

Critical Steps in the Production of Polyclonal and Monoclonal Antibodies: Evaluation and Recommendations

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Abstract

Antibodies are valuable tools in the laboratory and clinic. Antibodies include those secreted by a single clone of B lymphocytes, termed monoclonal antibodies, and those produced by a mixture of various B lymphocyte clones, termed polyclonal antibodies. Both products have become essential instruments in fundamental immunological research, immunohistochemistry, diagnostic testing, and vaccine quality control. Antibody production requires a substantial number of animals, and the animals are subjected to a number of invasive procedures such as antigen injection and blood collection. However, by carefully designing an immunization protocol and by optimizing the immunization response, it is possible to minimize animals' pain and distress while obtaining optimal immune responses. In this article, the critical steps in the production of polyclonal and monoclonal antibodies are described, specifically including selection of the animal species and its age, injection protocol, and ascites tapping. Recommendations are provided for optimizing the immunization response.

Key Words: critical steps; guidelines; immunization protocols; monoclonal antibodies; polyclonal antibodies; production

General Introduction: Protocols for Antibody Production

Antibodies are serum immunoglobulins (Igs¹) that have binding specificity for particular antigens. Antibodies are therefore of enormous utility in applications such as experimental biology, medicine, biomedical research, diagnostic testing, and therapy. Polyclonal antibodies (PABs¹) and monoclonal antibodies (MAbs¹) can be used for these purposes, although the production of these antibodies requires the use of substantial numbers of animals with considerable animal welfare consequences. In the

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¹Abbreviations used in this article: AVMA, American Veterinary Medicine Association; FCA, Freund's complete adjuvant; FIA, Freund's incomplete adjuvant; Ig, immunoglobulin; MAb, monoclonal antibody; PAB, polyclonal antibody.

case of PABs, animals are given injections of antigen or antigen/adjuvant mixtures for the induction of effective antibody responses, and it is usually necessary to collect blood to monitor antibody response during the experiment and to obtain the antibodies. In the case of MAbs, animals are given injections of antigen or antigen/adjuvant mixtures to induce specific B cells that are obtained from the spleen or lymph nodes to establish hybridomas. When in vitro production is not feasible, it is necessary to use animals for the production of antibodies by hybridomas in the abdomen cavity.

In making a choice between producing PABs or MAbs, the desired application of the antibody and the time and money available for production should be considered. The fact that a polyclonal antiserum can be obtained within a short time (4-8 wk) with little financial investment favors its use, whereas it takes about 3 to 6 mo to produce MAbs. Many research questions can be answered by using a polyclonal antiserum. MAbs are specific for an epitope, which can be essential in specific cases. For additional information, we refer readers to the detailed comparison of PABs and MAbs elsewhere in this issue of *ILAR Journal* (Lipman et al. 2005).

In the production of polyclonal and monoclonal antibodies, a number of critical steps can be identified that may influence the outcome of the animal experiment (immunological results and the pain and suffering of the animals). The goal of this article is to evaluate critical steps in the production of these antibodies, and to provide recommendations to optimize production protocols that will ultimately result in effective antibody responses and minimal pain and suffering for the animals.

Evaluation of Critical Steps in PAB Production

The several critical steps involved in the production of PABs include the following: (1) preparation of the antigen, (2) selection of the animal species, (3) selection and preparation of the adjuvant, (4) injection protocol, (5) postinjection observation, and (6) collection of the antibodies. Each step is described in the text below.

Preparation of the Antigen

When antibodies are produced, it is important to consider antigen features, which include the quality and quantity of

the antigen and the antigen preparation. The specificity of the immune response obtained depends on the purity of the antigen applied. Minute impurities (<1%) may prove to be immunodominant (e.g., with many bacterial antigens) and may result in antibodies that have more activity against the impurity than against the antigen of interest. Purification of antigen is time consuming and laborious but is potentially very worthwhile. Purification results in an increased number of specific antibodies and the ability to avoid removing many unwanted antibodies by extensive absorption procedures (Leenaars et al. 1997).

Before proceeding with the immunization, the investigator should consider the toxicity of the antigen preparation due to, for example, contamination with endotoxins such as lipopolysaccharide or chemical residues used to inactivate the microorganism, or an extreme pH level (Hendriksen and Hau 2003). The diluents should be endotoxin free, and the pH should be adjusted within physiological limits. Upon administration, these factors are important because they may have a negative effect on the welfare of the animal as well as on the immunological results. Other clearly important factors include the working conditions during antigen preparation, which must be sterile, animal preparation, and the quality control of injection product. The quantity of injected antigen determines the immune response that is evoked. Too much or too little antigen may induce suppression, sensitization, tolerance, or other unwanted immunomodulation (Hanly et al. 1995). The antigen quantity depends on the inherent properties of the antigen, whether the antigen is purified or a component in a mixture of antigens, the animal species to be immunized, the adjuvant used, and the route and frequency of injection. In general terms, microgram to milligram quantities of protein antigen are needed in conjunction with an adjuvant to elicit high-titer serum responses in laboratory animals (Harlow and Lane 1988). As Hanly and coworkers (1995) have reported, the usual dose of a soluble protein administered with Freund's adjuvant for rabbits is in the range of 50 to 1000 µg; for mice, 10 to 200 µg; for goats and sheep, 250 to 5000 µg.

Preinjection Recommendations:

- Carefully prepare the antigen.
- Maintain quality control of the antigen (antigen/adjuvant mixture).

Selection of the Animal Species

When selecting the animal species for PAb production, it is important to consider the following: (1) the amount of PAb needed, (2) the ease of obtaining blood samples, (3) the phylogenetic relationship between the antigen and the animal species, and (4) the intended use of the PAb.

The most frequently used animal species for PAb induction in the laboratory setting are the rabbit, mouse, rat, hamster, guinea pig, goat, sheep, and chicken (Hanly et al. 1995). For the production of PAbs, rabbits are used most

often because of their convenient size, ease of handling and bleeding, relatively long life span, and adequate production of high-titer, high-affinity, precipitating antiserum (Stills 1994). When larger amounts of PAbs are needed, farm animals such as sheep, goats, and horses are usually used. In some cases requiring large amounts of PAbs, chickens may be used (Erhard et al. 2000; Schade et al. 1996). After the immunization of chickens, antibodies pass from the blood to the egg yolk. Chicken egg antibodies (IgY) can be extracted from the egg to concentrations of approximately 100 to 250 mg of IgY/egg (CCAC 2002; Erhard et al. 2000). Compared with the IgG productivity in rabbits (approximately 250 mg per bleed), more IgY can be obtained from chickens due to the continuous secretion of IgY in the eggs. We refer readers to the detailed information on IgY production elsewhere in this issue (Hau and Hendriksen 2005).

From a practical as well as an animal welfare point of view, the ease of obtaining blood samples influences the selection of an animal species. When there is no identified need for a specific animal species, the animals from which samples of blood are relatively easy to obtain should be preferred over those that are difficult to bleed. In other words, the use of rabbits should be preferred over guinea pigs. Because laying hens do not require blood supplies (because the antibody is in the yolk), the stress associated with blood collection is reduced. Nevertheless, there are disadvantages of using chickens. The production of IgY is not widespread, probably due to several characteristics that include the following: specific housing requirements, limited availability of conjugated antibodies, lack of investigators' experience with chickens and chicken antibodies, and isolation and purification problems (Schade et al. 1996).

The selection of an animal species is also influenced by the species from which the antigen is taken. The greater the phylogenetic distance between the animal species that is the source of the antigen and the species of the animal to be immunized, the better the immune response that will be evoked. In other words, antibodies to mouse-specific antigens cannot be produced easily in mice. As a result, IgY chicken PAb production might be considered because of the chicken's phylogenetic distance to mammals.

It is very important to consider the intended use of the PAb when selecting an animal species. One example is the use of PAb in an enzyme-linked immunosorbent assay. The antibody that binds to the antigen—the primary antibody—should be from an animal species that is different from the conjugated (secondary) antibody used in the next step of the assay.

The age of the animals is another important consideration because that factor can influence the outcome of the immunization. It is important to use young adults, for whom the immune response is fairly robust and not affected by previous immune challenges. The robustness of the immune response decreases with age after the period of young adulthood. When chickens are immunized, they should be of egg-laying age by the time antibody is to be harvested. Recommendations related to the age at which animals

should be used for PAb production are given in several publications (Hanly et al. 1995; Hendriksen and Hau 2003) and are summarized in Table 1.

In addition to the species, the sex of the animals must also be decided. Traditionally, female animals are used for PAb production inasmuch as these animals can be group housed more successfully than males because females are more docile and less aggressive in social interaction (Hendriksen and Hau 2003). There are, however, no overriding scientific reasons for not using male animals for PAb production. Castrated male rabbits can be group housed, although this arrangement may pose ethical problems because the animals have been castrated to facilitate the group housing.

Finally, it is important (albeit obvious) to consider the health status of animals used for the production of antibodies. Infectious agents exist that may suppress, modulate, or stimulate the immune system. The use of disease-free animals minimizes the likelihood of cross-reactivity to other antigens the animal's immune system may have encountered.

Selection and Preparation of the Adjuvant

When the antigen to which antibodies are to be evoked is poorly immunogenic, the immune system requires a stimulus to induce an effective immune response. Adjuvants can be used for this purpose, and can direct an immune response against a more cellular or humoral response (Cox and Coulter 1997). More than 100 adjuvants have been described, and several reviews on adjuvants have been published (e.g., Stewart-Tull 2000; Vogel and Powell 1995). Only a few adjuvants are routinely utilized for polyclonal antibody production. Detailed information on adjuvants used for PAb production appears in this *ILAR Journal* issue (Stills 2005). Adjuvants used for PAb production include

Freund's complete adjuvant (FCA¹), Freund's incomplete adjuvant (FIA¹), aluminum salts (e.g., Al(OH)₃, AlPO₄), Quil A, Iscoms, Montanide, TiterMax™, and RIBI™.

FCA is frequently used for the production of polyclonal antibodies because high antibody titers are induced to almost all types of antigens. However, many investigators have reported severe side effects after injection of FCA. For this reason, the available guidelines and recommendations on PAb production (e.g., CCAC 2002; Home Office 1991; NIH 1988) focus on FCA, and many universities in the United States have their own guidelines. Amyx (1987) has suggested that most undesirable effects of FCA can be eliminated by careful control of injection quantity and site selection. In an international workshop in New Orleans in 2002, it was concluded that FCA is not as problematic as a cause of pain and distress as previously suggested when injection volumes are minimized (HSUS Challenges Researchers to Reduce Animal Pain and Distress in Antibody Production: Satellite meeting held in conjunction with the Fourth World Congress on Alternatives and Animal Use in the Life Sciences in New Orleans, LA, August 11, 2002; C.F.M.H., personal communication) (<http://www.hsus.org/search.jsp>). The fact that FCA is currently less problematic may be due to its improper former use (i.e., high volumes were used at unacceptable injection sites). In addition, according to the earlier literature, the FCA that was used was much less pure and more toxic, and it therefore induced more severe inflammatory reactions (Stewart-Tull 1995). In a more recent report (Leenaars et al. 1998), correct injection of FCA was reported to have resulted in no clinical or behavioral changes.

FCA is not the only adjuvant to induce pathological changes upon administration. Severe pathological changes have been reported after administration of TiterMax and RIBI adjuvant (Hendriksen and Hau 2003). The severity of pathological changes depends not only on the adjuvant but also on the type of antigen used. Moreover, the alternative adjuvants have not often induced effective antibody responses.

Based on the factors described above, the selection of an alternative adjuvant for PAb production that induces minimal side effects and high antibody titers is not easy. When a weak immunogenic antigen is used frequently for PAb production, it may be helpful to perform a comparative study using different adjuvants combined with the antigen of interest for selecting the best adjuvant. It is important to include a pathological examination in these studies because several investigators (Johnston et al. 1991; Leenaars et al. 1998; Smith et al. 1992) have reported that although clinical and behavioral changes were not observed after injection of FCA, severe pathological changes were observed. Pathological changes are indeed unwanted. When antibodies to a weakly immunogenic antigen are to be produced only once, comparative studies are not recommended because the chances of success (high antibody levels) are very limited, and the presence of side effects cannot be excluded.

Table 1 Recommended age of animals for polyclonal antibody production^a

Animal	Age
Mice	6 wk
Rats	6 wk
Rabbits	3 mo
Guinea pigs	3 mo
Chickens	18-20 wk
Goats	6-7 mo
Sheep	7-9 mo

^aData adapted from Leenaars PPAM, Hendriksen CFM, de Leeuw WA, Carat F, Delahaut Ph, Fischer R, Halder M, Hanly WC, Hartinger J, Hau J, Lindblad EB, Nicklas W, Outschoorn IM, Stewart-Tull DES. 1999. The Production of Polyclonal Antibodies in Laboratory Animal. The Report and Recommendations of ECVAM Workshop 35. *ATLA* 27:79-102. (Available online: <http://altweb.jhsph.edu/publications/ECVAM/ecvam35.htm>).

In addition to the selection of the adjuvant, the preparation of the antigen/adjuvant mixtures may influence the outcome of the immunization experiment. With aseptic preparations, the first critical step is to minimize potential contamination, which may affect the quantity and quality of the antibodies as well as the welfare of the animal. The second step in the case of an emulsion (e.g., FCA and FIA are water-in-oil emulsions) is to check the stability and quality of the emulsion. When FCA or FIA is used, a thick emulsion is the result of mixing the antigen and adjuvant (Lindblad 2000). The advantage of a thick emulsion is that it effectively protects the antigen against a quick degradation. The antigen is instead slowly released out of this emulsion and is therefore present a relatively long time for endocytosis and antigen presentation. The result is a long-lasting adjuvant effect. Water-in-oil emulsions that are not properly prepared are ineffective as adjuvants (Hendriksen and Hau 2003). In the case of a water-in-oil emulsion, the stability and quality of the emulsion can be evaluated by placing a drop of the mixture on the surface of water. When the drop remains as a discrete white drop on or just below the surface, the emulsion is considered a stable water-in-oil emulsion. When the drop forms a cloud of tiny particles, it is an oil-in-water emulsion.

Antigen-Adjuvant Preparation Recommendations:

- Prepare mixtures aseptically.
- Carefully monitor the stability and quality of the emulsion.
- Carefully select the injection route and volume, especially when oil adjuvants are used.

Injection Protocol

The immunization protocol can be prepared after selection of the animal species and the adjuvant. This protocol, which differs according to animal species and adjuvant, includes several critical steps that depend on each selection and are described below.

Route of Injection

The choice of injection route is shaped to some extent by the choice of the animal species and adjuvant, as well as by the character, quantity, and volume of the antigen. The most frequently used routes of injection for PAB production are subcutaneous (s.c.), intradermal (i.d.), intramuscular (i.m.), intraperitoneal (i.p.), and intravenous (i.v.). Hendriksen and Hau (2003) have recently described the advantages and disadvantages of the different injection routes, and this information is summarized in Table 2.

Footpad, intrasplenic, and intra-lymph node injection are usually not necessary for routine PAB production and should be justified on a case-by-case basis. The Canadian Council on Animal Care (CCAC 2002) discourages the use of the forenamed routes of injection. Indeed, because injection into any closed space is painful, the choice of i.m. and i.d. routes warrants examination (CCAC 2002). The August

2002 international workshop participants mentioned above recommended discouraging the use of i.p., intrasplenic, and food pad injections, irrespective of the adjuvant, based on published literature and investigators' personal experience (C.F.M.H., personal communication). Oil adjuvants are best administered s.c. to utilize the depot effect. Some injection routes may be eliminated by the choice of the adjuvant. The i.v. route for water-in-oil emulsions (e.g., FCA and FIA) may be lethal, and the i.v. route is not advised because of the risk of embolism for large particulate or viscous gel adjuvants (e.g., aluminum salts) (Hanly et al. 1995). For injections of FCA, the i.p. route is not recommended for PAB production because it is known to induce inflammation, peritonitis, and behavioral changes (CCAC 2002; Griffen et al. 2003).

The choice of animal species may also eliminate some injection routes. The i.d. route is not recommended in small rodents (mouse, rat, and hamster), and the i.p. route is not recommended in larger animals (rabbit and larger) (Hendriksen and Hau 2003). Although no clinical or behavioral changes have been observed after s.c. injection of minimal volumes of FCA (0.1 mL in mice and 0.25 mL in rabbits), considerable pathological changes have been reported (Leenaars et al. 1998). The s.c. injection of FCA has been shown to be immunologically effective and to induce side effects that were relatively more acceptable (encapsulate depot) than i.p. (in mice) or i.m. (in rabbits) injection.

When the i.v. or i.p. route is used for booster injections, there is a risk of inducing anaphylactic shock in the animals (Hendriksen and Hau 2003). The site of injection should not interfere with subsequent handling of the animals for blood sampling. Recommended routes of injection according to animal species and adjuvant are provided in Table 3. Routes other than those identified in Table 3 warrant strong scientific justification.

Volume of Injection

Regardless of the mixture to be injected, it is imperative to use the smallest possible volume that induces a sufficient antibody response. The minimal volume depends on particular characteristics of the antigen. When the antigen is dissolved in a large volume (and cannot be concentrated), a large volume must be injected. The injected volume has been found to have an effect on the extent of the lesions produced (Stills and Bailey 1991); higher volumes of FCA produced larger lesions. Amyx (1987) has also suggested that the negative effects of FCA depend on the injection volume. The maximum injection volumes depend on animal species, injection route, and injection mixture. Maximum injection volumes are given in Table 4a (oil and viscous gel adjuvants) and Table 4b (no or aqueous adjuvants).

Injection Protocol Recommendations:

- Use the smallest possible volume, with the maximal limitations provided in Tables 4a and 4b.
- Carefully select the injection route (recommendations are provided in Table 3).

Table 2 Adjuvant injection routes for research animals: details, advantages, and disadvantages^a

Injection route	Details	Advantages	Disadvantages
s.c.	Most frequently used route Preferred route Do not inject material in part of animal used for restraint Limit location to less than 4 sites	Relatively large volumes can be administered. Inflammatory processes can be easily monitored.	Slow absorption
i.m.	Skeletal muscles are well vascularized Not recommended for injection of oil adjuvant in rodents	Rapid adsorption, in particular, with muscular activity In large animals, relatively large volumes can be administered.	Injection into closed space, which is painful. Antigen and adjuvant can spread along interfacial planes and nerve bundles and may damage sciatic nerve and have other serious side effects. Local reactions can be easily overlooked
i.p.	Not recommended for injection of oil adjuvant Efficient route for antigen delivery	Relatively large volumes of inoculum can be accommodated.	Relative high percentage of injection failure Oil adjuvant induces peritonitis. Risk for anaphylactic shock at booster injection
i.v.	Not recommended for insoluble antigens Not recommended for injection of oil adjuvant Antigen is delivered primarily to spleen and secondary lymph nodes	Rapid distribution of antigen	No oil or viscous gel adjuvant can be used. High risk for anaphylactic shock at booster injection
i.d.		Efficient processing of antigen due to high density of Langerhans dendritic cells Small quantities of antigen already effective	Injection into close space in the dermis, which is painful Use of oil adjuvants leads to ulcerative processes.

^aAdapted and modified from Hendriksen C, Hau J. 2003. Production of polyclonal and monoclonal antibodies. In: Handbook of Laboratory Animal Science. 2nd ed. City: CRC Press LLC. p 391-411.

Number of Injection Sites

The antigen/adjuvant volume can be administered as a single injection or as multiple injections of low volumes. From the animal welfare perspective, there is a possible dilemma between the two options. Injection at one site of a large injection volume might be painful, particularly if there is limited space; however, multiple injections result in additional suffering due to the frequency of inoculation and the number of potentially painful sites. It is difficult to provide clear-cut recommendations because the available data are limited. In several European countries, a maximum of four injection sites is suggested when emulsions are used (Leenaars et al. 1999), but it is preferable to administer antigen/adjuvant mixtures at one site only.

Booster Injections

The boosting protocol can have a decisive impact on the result of the immunization. The time between two immuni-

zation steps can affect both the induction of B memory cells and the class switch of B cells. Animals can be rested for long intervals (months) between boosting. In general, a booster can be considered after the antibody titer has

Table 3 Injection route recommendations for different adjuvant types per animal species^a

Animal	Oil adjuvants and viscous gel adjuvants	No or aqueous adjuvants
Mice, rats, hamsters, guinea pigs	s.c.	s.c., i.p., i.v. ^b
Rabbits, sheep, goats	s.c., i.d.	s.c., i.d., i.m., i.v. ^b
Horses	—	s.c., i.d., i.m., i.v. ^b

^aBased on the available guidelines and recommendations.

^bi.v. only with soluble antigens.

Table 4a Recommended maximum injection volumes (in mL) used for injection of oil and viscous gel adjuvants per injection route for different animal species^a

Animal	Injection route ^b	
	s.c.	i.d.
Mice	0.1	NR ^c
Rats	0.1-0.2	NR
Guinea pigs	0.2	NR
Rabbits	0.1-0.25	0.025-0.05
Sheep/goats	0.5	0.05
Cattle	0.5	0.05
Poultry	0.25	0.05

^aAdapted and modified from the following: (1) CCAC [Canadian Council on Animal Care]. 2002. CCAC Guidelines on: Antibody Production, Ottawa ON: CCAC. (Available online: <http://www.ccac.ca/english/gdlines/antibody/antibody.pdf>); and (2) Hendriksen C, Hau J. 2003. Production of polyclonal and monoclonal antibodies. In: Handbook of Laboratory Animal Science. 2nd ed. Boca Raton: CRC Press LLC. p 391-411.

^bi.p. and i.m., not recommended; i.v., not acceptable.

^cNR, not recommended.

reached a plateau or begins to decline. When the first immunization is performed without a depot-forming adjuvant, the antibody titer usually peaks 2 to 3 wk after immunization. When a depot-forming adjuvant is used, a booster injection likely follows at least 4 wk after the first immunization (CCAC 2002).

An adjuvant is not always needed for booster injections. Booster injection with very small amounts of antigen may improve antibody affinity. Antibody titers after a booster injection can be measured to determine whether or not additional booster injections are needed. The number of booster injections should be limited for the welfare of the animal. Usually, a maximum of two or three booster injections are recommended (Hendriksen and Hau 2003). FCA should be used only once because repeated injection of FCA (or *Mycobacteria* proteins) may lead to severe tissue reactions. FIA should be used for booster injections. As described above, a risk of i.v. and i.p. booster injections is the induction of anaphylactic shock in the animals.

Emulsion Use Recommendations:

- Administer the boost at least 4 wk after the prime.
- Use the suggested maximum of two or three boosters.

Postinjection Observation

After immunization, animals should be monitored daily and examined for specific side effects at least three times per

Table 4b Recommended maximum volume of injection (in mL) used for injection of aqueous antigen/adjuvant mixture per injection route for different animal species^a

Animal	Injection route				
	s.c.	i.d.	i.m.	i.p.	i.v.
Mice	0.5	NR ^b	NR	1.0	0.2
Rats	0.5-1.0	NR	NR	5.0	0.5
Guinea pigs	1.0	NR	NR	5.0-10.0	0.5-1.0
Rabbits	1.5	0.05	0.2-0.5	10.0-20.0	1.5
Sheeps/goats	2.0	0.05	2.0	NA ^b	30.0
Cattle	2.0	0.05	2.0	NA	NG ^b
Poultry	0.5	0.05	1.0	NA	0.5

^aAdapted and modified from the following: (1) CCAC [Canadian Council on Animal Care]. 2002. CCAC Guidelines on: Antibody Production, Ottawa ON: CCAC. (Available online: <http://www.ccac.ca/english/gdlines/antibody/antibody.pdf>); and (2) Hendriksen C, Hau J. 2003. Production of polyclonal and monoclonal antibodies. In: Handbook of Laboratory Animal Science. 2nd ed. Boca Raton: CRC Press LLC. p 391-411.

^bNR, not recommended; NA, not acceptable; NG, not given.

week. Examination and palpation of the injection site are essential to evaluate side effects of the injected mixture. Clinical observations do not always predict severe pathology at the site of injection (Leenaars et al. 1998). Several investigators have described severe pathological changes despite the absence of observed clinical or behavioral changes. After booster injection via the i.v. or i.p. route, it is imperative to monitor the animals during subsequent hours for any anaphylactic reactions.

Monitoring Recommendation:

- Palpate the injection site to evaluate side effects of the injection.

Monitoring of the Antibody Response

In mammals, antibody responses during the experiment can be monitored by obtaining and evaluating a blood sample for antibodies in the serum. In chickens, because antibodies are excreted in the eggs, titers can be studied in the egg without an invasive action. The frequency of blood collection and the maximum blood volume that can be removed safely from the animal are limited. If too much blood is withdrawn too rapidly or too frequently without replacement, an animal may go into short-term hypovolemic shock and become anemic.

As a rough guide, up to 10% of the circulating blood volume can be taken on a single occasion from healthy animals (BVA 1993); in practice, an amount up to 1% of total body weight can be removed safely (McGuill and

Rowan 1989). After this maximum blood volume has been collected, animals need to rest for 3 to 4 wk (BVA 1993). Maximum blood volumes that can be collected from different animal species safely are provided in Table 5.

Collection of the Antibodies: Exsanguination and Euthanasia

Exsanguination must be performed under general anesthesia and is best carried out by heart puncture. It should result in the death of the animal. When there is uncertainty about death, small rodents can be subjected to cervical dislocation, and larger animals can be euthanized by an overdose of an appropriate anesthetic agent. Euthanasia should be in accordance with the 2000 Report of the American Veterinary Medicine Association (AVMA¹) panel on euthanasia (AVMA 2000).

Evaluation of Critical Steps in MAb Production

MAbs are antibodies produced by a single clone of B cells. Köhler and Milstein (1975) discovered that these cells can be immortalized by fusion with myeloma cells, resulting in hybridoma cells that are able to produce virtually unlimited quantities of monoclonal antibodies. Since the Nobel price-winning work of these researchers, MAbs have become essential tools in basic research as well as in diagnostic testing and medical treatments.

The process of MAb development includes the following successive working phases: the generation of antigen-specific B cells, the fusion of these cells with myeloma

cells, the cloning and selection of the specific hybridoma clone by “limiting dilution,” and the up-scaling of MAb production (Johnson 1995). Traditionally, two working phases in this process are based on procedures involving the use of laboratory animals—the generation of B cells and the MAb mass production by the ascites induction method. In the text below, we focus on these two steps.

Immunization to Generate Antigen-specific B Cells

Production of specific B cells requires immunization of the animal with the antigen under study. Critical aspects that should be considered include antigen selection, aseptic antigen processing, adjuvant selection, preparation of antigen-adjuvant mixture, choice of immunization route and injection volume, and aseptic inoculation, as discussed above in relation to the production of polyclonal antibodies. For immunization, BALB/c mice are typically used because many of the myeloma cells available for fusion have a BALB/c origin. In specific cases, immunocompromised (e.g., severe combined immunodeficient [SCID¹]) mice or other animal species such as the rat and hamster, and even human cases may also be used. Recommendations regarding immunization protocols for B cell generation are difficult to devise because protocols differ based on the type of antigen and adjuvant used. Generally, several booster immunizations are needed, with intervals of 14 to 28 days between boosters, although other protocols exist. It may be possible to use serum antibody titer information to guide booster protocols. To address that possibility, it is advisable to perform test bleeds before immunization. Often a final booster with antigen only (without adjuvant, i.p or i.v. route) is administered a few days before the animal is sacrificed. B cells are harvested from the spleen, although other lymphoid tissue can also be used.

Methods to generate antigen-specific B cells after in vitro “immunization” have been described (Guzman et al. 1995; Stadler 1999) but have not been very successful to date because the vast majority of the B cells produce IgM rather than IgG antibodies (Borrebaeck et al. 1988). Antibody-producing B cells from the spleen or other lymphoid tissue are fused with nonsecreting myeloma cells (hybridoma cells), and the product is then cloned and selected for antigen specificity. Selection for further growth characteristics and specificity of MAbs is crucial. Failures related to in vitro MAb production are often attributable to the improper selection and subcloning of hybridoma cells.

The next step, the up-scaling of MAb production, is traditionally accomplished first by administering injections of MAb-producing hybridoma cells in the abdominal cavity of mice and then by collecting the ascites that develops after the next 7 to 14 days. The abdominal cavity is indeed an optimal growth chamber for the hybridoma cells because it guarantees a constant temperature, an optimal nutrient and

Table 5 Maximum blood volumes to be collected from animals during experimentation^a

Animal	Maximum blood volume (mL)
Rabbits	15 ^b
Mice	0.3
Rats	2
Guinea pigs	5
Hamsters	0.3
Sheeps	200-600 ^b
Goats	150-400 ^b
Horses	500-7000 ^b

^aAdapted from BVA/FRAME/RSPCA/UFPAW [British Veterinary Association/Fund for the Replacement of Animals in Medical Experiments/Royal Society for the Prevention of Cruelty to Animals/Universities Federation for Animal Welfare]. 1993. Joint working group on refinement: Removal of blood from laboratory mammals and birds. *Lab Anim* 27:1-22.

^bDependent on body weight.

oxygen supply, and the optimal removal of CO₂ and metabolic waste products (Hendriksen and de Leeuw 1998). Hybridoma cells grow to high densities, and the resulting MAb concentration levels are consequently high. Nevertheless, for several reasons, the ascites method has fallen into disfavor. The most important reasons relate to the following: (1) Pain and distress are likely to be involved with ascites production (NRC 1999). (2) High-quality in vitro production systems are increasingly available, and new production approaches are progressing. (3) There is evidence of contamination with other immunologically active compounds and the risk of MAb contamination with viruses and other microorganisms. For further information, we refer readers to discussions of in vitro systems and methods provided elsewhere in this issue (Dewar et al. 2005; Lipman et al. 2005). Also in this issue, Peterson (2005) discusses new approaches to MAb production.

Based on various reports, the most important of which is the 1999 NRC Report, there is now consensus that ascites production should be the exception, requiring rigorous and well-documented justification. Exceptional circumstances that might justify the use of ascites production include the following: emerging therapeutic applications; existing regulatory approval for diagnostic and therapeutic products (until the approval expires); and poor growth of hybridoma cells in vitro, when subcloning fails to improve the growth (Marx et al. 1997). For those limited specific cases in which the ascites method can be justified, background information is provided about the underlying procedure, and investigators must conform to the standards of practice given, in the *Guide for the Care and Use of Laboratory Animals* (NRC 1996).

MAb Production by Ascites Induction in Laboratory Animals

BALB/c mice are generally preferred for ascites production because this strain of animals is syngeneic for the myeloma cells most frequently used for fusion. Animals are first given an i.p. injection with a priming agent. The primer most frequently used is pristane (2,6,10,14-tetramethyl pentadecane), but FIA is also applied (Gillette 1987). The effect of the primer is three-fold: (1) suppression of the immune system, thus preventing impairment of hybridoma cell growth; (2) toxic irritation of peritoneum, resulting in peritonitis and the secretion of serous fluid; and (3) retardation of hybridoma cell clearance from the peritoneal cavity (Moore and Rajan 1994).

A total of 7 to 10 days after priming, the hybridoma cells are administered by i.p. injection. Tumor development and ascites production begin within a few days after inoculation and result in an increase of abdominal distention. Ascites induction is a life-threatening procedure due to tumor growth, metastatic spreading, infiltrative growth, and, ultimately, respiratory distress. Some tumor cells tend to grow rapidly and form large solid tumors in the peritoneum, with-

out significant production of ascites. Usually, however, soft tumors develop with ascites production ranging from 0 to 10 mL/mouse, with an average of 2 to 4 mL/mouse and a MAb concentration of 1 to 28 mg/mL (Hendriksen 1998). Factors that might affect the yield of ascites or the MAb concentration per milliliter are shown in Table 6.

During ascites development, animals should be observed at least three times per day for the first week and daily thereafter (including weekends and holidays) to monitor the degree of abdominal distention and signs of illness (<http://medicine.ucsd.edu>). An overview of clinical, pathophysiological, and pathological effects of ascites production is given in Table 7. It is important to note that side effects of tumor growth can be more severe due to incorrect i.p. injection of hybridoma cells as a result of inoculation of hybridoma cells in abdominal organs, such as urinary bladder or intestines (Walvoort 1991). Peterson (2000) assessed the effects on well-being of pristane injection and ascites production using parameters such as wheel-running activity, food and water consumption, open-field box activity, clinical observation, and plasma corticosterone concentration. No significant evidence of distress was observed in the animals studied.

Ascites Collection

Ascites fluid must be collected before the abdominal distention leads to significant health problems, which usually develop 1 to 2 wk after injection of hybridoma cells. An 18- to 22-gauge syringe needle is inserted into the lower abdomen and is tapped by “massaging” the ascites. Shock from hypovoluminae may be prevented by s.c. injection of 2 to 3 mL of warm saline or lactated Ringer’s solution (<http://iacuc.cwru.edu>). With each successive tap, the quantity of ascites decreases; however, while the antibody concentration increases, the survival of animals decreases (from 98% for tap 1 to 35-100% for tap 3 [Jackson et al. 1999]).

Different policies exist with regard to the number of taps. According to the guidelines of the UK Co-ordinating Committee on Cancer Research (UKCCR 1998), ascites tumors should be drained only once. Some institutions allow removal of ascites fluid more frequently; however, repeated draining increases the risk of solid tumor deposit development, bleeding to the peritoneal cavity, and cachexia. Therefore, it is generally recommended that the number of taps should be limited to a maximum of three, or at any point if there is evidence of debilitation, pain, or distress, and that animals should be euthanized humanely according to the 2000 Report of the AVMA Panel on Euthanasia (AVMA 2000).

MAb Production: Guidelines

Due to concern for the welfare of animals, regulations and guidelines related to ascites production are now included in

Table 6 Parameters affecting ascites production^a

Parameter	Specification	Comment	Reference (see text)
Animal	Age	Highest Mab ^b concentrations in mice aged 43-75 days	Brodeur et al. 1984
	Sex	Males have a longer period of secretion, higher concentration/mL and greater volume of ascites, probably due to testosterone. Retired breeders.	Takakura et al. 1967
	Strain	Nude mice (irradiated mice) and SCID ^b mice can be used in case of nonsyngeneic hybridoma cells. Yields can be increased by use of BALB/c-derived cross-bred F1 hybrids.	http://iacuc.cwru.edu Pistillo et al. 1992 Brodeur and Tsang 1986 Stewart et al. 1989
Hybridoma cells	Type of cell	Some cells show a large variety in growth pattern in animals and tend to grow poorly in some animals. Some cells have an aggressive growth pattern and are often poor MAb producers.	C.F.M.H., personal communication (see text) C.F.M.H., personal communication (see text); Brodeur et al. 1984
	Number of cells	Cell number affects the duration of secretion. Recommended number of cells is between 6×10^5 and 5×10^6 per mouse.	Johnson 1995 Brodeur et al. 1984
Primer	Product	By using FIA, ^b the interval between priming and hybridoma cell inoculation can be shortened and the animals survive more taps.	C.F.M.H., personal communication (see text)
	Volume	Optimum volume is 0.5 mL, but a lower volume (0.1-0.2 mL) has also been shown to be effective and to cause less distress.	C.F.M.H., personal communication (see text); Gillette 1987; FSU 1998

^aAdapted from Hendriksen CFM, de Leeuw W. 1998. Production of monoclonal antibodies by the ascites method in laboratory animals. Res Immunol Forum 6 149:535-542.

^bMAB, monoclonal antibody; SCID, severe combined immunodeficient; FIA, Freund's incomplete adjuvant.

the policies of individual institutes, as well as those at a national level in several countries. Ascites production is strongly discouraged in several European countries such as the United Kingdom, Germany, and The Netherlands. Insti-

tutional policies exist in the United States, and many examples of policies can be found on the internet (e.g., <http://medicine.ucsd.edu> and <http://iacuc.cwru.edu>). In 1997, a "Dear Colleague" letter sent by the US National Institutes of

Table 7 Clinical, pathophysiological, and pathological effects of ascites production^a

Clinical effects	Pathophysiological effects	Pathological effects
Abdominal distension	Anorexia	Peritonitis
Decreased activity and body mass	Anemia	Infiltrative tumor growth
Shrunken eyes	Dehydration	Adhesions in the abdomen
Difficulty with 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	

^aAdapted from Anon. 1989. Code of Practice for the Production of Monoclonal Antibodies. Rijswijk, The Netherlands: Veterinary Public Health Inspectorate.

Health Office for Protection from Research Risks referred to the evidence that the ascites method causes discomfort, distress, or pain and that in vitro methods for MAb production should be preferred (NIH 1997). Based on documented evidence, in addition to the advances described in the foregoing text, it is reasonable to conclude that new developments in in vitro MAb production gradually will limit the use of animals for this purpose.

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