclonal antibodies in Balb/c and Balb/c derived cross-bred mice. *Journal of Immunological Methods* **119**, 269–275.
19. Marx, U. & Merz, W. (1995). *In vivo* and *in vitro* production of monoclonal antibodies. Bioreactors versus immune ascites. In *Methods in Molecular Biology, Vol 45, Monoclonal Antibody Protocols* (ed. W.C. Davis), pp. 169–176. Totowa, NJ, USA: Humana Press.
20. Sjörgren-Jansson, E., Ohlin, M., Borrebaeck, C.A.K. & Jeanson, S. (1991). Production of human monoclonal antibodies in dialysis tubing. *Hybridoma* **10**, 411–419.
21. Fromer, M.J. (1997). NIH denies petition to ban *in vivo* mAb production; lawsuit threatened. *Oncology Times* **19**, 37–40.
22. Spicer, S.S., Spivey, M.A., Ito, M. & Schulte, B.A. (1994). Some ascites monoclonal antibody preparations contain contaminants that bind to selected Golgi zones and mast cells. *Journal of Histochemistry and Cytochemistry* **42**, 213–221.
23. Appelmelk, B.J., Verweij-Van Vught, A.M., Maaskant, J.J., Thijs, L.G. & MacLaren, D.M. (1992). Murine ascites fluids contain varying amounts of an inhibitor that interferes with complement-mediated effector functions of monoclonal antibodies. *Immunological Letters* **33**, 135–138.
24. McArdle, J. (1997). Alternatives to ascites production of monoclonal antibodies. *Animal Welfare Information Center Newsletter* **8**, 1–18.
25. Shalev, M. (1998). European and US regulation of monoclonal antibodies. *Laboratory Animal* **27**, 15–17.
26. Anon. (1998). Animal Research Review Panel: Monoclonal Antibodies. New South Wales Government, 6pp. Sydney: ANSW Agriculture.
27. Matfield, M. (1997). ECVAM report: monoclonal AB production. *EBRA Bulletin*, **August 1997**, 9.
28. Anon. (1994). Richtlinien zur Herstellung von monoklonalen Antikörpern. (Guidelines for the production of monoclonal antibodies.) *Richtlinie Tierschutz 5.01*, Liebefeld-Bern: Bundesamt für Veterinärwesen.
29. Falkenberg, F.W., Hengelage, T., Krane, M., Bartels, I., Albrecht, A., Holtmeier, N. & Wüthrich, M. (1993). A simple and inexpensive high density dialysis tubing cell culture system for the *in vitro* production of monoclonal antibodies in high concentrations. *Journal of Immunological Methods* **165**, 193–206.
30. Falkenberg, F.W., Weichert, H., Krane, M., Bartels, I., Palme, M., Nagels, H.O. & Fiebig, H. (1995). *In vitro* production of monoclonal antibodies in high concentrations in a new and easy to handle modular minifermentor. *Journal of Immunological Methods* **179**, 13–29.
31. Heidemann, R., Riese, U., Lütkemeyer, D., Büntemeyer, H. & Lehmann, J. (1994). The superspinner: a low cost animal cell culture bioreactor for the CO_2 incubator. *Cytotechnology* **14**, 1–9.
32. Pannell, R. & Milstein, C. (1992). An oscillating bubble chamber for laboratory scale production of monoclonal antibodies as an alternative to ascites tumours. *Journal of Immunological Methods* **146**, 43–48.
33. Jackson, L.R., Trudel, L.J., Fox, J.G. & Lipman, N.S. (1996). Evaluation of hollow fibre bioreactors as an alternative to murine ascites production for small scale monoclonal antibody production. *Journal of Immunological Methods* **189**, 217–231.
34. Pesson, B. & Emborg, C. (1992). A comparison of three different mammalian cell bioreactors for the production of monoclonal antibodies. *Bioprocess Engineering* **8**, 157–163.
35. Fischer, R.W. & Ferber, P.C. (1992). Monoklonale Antikörper: *In vitro*-Produktionsmethoden im Vergleich. (Monoclonal antibodies: comparison of *in vitro* methods.) *Alternativen zu Tierexperimenten* **16**, 15–24.
36. Van der Kamp, M. & de Leeuw, W. (1996). Short

review of *in vitro* production methods for monoclonal antibodies. *NCA Newsletter* **3**, 10–11.
37. Van der Velden-de Groot, C.A.M. (1990). Monoclonale antistoffen. Produktie *in vivo* oder *in vitro*. (Production of monoclonal antibodies. Production *in vivo* or *in vitro*.) *Biotechniek* **29**, 73–74.
38. Kurkela, R., Fraune, E. & Vihko, P. (1993). Pilot-scale production of murine monoclonal antibodies in agitated, ceramic-matrix of hollow-fiber cell culture systems. *BioTechniques* **15**, 674–683.
39. Chirbik, R.J., Rosen, S.M., Trunfio, D.M., Fischberg-Bender, E.W. & Palmer, S.M. (1996). Factors affecting antibody production efficiency in hollow-fiber bioreactors. *IVD Technology Magazine*. Web site: http://www.devicelink.com/ivdt/archive/96/07/007.html.
40. Lowrey, D., Murphy, S. & Goffe, R.A. (1994). A comparison of monoclonal antibody productivity in different hollow fibre bioreactors. *Journal of Biotechnology* **36**, 35–38.
41. Anon. (1997). "Alternatives in Monoclonal Antibody Production" a workshop of The John Hopkins Center for Alternatives to Animal Testing and The Office for Protection from Research Risks, National Institute of Health, Baltimore, MD, USA, 24–25 September 1997. Web site: http://www.sph.jhu.edu/~altweb/science/meetings/mab/proceedings.htm.
42. Kagan, E. Vieira, E. & Petrie, H.T. (1997). Comparison of hollow fiber bioreactors and modular minifermentors for the production of large numbers of monoclonal antibodies *in vitro*. In "Alternatives in Monoclonal Antibody Production", a workshop of The John Hopkins Center for Alternatives to Animal testing and The Office for Protection from Research Risks, National Institutes of Health, Baltimore, MD, 24–25 September. Web site: http://www.sph.jhu.edu/~altweb/science/meetings/mab/ proceedings.htm.
43. Jochems, C. (1997). *Use, Trade and Harvest of Livestock Sera*. Thesis, Utrecht University.
44. Fike, R.M., Jayme, D.W. & Weiss, S.A. (1991). Monoclonal antibody enhancement in protein-free and serum-supplemented hybridoma culture media. *American Biotechnology Laboratory* **9**, 40–42.
45. Goodall, M. (1997). Production of monoclonal antibodies: *in vivo* versus *in vitro* methods. In *Animal Alternatives, Welfare & Ethics* (ed. L.F.M. van Zutphen & M. Balls), pp. 965–972. Amsterdam: Elsevier.
46. Patel, P.T., Parekh, R.B., Moellering, B.J. & Prior, C.P. (1992). Different culture methods lead to differences in glycosylation of a murine IgG monoclonal antibody. *Biochemical Journal* **285**, 839–845
47. Stoll, T., Ruffleux, P-A., Lüllau, E., von Stockar, U. & Marison, I.W. (1995). Production of immunoglobulin A in different reactor configurations. *Cytotechnology* **11**, 608–614.
48. De Kruif, J., Van der Vuurst de Vries, A.R., Cilenti, L., Boel, E., Van Ewijk, W.C. & Logtenberg, T. (1996). New perspectives on recombinant human antibodies. *Immunology Today* **17**, 453–455.
49. Devlin, J.J., Panganiban, L.C. & Devlin, P.E. (1990). Random peptide libraries: a source of specific protein binding molecules. *Science, New York* **249**, 404–406.
50. Ma, J.K-C., Hiatt, A., Hein, M., Vine, N.D., Wang, F., Stabila, P., van Dolleweerd, C., Mostov, K. & Lehner, T. (1995). Generation and assembly of secretory antibodies in plants. *Science, New York* **268**, 716.
51. Carayannopoulos, L., Max, E.E. & Capra, J.D. (1994). Recombinant human IgA expressed in insect cells. *Proceedings of the National Academy of Sciences USA* **91**, 8348–8352.
52. Plueckthun, A. (1992). Mono and bivalent antibody fragments produced in *Escherichia coli*: engineering, folding and antigen binding. *Immunological Reviews* **130**, 151–188.
53. Eldin, P., Pauza, M.E., Hieda, Y., Lin, G., Murtaugh, M.P., Pentel, P.R. & Pennell, C.A. (1997). High level secretion of two antibody single chain Fv fragments by *Pichia pastoris*. *Journal of Immunological Methods* **201**, 67–75.
54. Anon. (1997). *Supplementary Note to the Home Secretary's response to the Animal Procedures Committee Interim Report on the Review of the Operation of the Animals (Scientific Procedure) Act 1986*, 6pp. London: HMSO.
55. Ellis, G.B. & Garnett, N.L. (1997). Production of monoclonal antibodies using mouse ascites method. *Animal Welfare Information Center Newsletter* **8**, 19.
56. Schulhof, J. & Lamborn, C. (1998). Jumping from the Banned Wagon. *Laboratory Animals* **27**, 9.
57. Anon (1986). Council Directive 86/609/EEC of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes. *Official Journal of the European Communities* **L358**, 1–29.
58. Anon. (1986). *European Convention for the Protection of Vertebrate Animals Used for Experimental and Scientific Purposes*, 51 pp. Strasbourg: Council of Europe.
59. Anon. (1993). *Tierschutzbericht 1993.* Drucksache 12/4242, pp. 49–50. Bonn: Bundesministerium für Ernährung, Landwirtschaft und Forsten.
60. Anon. (1989). *Code of Practice for the Production of Monoclonal Antibodies*, 6pp. Rijswijk, The Netherlands: Veterinary Public Health Inspectorate.
61. Anon. (1994). General recommendations of the National Board for Laboratory Animals on the treatment of certain matters relating to ethical reviews of the use of animals for scientific purposes (LSFS/Statute Book of the National Board of Agriculture/1990:21, Subject no. L29). In *Provisions and General Recommendations Relating to the Use of Animals for Scientific Purposes* (ed. O. Lundgren), pp. 43–46. Stockholm: Karl Olov Ösler.
62. Anon. (1992). *Report of the Animal Procedures Committee for 1991*, Appendix II, Cmnd 2048, 37pp., London: HMSO.

Appendix 1

Recommendations from Recent Congresses and Workshops

A number of congresses and workshops have been held over the last few years to evaluate the opportunity for replacement of *in vivo* ascites production by *in vitro* systems. The conclusions of these meetings are briefly summarised below.

At a national hearing held at ZEBET (National Centre for the Documentation and Evaluation of Alternatives to Animal Experiments, Berlin, Germany) in 1989, national experts evaluated the current *in vitro* methods. The consensus of opinion among the experts was that the *in vivo* production of monoclonal antibodies (mAbs) should only be permitted in the following exceptional cases: when the mAbs are intended for diagnostic and therapeutic purposes in humans, and provided that no other options are available; when hybridoma cells need to be rescued, because they have either failed to grow *in vitro* or they have become infected; and when mAbs are needed to investigate new scientific problems (1).

A symposium entitled *Monoclonal antibody production: are animals still needed?* (Bilthoven, The Netherlands, November 1995), was held to evaluate the 1989 Dutch Code of Practice for the Production of Monoclonal Antibodies (2). This Code strongly supports *in vitro* mAb production and limits the number of animals per hybridoma to 5–10. The symposium was attended by about 120 researchers, bioprocess technologists and animal welfare officers. From the discussions, it became clear that several institutes in The Netherlands have established core facilities for *in vitro* mAb production. In some institutes, ascites production had been completely replaced by *in vitro* production. There was a general consensus at the symposium about the validity of *in vitro* production systems (3). Based on this outcome, and referring to Article 7.2 of the European Union (EU) *Directive 86/609/EEC* (4), the Veterinary Public Health Inspectorate ordered a ban on ascites production, on the condition that, based on good scientific justification, exemptions could be granted (Official letter of the veterinary Public Health Inspectorate. VHI/D/U/-967/jw dated 5 January 1996). A national committee of experts was set up to evaluate protocols for approval.

The ECVAM workshop on monoclonal antibody production (Angera, Italy, 1996), attended by 12 experts, was held to review the status of various types of *in vitro* tests and to make recommendations about the best way forward (5). The main conclusions and recommendations of this workshop were as follows.

— 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.

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

— 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 (6).

— 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 two 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.

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

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

— 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 them, at the level of individual user establishments.

— Project reviews and inspection systems should be subject to approval. In countries where there is no project review system, one should be introduced. During the review of applications, advice should be sought from those with experience in *in vitro* methods.

Conclusions from the symposium, *Alternatives in Monoclonal Antibody Production*, organised by The Johns Hopkins Center for Alternatives to Animal Testing and The Office for Protection from Research Risks, National Institutes of Health (24–25 September 1997, Baltimore, MD, USA), clearly stated that *in vitro* methods for mAb production should be the accepted methods, especially since core facilities are available to provide mAbs to investigators who are uncomfortable with the *in vitro* methods or who believe mAbs could not be produced for a reasonable cost in their laboratories (7). The use of the ascites method should be the exception and should be cautiously justified. In the US, Institutional Animal Care and Use Committees are responsible for advising investigators about proper alternatives to the ascites method. If, after careful consideration, it is determined that the ascites method must be used to produce mAbs, the investigator should do everything possible to minimise stress, pain, etc.

The Second World Congress on Alternatives and Animal Use in the Life Sciences (20–24 October 1996, Utrecht, The Netherlands) included a workshop on the production of monoclonal and polyclonal antibodies. Workshop participants felt that neither scientific nor economic arguments could justify the continued use of *in vivo* ascites production, except when it is documented that *in vitro* production fails to produce the desired mAbs. In these cases, permission should be given to use animals (8).

References

1. Anon. (1993). *Tierschutzbericht 1993*. Drucksache 12/4242, pp. 49–50. Bonn: Bundesministerium für Ernährung, Landwirtschaft und Forsten.
2. Anon. (1989). *Code of Practice for the Production of Monoclonal Antibodies,* 6 pp. Rijswijk, The Netherlands: Veterinary Public Health Inspectorate.
3. Hendriksen, C., Rozing, J., Van der Kamp, M. & de Leeuw, W. (1996). The production of monoclonal antibodies: are animals still needed? *ATLA* **24**, 109–110.
4. Anon (1986). Council Directive 86/609/EEC of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes. *Official Journal of the European Communities* **L358**, 1–29.
5. Marx, U., Embleton, M.J., Fischer, R., Gruber, F.P., Hansson, U., Heuer, J., de Leeuw, W.A., Logtenberg, T., Merz, W., Portelle, D., Romette, J-L. & Straughan, D.W. (1997). Monoclonal antibody production. The report and recommendations of ECVAM workshop 23. *ATLA* **25**, 121–137.
6. Anon. (1986). *European Convention for the Protection of Vertebrate Animals Used for Experimental and Scientific Purposes*, 51 pp. Strasbourg: Council of Europe.
7. Anon. (1997). "Alternatives in Monoclonal Antibody Production" a workshop of the John Hopkins Center for Alternatives to Animal Testing and The Office for Protection from research Risks, National Institutes of Health, Baltimore, MD, USA, *24–25 September 1997*. Web site: http://www.sph.jhu.edu/~altweb/science/meetings/ mab/proceedings.htm.
8. Hendriksen, C.F.M. & Claassen, E. (1997). Polyclonal and monoclonal antibodies: synopsis of the workshop. In *Animal Alternatives, Welfare and Ethics* (ed. L.F.M. van Zutphen & M. Balls), pp. 1071–1071. Amsterdam: Elsevier.

Appendix 2

Comments on General Objections Against a Prohibition of the Use of Animals for Ascites Production

Some general issues have been raised against a prohibition of the use of ascites production:

". . . a total ban on using mouse ascites methods would impede or stop the progress of important research." (1)

Experience from those European countries which prohibit the use of animals for ascites production shows that the change from *in vivo* to *in vitro* production did not have a negative effect on biomedical research programmes. On the contrary, it is obvious that the increased knowledge and expertise in *in vitro* monoclonal antibody (mAb) technology has additional benefits to other areas of *in vitro* research, for example, vaccine production.

"Industry is comfortable with ascites production and is not likely to switch to *in vitro* methods as time and money should be devoted to products, not process." (2)

Introducing *in vitro* production technology into a laboratory requires an initial outlay for equipment, for training and for building up expertise, whereas animal facilities are already available in most laboratories. This might be an obstacle to the implementation of *in vitro* technology. However, it should be kept in mind that: a) even in the case of ascites mAb production, cell culture facilities are needed for the fusion and subcloning of hybridoma cells; b) costs for animal experiments are likely to increase in the future, while costs for *in vitro* production technology are likely to decrease; c) cell culture facilities are increasingly being established in laboratories, for different kinds of purposes, and the spin-off of *in vitro* production technology is extremely helpful in improving these facilities; and d) from a public relations point of view, *in vivo* ascites production is counterproductive.

Experience in The Netherlands has shown that economic aspects are not the main issues in the implementation of *in vitro* techniques. The pharmaceutical companies in The Netherlands were the first to replace ascites production with *in vitro* systems. Most of the mAbs produced by these companies were for bench-related activities and not for commercial purposes. Larger obstacles are the small independent research groups, often found at universities, which are biased in favour of the ease of using animals, which lack experience in cell culture techniques and cell culture facilities, or which are led by traditions and old habits within their research setting. Monoclonal antibodies are tools in experimental studies and hardly ever the objective of the study itself. Scientists quite often do not try to replace their study tools, if this is not profitable for the study itself. A way out of these situations is the establishment of core facilities. This means a win-win situation in providing investigators with specialised laboratory services and obtaining the product in a cost-effective way.

"A prohibition of ascites production in Europe can easily be by-passed by subcontracting ascites production to non-European countries."

An important ramification is that European scientists might by-pass the regulations by subcontracting ascites production to non-European countries. For example, addresses can be found on the Internet of commercial companies offering *in vivo* mAb production, even in multiple litre quantities.

Although it would be impossible to completely prevent subcontracting, some approaches have been suggested (3): a) labelling commercially available mAbs to show whether they were produced *in vivo* or *in vitro*; b) labelling ascites-produced mAbs imported into the EU to indicate their country of origin; and c) indicating in scientific journals how mAbs were produced, and including this requirement in the *Instructions to Authors*. An additional approach might be to request management boards of institutes to sign a memorandum of understanding that ascites mAbs can be used only with strong scientific justification.

It is strongly recommended that ECVAM should undertake activities (for example, with its US counterparts) to promote harmonisation in legislation and regulation with regard to mAb production.

"In vitro production techniques require more expertise and skills than ascites production. As most investigators only occasionally need mAbs and only in small amounts, it cannot be expected from these investigators to set up an *in vitro* production facility."

Firstly, it should be realised that the establishment of a new hybridoma cell line always needs cell culture facilities for selection and cloning. In addition, it can be argued that, for small-scale production, *in vitro* techniques are available which are simple to use and inexpensive. However, the best way forward is to establish centralised (core) facilities for mAb production in research laboratories. These core facilities can build up expertise and offer a wide range of techniques, for both small-scale and large-scale production. They can offer advice and be helpful in troubleshooting, and they can stay up-to-date with new developments. Experience has shown that core facilities are very cost-effective in terms of production. Their expertise in cell culture techniques has additional benefits for other, related, cell culture activities.

References

1. Agnew, B. (1997). NIH refuses to ban mouse ascites method of mAb production. *The Journal of NIH Research* **9**, 28–29.
2. Fromer, M.J. (1997). NIH denies petition to ban *in vivo* mAb production: lawsuit threatened. *Oncology Times* **19**, 37–40.
3. Marx, U., Embleton, M.J., Fischer, R., Gruber, F.P., Hansson, U., Heuer, J., de Leeuw, W.A., Logtenberg, T., Merz, W., Portelle, D., Romette, J-L. & Straughan, D.W. (1997). Monoclonal antibody production. The report and recommendations of ECVAM workshop 23. *ATLA* **25**, 121–137.