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Pain and Distress Associated with Polyclonal Antibody Production: Discussion and Recommendations

The Humane Society of the United States

Pain and Distress *Associated with* Polyclonal Antibody Production

Discussion and Recommendations

Promoting the protection of all animals

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Executive Summary

The Humane Society of the United States (HSUS) held a workshop in August 2002 in order to develop recommendations for minimizing pain and distress associated with polyclonal antibody (Pab) production. A small group of experts in the fields of antibody production, animal welfare, in vitro alternatives, and/or regulatory compliance participated in the roundtable discussion. The workshop was a scientifically based meeting, and recommendations were based on the extensive experience of the workshop participants as well as published literature regarding the relevant issues.

Participants recognized that insufficient attention has been paid to animal welfare aspects of Pab production, in part because this technique typically is a small part of larger research projects and is not, per se, directly related to the hypothesis being investigated. This lack of attention, as well as the pain and distress associated with Pab production, has led to our focus on this specific issue. Additionally, information in the published literature regarding Pab production varies greatly, further prompting the need to determine and harmonize recommendations.

Antibodies are produced by injecting an adjuvant (antigen) into the animal (human or nonhuman), thereby eliciting an antibody response by the immune system. The challenge involved in Pab production is to obtain high antibody yield while minimizing pain and distress to the animals. Several aspects of Pab production were considered by the group, including the determination of appropriate adjuvants, optimal volume of adjuvant per species, and optimal route of immunization; use of booster injections; consideration of available alternatives; and measurement of animal welfare. Each of these topics was considered in regard to minimization of pain and distress, and recommendations were generated. The participants also briefly discussed monoclonal antibody (Mab) production and corresponding alternatives.

The workshop resulted in both general and specific recommendations. General recommendations addressed outsourcing to Pab suppliers; increasing and improving training; increasing consideration of alternative production techniques; improving pain and distress assessment via score sheets; harmonizing guidelines; keeping abreast of and incorporating recent developments; minimizing the number of animals used when possible; and including relevant Pab production information in published papers. Some of the specific recommendations addressed were using the chicken egg yolk technique as a refinement and reduction procedure; choosing an adjuvant that produces high antibody yield while minimizing pain and distress; considering the use of Freund's complete adjuvant (FCA) for the first injection; using the smallest volume of adjuvant possible; and determining appropriate use of booster injections. Finally, areas in which additional research is needed were discussed—such as proper pain and distress assessment, formulation of new adjuvants, and examination of the roles that pain and enrichment may play in Pab production. The recommendations from this workshop will be widely distributed in order to press the debate on this issue and to increase attention to the pain and distress associated with Pab production.

Introduction

The Humane Society of the United States (HSUS) hosted a workshop on refinements to polyclonal antibody (Pab) production in conjunction with the Fourth World Congress on Alternatives and Animal Use in the Life Sciences in New Orleans, Louisiana, on August 11, 2002. A small group of experts in the fields of antibody production, animal welfare, in vitro alternatives, and the regulation of the use of animals for experimental and other scientific purposes participated in a roundtable discussion of Pab and monoclonal antibody (Mab) production; their recommendations are presented here. Coenraad Hendriksen, D.V.M., Ph.D., an expert in vaccine and biologicals quality control, chaired the workshop. The recommendations made in this document were based on the extensive experience of the workshop participants as well as on relevant published literature. See Appendix I for a list of participants.

Dissemination of information within the scientific community regarding good practices and techniques is essential to reduce pain and distress in animal research. The HSUS, through its Pain & Distress Initiative, works toward the goal of eliminating animal pain and distress in research and testing. Conducting technical workshops and disseminating the resulting information is one aspect of this initiative. The first such HSUS workshop addressed the use of animals in toxicity testing (see *www.hsus.org/ace/11447*).

The main aim of the antibody workshop was to review the current "state of the art" and develop recommendations on techniques involved in the production of Pabs. Pab production is typically not the primary focus or goal of research projects, but only comprises one step in the process. Available information on animal welfare aspects of Pab production tends to be relatively generic or consists more of anecdotal reports than careful scientific studies. This workshop was convened to identify and resolve conflicts regarding animal welfare recommendations and to suggest topics for further investigation and clarification.

Additional aims of the workshop were to identify components of "best practices" and encourage examination of the inconsistencies in the various published guidelines regarding Pab production, as well as to encourage the U.S. National Institutes of Health to allocate funding to research unanswered questions. Overall, the workshop sought to refine procedures so that less pain and suffering is caused during Pab production and optimum immune responses are obtained.

The Canadian Council on Animal Care (CCAC) produced a comprehensive monograph on the subject of antibody production in 2002 (*www.ccac.ca/english/gdlines/antibody/antibody.pdf*). The HSUS workshop did not seek to replicate or replace the CCAC document, but sought to complement it and discuss specific areas in greater depth. However, there were instances in which workshop participants questioned aspects of the CCAC guidelines, further supporting the need for increased examination of these issues.

Polyclonal Antibody Production: Overview

In the laboratory, antibodies are produced by injecting an immunogen (a chemical that is foreign to that species, such as a protein or glycoprotein from another species or microorganism) usually in combination with an adjuvant (antigen) into the animal, stimulating an antibody response by the immune system. Mabs are produced by cells that are derived from a single clone of an antibody-producing cell (B cell or plasma cell); they produce identical antibodies that all react to the same site (epitope) on the immunogen or a cross-reacting antigen. In contrast, Pabs are derived from many different clones, producing many sets of antibodies that will bind to various sites on the antigen; therefore, they have decreased specificity in comparison with Mabs, but a range of differing avidities (i.e., strength of binding).

Rabbits are the most commonly used species in laboratory Pab production largely because they are easy to bleed, house, and handle and their bodies can take adequate volumes of serum. However, mice, rats, guinea pigs, and large mammals such as horses, sheep, and goats are used for antibody production as well (Schade et al., 1996).

Chickens are also used for this purpose (producing immunoglobulin Y [IgY] in egg yolk), but the procedures differ from those used in mammals, as will be discussed later.

In addition to housing and care issues, there are two stages in the production of Pabs that raise animal welfare concerns: the immunization procedure and the bleeding of the animal. The antigen challenge, part of the immunization procedure, is to obtain high antibody yield of good quality while minimizing pain and distress to the animals. In this document, we focus primarily on immunization procedures, including determination of appropriate adjuvant, optimal volume of adjuvant per species, optimal route of immunization, permissibility of booster injections, and measurement of animal welfare. In the workshop, each of these topics was considered in relation to minimizing any pain or distress. The participants also discussed Mab production as well as alternatives to Mab and Pab production. The workshop recommendations are given below. For information regarding bleeding protocols and determination of appropriate species, please refer to the CCAC guidelines on antibody production (2002) and Leenaars et al. (1999).

Recommendations

Require Appropriate Training

- **Recommendation: Only experienced personnel should be allowed to conduct antibody production.**

Personnel should be properly trained in all relevant aspects of antibody production, including: preparation of immunogen-adjuvant mixture (emulsion); preparation of noninfective injectates; injection technique; bleeding; assessment of welfare (such as any pain and distress); and, finally, any subsequent animal care. Causing minimal animal pain and distress during the process of producing antibodies is an important task and requires a well-trained staff.

Determine Needs and Consider Alternatives

- **Recommendation: Determine on a case-by-case basis when it is more appropriate to use Mabs instead of Pabs.**

Investigators should carefully determine whether Pabs or Mabs are necessary in order to meet the needs of the research or application (e.g., for diagnostic testing). If it is determined that Mabs would be preferable over (or equivalent to) Pabs, then Mab production should be done via in vitro techniques. In vitro Mab production still involves mice in the initial immunization stage, but in vitro techniques can be used for the propagation and harvesting of Mabs from the hybridoma—therefore causing significantly less pain and distress in comparison with the use of mice for the entire production process (known as the ascites method). For additional information on Mab production, see page 10.

- **Recommendation: Institutions should utilize the chicken egg yolk (IgY) technique for Pab production when possible.**

Pabs can be obtained from IgY in higher quantities compared to mammals such as rabbits. There are various animal welfare advantages to using this technique. For example, since significantly larger quantities of antibodies can be produced per animal, this technique requires fewer animals overall (reduction). This technique is also considered to be a refinement method because no bleeding of the chicken is necessary, as it is with the use of other species; therefore, the elimination of one of two invasive steps leads to a substantial reduction in distress when hens are used (Schade et al., 1996). Additionally, as was indicated by Gassmann et al. (1990) in regard to the use of Freund's complete adjuvant (FCA) in chickens, immunization sites in chickens may not be associated with marked local inflammatory responses as is the case in mammals.

There was some speculation as to why the IgY technique is not more widely used, given that it addresses both reduction and refinement. One factor may be that an appropriate facility for housing chickens is necessary and institutions may not have such facilities. Scientists may be hesitant to use procedures that are different from those they are used to and they may lack training in the technique. It typically takes 24–72 hours for the IgY antibodies to bind, and this may be a technical barrier (immunoglobulin G [IgG] can take less time, but no published studies have compared IgG and IgY in regard to optimal binding time). Finally, IgY may not be acceptable in cases where the antibody may be used in an in vitro mammalian culture.

There are, however, benefits to the use of the chicken egg yolk technique beyond animal welfare concerns that are important to consider. For example, a hen produces 10 times more IgY in one month than comparable IgG obtained from a rabbit. Additionally, the hen's immune system "has properties favorable for producing antibodies against highly preserved mammalian antigens" (Schade et al., 2001), which may be caused by phylogenetic differences between avian and mammalian species; therefore, IgY antibodies are particularly advantageous when antibodies to highly conserved sites or epitopes are required. Finally, false positive reactions in certain immunochemical assays are unlikely (Schade et al., 1996). There is sufficient evidence, therefore, that IgY meets both scientific needs and animal welfare concerns.

For additional information, the European Centre for the Validation of Alternative Methods published a report and recommendations on IgY (Schade et al., 1996; *http://altweb.jhsph.edu/publications/ECVAM/ecvam21.htm*), and Schade et al. (2001) published a book on the topic. These publications include information on appropriate housing of chickens, immunization techniques (i.e., type of adjuvant, dose, and volume), and isolation and purification methods.

Outsourcing

- **Recommendation: Investigators should utilize qualified commercial suppliers when possible.**

The difficulty of producing Pabs and the level of expertise needed for such production were discussed. The use of reputable and qualified commercial suppliers experienced in the development and use of humane production procedures was recommended, but with certain stipulations. One incentive for using commercial suppliers is that Pabs are less expensive when one considers the real costs of animal care, staff time, and equipment needed for Pab production. In the United States, investigators should be aware that if their institution has an assurance on file with the Public Health Service (PHS), then the antibody supplier must also have PHS assurance. Workshop participants also recommend that these qualified commercial suppliers be accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Appendix II lists websites with information on antibody suppliers. Listing of specific companies in this appendix in no way signifies endorsement of these companies by The HSUS.

Routes of Injection

- **Recommendation: Intramuscular (IM), intraperitoneal (IP), intrasplenic, intravenous (IV), and footpad injections should be discouraged.**

The workshop participants agreed that there is sufficient evidence, both in the published literature and from their own experience, that IM, IP, intrasplenic, and footpad routes of injection result in increased pain and distress and increased risk of lesions, and should therefore be discouraged. IV injection, which is not often used, can lead to an anaphylactic response. If these routes are used, strong scientific justification should be provided.

Diligent care should be performed for an aseptic, noncontaminating injection. Preparation of the injection site that includes the removal of fur and a wipe with an appropriate antiseptic (povidine, chlorhexidine) is normally sufficient. For intradermal sites, the hair should be clipped; this may not always be necessary for other routes of injection. For additional information regarding preparation of the injection site, please refer to Leenaars et al. (1999).

Adjuvants that can be coadministered with antigens orally and nasally are new developments (Eriksson & Holmgren, 2002; Foss & Murtaugh, 2000); researchers are encouraged to monitor the emerging literature in this area, as oral and nasal routes of administration may produce an appropriate antibody response and may be preferred in regard to animal welfare. A major problem is that a local immunity (IgA) response is elicited rather than a humoral (IgG) response.

- **Recommendation: Subcutaneous (SC) and intradermal (ID) were considered to be acceptable routes of injection. However, additional research is needed in order to determine whether either SC or ID is the preferable injection route in regard to animal welfare.**

Although SC and ID were the recommended injection routes, there was discussion concerning which is the best in terms of animal welfare. For example, one participant argued that SC injections cause abscesses and more granulomas when compared with ID injections. It was further argued that ID is the preferred route because the resulting lesions are readily visible and can be easily treated and monitored. The participant conducted a study (unpublished) that examined the ID route by measuring white cell counts, inflammatory response, and body temperature, and used an imaging technique (behavior was not examined). Following this study, the participant feels confident in the use of ID injections.

A second participant disagreed that the ID route is preferable and reported fewer problems with the SC method in terms of animal welfare. For example, on palpation of the subcutaneous nodules due to SC injection of FCA, the rabbits do not show signs of pain. When the ID route is used, the skin on the rabbits' backs is very sensitive and the animals twitch even when not being touched, suggesting that the lesions cause discomfort in terms of pain or itching. The rabbits also appear to have a stronger aversive reaction to being handled.

During this discussion, it was pointed out that SC injections may appear to be acceptable because no lesions are visible, but appearances may deceive. The manifestation of draining lesions may be upsetting to the personnel working with the animals and may therefore prevent them from using ID injections.

The use of the ID route seems to be more common in the United States while SC appears to be preferred in Europe. The CCAC guidelines on antibody production (2002) discourage the use of ID injection for small animals such as mice and rats. The participants generally agreed with this recommendation. In any event, it would be very difficult to ensure that an injection is an ID route in thin-skinned animals such as mice and rats.

Pain and Distress Assessment

- **Recommendation: Daily observations should be made by properly trained personnel in order to assess pain and distress during Pab production, and if necessary, adjustments to the protocol should be made accordingly.**

Pain is an unnecessary and unwanted side effect in immunization procedures (Amyx, 1987), and steps can be taken to minimize the pain and distress associated with these procedures. The use of score sheets was discussed as a useful approach to pain and distress assessment (Morton, 2000). These sheets could include categories such as activity level, gait, hair coat, appetite, grooming, pruritis, scratching, signs of inflammation, twitching of skin, posture (lying stretched out or not), and the presence of lesions. Again, proper training of personnel is an important factor. Appendix III is an example of a score sheet created and used by the University of Birmingham, United Kingdom.

Welfare assessments of animals used for Pab production should occur in a setting in which the animals can move about freely so that clinical signs of lameness can be seen, especially if an IM injection site is used. Lameness is obvious when animals are kept in pens where they can normally move around and express their natural behaviors. Rabbits housed in a standard rabbit cage may appear to be fine; only when they are placed in a run may it be noticeable that they do not move or interact socially to the extent that control animals do. One participant pointed out, however, that a run may be unfamiliar and intimidating to laboratory rabbits; therefore, placement in a run may not be useful for welfare assessment. In this case, the rabbits should be encouraged to move within their home cages (without causing stress to the animals), and reluctance to move can be interpreted as abnormal.

In regard to pain and distress relief, treatment of dermal reactions under veterinary direction is recommended and poses no interference with the production of Pabs. For example, topical preparations with and without antibiotics, topical antibiotics combined with topical cortisone that are minimally absorbed, and topical anesthetics can each be effective.

Overall, it was agreed that pain and distress associated with Pab production is a topic that needs closer examination and more data.

Adjuvants

- **Recommendation: The chosen adjuvant should ideally induce high antibody titers in serum and/or egg yolk while minimizing pain and distress. Adjuvant choice should be carefully determined and pilot studies are suggested as one way to determine the best adjuvant for a particular immunogen or class of antigens.**

There is an abundance of published information on the different adjuvants available, and there are many conflicting claims. The various available adjuvants include FCA (discussed further below), incomplete Freund's adjuvant (IFA), Ribi®, Titremax®, mineral salts (such as aluminum hydroxide), microbial products (such as glycopeptides), surfactants (such as liposomes), cytokines, and others. As a result of this range of available adjuvants and in the case of unknown immunogens, pilot studies (with the use of score sheets) are one means of determining which adjuvant to use. Institutional Animal Care and Use Committees (IACUCs) should carefully scrutinize the choice of adjuvant when assessing protocols that involve Pab production, request actual data (e.g., titer, class of antibody, avidity of antibody) from the investigator, and require animal care staff to monitor the animals and report back to the IACUC regarding their condition. Overall, an informed choice has to be made based on the nature of the immunogen and the class of immunoglobulin required to maximize the antibody yield and to minimize the welfare cost.

In addition to determining which adjuvant to use, the amount of mycobacteria, the molecular length (or molecular weight) of the oil component, and the adjuvant/antigen ratio can lead to different scientific and welfare outcomes with different batches of the same adjuvant. This again highlights the importance of keeping up with the literature, seeking expert advice, performing pilot studies, and developing experience and expertise among animal care staff and those carrying out the procedure.

- **Recommendation: FCA may not be as problematic with regard to pain and distress as has been suggested and may have advantages over other adjuvants when used properly (more details regarding proper use follow). However, when using FCA, it is crucial that the volume used be minimized (based on route of injection and animal species), the sample for injection is properly prepared, and the administration competently performed. Under no circumstances should an animal be given a second injection of FCA.**

There is broad agreement that FCA gives a strong antibody response in comparison with most of the other adjuvants; this is why FCA is often the adjuvant of choice. However, there

has been much debate over the use of FCA due to reports of resulting local inflammatory response (Amyx, 1987; Broderson, 1989; and others). Consideration of scientific needs and animal welfare must be done with caution and be carefully balanced in order to minimize pain and distress while also minimizing the numbers of animals used.

It has been suggested that much of the concern regarding adverse FCA reactions involved studies of humans with prior sensitivity to mycobacteria, such as tuberculosis (Chapel & August, 1976). Any mammal with prior sensitivity will react very strongly to FCA, and this is the reason FCA should not be used for booster immunizations. A booster injection should not be necessary when using FCA because the FCA titer does not plateau for six weeks (much later than most other adjuvants). However, if a booster is determined to be necessary, IFA or any other adjuvant not including mycobacteria can be used.

The volume of FCA injected per site is a critical factor influencing the levels of pain and distress and should be given careful consideration (Amyx, 1987; Halliday et al., 2000; also see recommendation regarding volumes below). It is also important to note that the majority of published studies that used FCA to specifically induce an inflammatory response involved injection of FCA into the hind paw—an injection route that is not recommended.

Again, anecdotal reports and the few scientific reports investigating FCA use give conflicting results. In this context it must be realized that there are differences between the protocols and emulsions used. This is another area that needs to be examined closely.

Cytokines

- **Recommendation: If cytokines are used as adjuvants, only small levels are necessary for an adequate antibody response, and this will also avoid adverse effects.**

The use of cytokines (proteins that affect immune response) as adjuvants is expensive and, therefore, uncommon. However, when used, cytokine adjuvants typically result in a rapid high titer followed by a rapid drop in titer. The response is species-specific, and an animal's reaction and pathology depend on the concentration of cytokines. Therefore, when using cytokine adjuvants, the animals should be closely monitored and pilot studies are recommended.

Volume and Number of Sites

- **Recommendation: Regardless of the adjuvant chosen, the smallest volume possible that produces an adequate antibody response should be used. It is recommended that very small volumes be injected into multiple sites for an enhanced antibody response.**

Participants agreed that the route of injection and the volume used are equally important as the actual type of adjuvant used in minimizing pain and distress associated with the procedure. It was agreed by most participants that the greater the number of sites, the greater the antibody response. Larger volumes per site may lead to increased ulceration and welfare problems; therefore, small volumes are preferable and recommended (ANZCCART, 1998). The CCAC guidelines on antibody production (2002) specify recommended volumes for different species. It should be noted that one participant expressed concern with the use of greater than four injection sites (which, properly sited, will drain to the four major lymph node groups) on scientific and welfare grounds.

Please see Morton et al. (2001) and Diehl et al. (2001) for additional information regarding this issue.

Additional Recommendations

Harmonization of guidelines: There are many guidelines regarding Pab production available from various organizations and agencies, and some of these conflict. We suggest that appropriate organizations hold a workshop of all stakeholders in order to address harmonization around best contemporary practice. Inconsistencies

should be examined and reasons for disagreements or "special needs" should be addressed and understood.

Choice of species: For information regarding choice of species for Pab production, see Leenaars et al. (1999) and CCAC (2002).

Areas for Further Research: Pab Production

This lengthy discussion of Pab production resulted in as many questions as there were answers. The following is a list of areas that were determined to need additional research and examination (in order of priority/importance). We emphasize that such research should, whenever possible, be "piggybacked" onto existing work in order to avoid the use of additional animals.

1. **Welfare and pain and distress assessments:** Such assessments are severely underrepresented in the literature and should be a major area of concern. Species-specific information regarding welfare and pain and distress is needed—including behavioral data which is often either discounted or ignored—particularly in the context of pair- and group-housing situations.

2. **Alternatives to adjuvants:** The first question should always be: Do I need an adjuvant? Based on the characteristics of the antigen, in many cases this question can be answered without performing a pilot study. A means of creating an immune response without using some of the current adjuvants should be studied, given that adjuvants are responsible for much of the pain and distress associated with antibody production.

3. **New adjuvants:** Additional research on new adjuvants and immunization methods (including DNA vaccines) is necessary as such adjuvants may result in decreased pain and distress.

4. **Pain:** It should be determined what role pain actually plays in the antibody response. It is possible that if pain is decreased, antibody production would increase—this would lead not only to refinement through decreased pain and distress, but reduction in the number of animals needed in order to meet scientific needs.

5. **Mycobacteria:** The effects of varying amounts of mycobacteria in adjuvants should be examined in order to determine if certain mycobacteria levels are preferable in regard to animal welfare.

6. **Injections:** ID versus SC routes of injection should be examined (particularly in regard to behavior and antibody yield) in order to determine the optimal injection route from the animal welfare perspective.

7. **Enrichment:** It should be determined whether cage enrichment and group housing would simplify the recognition and management of welfare problems as well as improve production of Pab and the animals' psychological well-being (see Turner et al. [1997] for one example).

8. **Pathology of animals following Pab production:** It should be determined whether there are pathological effects that may be indicative of pain and distress as a result of Pab production techniques.

Mab Production

In the 1990s, Mab production received a great deal of attention and there has since been an initiative to eliminate the use of animals for Mab production. In 2000, the National Institutes of Health issued a guidance notice on this issue and indicated that institutions should utilize the in vitro methods of Mab production when possible instead of utilizing the mouse ascites method, which involves a great deal of pain and distress. In 1997, surveys indicated that one-third of respondent U.S. facilities were involved in Mab production, and of those, 66% had one or more investigators utilizing the ascites method and 36% utilized the in vitro method (McArdle, 1997). Informal surveys indicate that most institutions in the United States have since changed to the in vitro procedure (McArdle, 1997).

It has been determined that in vitro methods have the potential for a 3–5% failure rate (Institute for Laboratory Animal Research, 1999); in these rare cases, the mouse ascites method would have to be used. Some workshop participants, however, believe (based on experience) that the actual failure rate may be lower than that reported.

The Office of Laboratory Animal Welfare (OLAW) of the National Institutes of Health requests that institutions prove that the in vitro method has failed if the ascites method is being used. The U.S. Food and Drug Administration requires use of the in vitro method, and the U.S. Department of Agriculture is supportive of the initiative to eliminate Mab production to the extent possible, as well. According to one workshop participant, business has also dramatically increased for in vitro Mab production companies—another sign that the use of the in vitro method has increased.

Some European participants voiced concern about the status of Mab production in some European countries. One participant who works at an institution in Europe indicated that her institution requires a signed statement indicating that the in vitro method was unsuccessful before the in vivo method is approved. Since the induction of this requirement, most people have stopped requesting the use of the mouse ascites method at that institution.

It should be noted, however, that some European countries, including the United Kingdom, the Netherlands, Germany, and Switzerland, have an official ban on ascites production, unless it can be demonstrated that in vitro production systems would not suffice. One of the consequences is that in vivo production may simply have shifted elsewhere, as there have been specific cases of this observed.

Due to the success of the initiative to end the use of the mouse ascites method, the participants did not devote additional attention to this issue.

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Appendix I
List of participants

Vera Baumans (D.V.M., Ph.D.) is a professor of Laboratory Animal Science at the Karolinska Institute. Her interests are housing, behavior, and welfare of laboratory animals.

Kathleen Conlee (B.Sc.) is director of Program Management for Animal Research Issues and coordinates the Pain & Distress Initiative for The HSUS. She worked with captive nonhuman primates in a research setting for several years before joining The HSUS.

Wim A. de Leeuw (D.V.M.) has been a Senior Veterinary Public Health Officer for the Inspectorate for Health Protection and Veterinary Public Health, Ministry of Public Health in the Netherlands since 1992. Dr. de Leeuw was also involved in the draft of the Dutch Code of Practice for the immunization of laboratory animals and the organization of an international workshop on polyclonal antibody production sponsored by the European Centre for the Validation of Alternative Methods.

Coenraad Hendriksen (D.V.M., Ph.D.) is an animal welfare officer and senior scientist at the Netherlands Vaccine Institute (NVI) and head of the Netherlands Centre for Alternatives to Animal Use (NCA) of Utrecht University.

David Johnson (D.V.M.), a laboratory animal veterinarian, is a scientific advisor for Harlan, Inc.

John McArdle (Ph.D.) recently founded the International Center for Alternatives Resources. He was director of the Alternatives Research and Development Foundation for approximately 20 years. Dr. McArdle was instrumental in the promotion of the in vitro method of Mab production in the United States.

David Morton (B.V.Sc., Ph.D.) is a laboratory animal veterinarian and professor and head of the Department of Biomedical Science at the University of Birmingham, United Kingdom. His research interests include the welfare of animals, particularly the recognition and assessment of animals' pain, distress, and suffering during their use in research and other areas.

Norm Peterson (D.V.M.) is an assistant professor in the Department of Comparative Medicine and a clinical veterinarian at Johns Hopkins University. His research interests include the development of improved and alternative technologies in hybridoma and Mab production. Dr. Peterson also coordinates the Refinement Program Project at the Center for Alternatives to Animal Testing (CAAT).

Jon Richmond (M.D., Ph.D.) is a physiology and medical graduate, currently employed as Chief Inspector of the Animals (Scientific Procedures) Inspectorate at the Home Office in the United Kingdom.

Margaret Rose (Ph.D.) is an associate professor at the University of New South Wales and specializes in laboratory animal care.

Andrew N. Rowan (Ph.D.) is executive vice president, Operations, at The HSUS and studied biochemistry for his doctorate degree. He has authored numerous scientific articles on animal research, alternatives, ethics of animal research, animal control, and human-animal interactions.

Harold Stills (D.V.M.) is director of Laboratory Animal Resources and professor of pathology at Wright State University School of Medicine.

Founded in 1954, The Humane Society of the United States (HSUS) is the nation's largest animal protection organization, with more than eight-and-a-half million members and constituents. The organization works to create a humane and sustainable world for all animals, including people, through education, advocacy, and the promotion of respect and compassion. For more information on The HSUS's Pain and Distress Initiative, visit *www.hsus.org/research*.

Appendix II
*Antibody suppliers**

Websites with links to various antibody suppliers

The Antibody Resource Page
www.antibodyresource.com

Altweb Special Section on Monoclonal Antibodies
http://altweb.jhsph.edu/topics/mabs/where.htm

Biocompare
www.biocompare.com

The Nature Biotechnology Directory
www.guide.nature.com

University of California Davis–Monoclonal Antibody Resources
www.vetmed.ucdavis.edu/Animal_Alternatives/biblio~1.htm

Individual suppliers

Biogenes
www.biogenes.de/frames/base_win.html
Phone: +49-030-6576-2396

Biotez
www.biotez.de/english/eestart.htm
Phone: +49-030-9489-3317

Calbiochem
www.emdbiosciences.com/html/CBC/home.html
Phone: +1-800-854-3417

Charles River Laboratories
www.criver.com
Phone: +1-978-658-6000

Covance Research Products
www.crpinc.com
Phone: +1-800-345-4114

Harlan Bioproducts for Science
www.hbps.com
Phone: +1-317-894-7521

Lampire Biological Laboratories
www.lampire.com/custom.html
Phone: +1-215-795-2838

Pharmacia Biotech
www.amershambiosciences.com/aptrix/upp01077.nsf/Content/na_homepage
Phone: +1-800-526-3593

Promega
www.promega.com
Phone: +1-608-274-4330

* *Listing of specific companies in this appendix in no way signifies endorsement of these companies by The HSUS.*

Appendix III
Immunisation

IMMUNISATION							
User:	**Sex:**		**Source:**				
Animal No.:	**Issue No.:**		**Species/Strain:**				
Date:							
Antigen							
Adjuvant (type)							
Volume per site							
Route							
Number of sites							
SIGNATURE							

TEST BLEED							
Date:							
Site of withdrawal							
Volume							
SIGNATURE							

ANAESTHETIC/SEDATIVE							
Type							
Volume/dose							
Site							

HEALTH AND WELFARE SCORE							
User:	**Sex:**		**Source:**				
Animal No.:	**Issue No.:**		**Species/Strain:**				
Date:							
Appearance abnormal							
Inactive							
Starey coat							
Huddling							
Isolated							
Eyes half closed							
Hollow flanks							
Not eating/ drinking							
Bodyweight (g)							
Group weight*							
Average weight*							
% Weight loss							
Body temp							
Injection site, e.g., abscess, ulceration							
Other:							
Action taken:							
SIGNATURE							

**Mice when immunized in groups. If an individual mouse is affected, it will be weighed separately.*

Humane endpoints and actions:

1) Any animal showing departure from normal growth rates or other deterioration in condition, attributable to the procedure, will be carefully monitored and killed if there is no response to treatment as soon as this becomes clear and always within 7 days.

2) Any animal showing signs of anaphylaxis will be killed immediately.

3) Any animal with confirmed anaemia will be rested until the problem is resolved, or if this is not possible, killed and exsanguinated as soon as possible.