The Production of Polyclonal Antibodies in Laboratory Animals

The Report and Recommendations of ECVAM Workshop 35[1,2]

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Preface

This is the report of the thirty-fifth of a series of workshops organised by the European Centre for the Validation of Alternative Methods (ECVAM). ECVAM's main goal, as defined in 1993 by its Scientific Advisory Committee, is to promote the scientific and regulatory acceptance of alternative methods which are of importance to the biosciences and which reduce, refine or replace the use of laboratory animals. One of the first priorities set by ECVAM was the implementation of procedures which would enable it to become well-informed about the state-of-the-art of non-animal test development and validation, and the potential for the possible incorporation of alternative tests into regulatory procedures. It was decided that this would be best achieved by the organisation of ECVAM workshops on specific topics, at which small groups of invited experts would review the current status of various types of *in vitro* tests and their potential uses, and make recommendations about the best ways forward (1).

This joint ECVAM/FELASA (Federation of European Laboratory Animal Science Associations) workshop on The Immunisation of

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[1]ECVAM — The European Centre for the Validation of Alternative Methods. [2]This document represents the agreed report of the participants as individual scientists.

Laboratory Animals for the Production of Polyclonal Antibodies was held in Utrecht (The Netherlands), on 20–22 March 1998, under the co-chairmanship of Coenraad Hendriksen (RIVM, Bilthoven, The Netherlands) and Wim de Leeuw (Inspectorate for Health Protection, The Netherlands). The participants, all experts in the fields of immunology, laboratory animal science, or regulation, came from universities, industry and regulatory bodies. The aims of the workshop were: a) to discuss and evaluate current immunisation procedures for the production of polyclonal antibodies (including route of injection, animal species and adjuvant); and b) to draft recommendations and guidelines to improve the immunisation procedures, with regard both to animal welfare and to the optimisation of immunisation protocols. This report summarises the outcome of the discussions and includes a number of recommendations and a set of draft guidelines (included in Appendix 1).

Introduction

The immunisation of laboratory animals to induce a humoral and/or cellular immune response, is a routine procedure performed worldwide. Consequently, the use of animals is substantial, although no precise figures are available. Researchers from many disciplines need to produce antibodies. Often, they do not have specific knowledge of immunology, and are not sufficiently experienced in procedures for immunisation of laboratory animals. Therefore, specific guidelines on immunisation protocols are required.

Although several animal species are used for the production of antibodies, rabbits and mice are the species most frequently used for the production of polyclonal antibodies (pAbs) and monoclonal antibodies (mAbs), respectively.

Certain immunisation procedures are currently under discussion for animal welfare reasons. For example, the adjuvant products used to enhance the immune response are known to cause local inflammation, and some immunisation protocols are associated with pain and distress.

Some European countries, individual organisations and institutes have issued guidelines setting out criteria, for example, on the maximum volume to be injected, the route of inoculation and the number of injections. The aim of these guidelines is to ensure proper immunisation procedures, which combine acceptable immunological results with minimal discomfort for the animals. The immunisation protocol (including primary and booster injections and blood collection) has a great impact on both aspects. It is therefore crucial to carefully design this protocol.

The workshop focused on the production of pAbs and did not address the cellular immune response. Various aspects were discussed which could influence the induction of pAbs and affect the welfare of the animals.

Polyclonal Antibodies Versus Monoclonal Antibodies

The immune systems of most mammals are believed to be comprised of approximately 1000 clonal populations of lymphocytes, as characterised by their antigen-receptor specificity. This diversity permits immune responses to a broad range of immunogens, for example, foreign proteins, carbohydrates, peptides and bacterial and viral components. The lymphoid organs (spleen, lymph nodes, and gut-associated lymphoid tissue, including tonsils) are the production sites of a vast range of antibodies by stimulated B lymphocytes (plasma cells). Each antibody molecule recognises a specific antigenic epitope, possibly as small as 5–6 amino acids or between one and two glucose or other monosaccharide units, and is able to bind to the immunogen. A polyclonal humoral response, making use of the entire range of antibodies (pAbs), results in high avidity (defined as the product of the affinity constants of all binding antibodies) and gives the organism the ability to defend itself successfully against pathogens.

Köhler & Milstein (2) were the first to make B-cell hybridomas by using a fusion technique with Sendai virus. Subsequent workers have carried out successful fusions by using other agents, for example, polyethylene glycol. The fusion technique permits the immortalisation of single lymphocytes. The mAbs produced by such hybridoma cell clones originating from a single B lymphocyte are identical, and specific for a single epitope. There are some cases in which the

extreme monospecificity of mAbs can be a disadvantage. If for any reason the antigenic site is altered, which could be the case in many experiments, the mAb might not continue to bind. Nor are individual mAbs of use in precipitation assays such as immunoelectrophoresis. In contrast, the specificity of pAbs depends on a combination of hundreds or even thousands of clonal products, which bind to a number of antigenic determinants. As a result, small changes in the structure of the antigen due to genetic polymorphism, heterogeneity of glycosylation or slight denaturation, will usually have little noticeable effect on pAb binding. Whether these characteristics are seen as a problem or an advantage will depend on individual circumstances.

The avidity of a mAb is generally equal to its affinity for a protein antigen, a fact which is sometimes considered to be a disadvantage. MAbs are sometimes used in combinations, to increase the heterogeneity.

In making a choice between the generation of mAbs or pAbs, the desired application of the antibody and the time and money available for its production should be considered. The production of mAbs is tedious and takes 3–6 months. Cell cultures are required in addition to the animal immunisation. Examples of the use of mAbs include the immunostaining of western blots, ELISA, the affinity purification of proteins, and the immunostaining of thin tissue sections visualised by light microscopy or electron microscopy. The immortalised hybridoma serves as an inexhaustible antibody source of standardised quality. The induction of pAbs usually takes 4–8 weeks. The serum is suitable for many applications, for example, the immunostaining of western blots, ELISA and immunoprecipitation complement fixation. In most cases, polyclonal sera are of high titre, and permit substantial dilution; however, there may be batch-to-batch variability. The fact that a polyclonal antiserum can be obtained within a short time with little financial investment favours its use. In research, many questions can be answered with the assistance of a polyclonal antiserum.

General Considerations

Of primary importance for pAb production are factors such as the antigen used, route of immunisation, animal species, type and quality of the adjuvant, and method of blood collection. However, a number of other aspects can also have an impact on the successful outcome of an immunisation procedure and/or on the welfare of the animals, including the health and genetic status of the animals, the expertise and competence of the staff, and the hygiene, diet and housing of the animals.

Immunosuppressive effects have been reported for various agents which infect rodents (for example, the Sendai virus, mouse hepatitis virus, minute virus of mice; 3) or rabbits (Encephalitozoon; 4). Infections in the donor animals might also reduce the specificity of the antiserum, and, in the case of zoonotic agents, pose a human health risk. In general, the workshop participants recommended the use of specific pathogen-free animals in the case of small laboratory animals, and the implementation of a microbiological monitoring system according to the recommendations of FELASA (5, 6). However, it was also felt that, in some cases, more-conventional housing might be acceptable. In addition, it was recommended that the end-user of the animals should ask the supplier for an animal health and diet record, since specific tolerance to an antigen of interest may be generated by an animal's early dietary exposure to the antigen of an analogue. Immunosuppressive effects of diets have also been reported (7, 8).

Stress in animals should be avoided, since this can result in immune suppression, as well as discomfort for the animals. Immune suppression can result from stress generated before, as well as during, the actual period of immunisation (9). The quality of immunisation procedures can be optimised by the establishment of central facilities or units with responsible scientists (for example, a veterinarian) with experience in animal husbandry and immunisation processes.

The workshop participants emphasised the importance of training programmes for the personnel engaged in immunisation procedures and, in particular, for the animal technician. Reference was made to FELASA recommendations on education, which are now available for animal caretakers (Category A) and researchers (Category C; 10), and are being drafted for animal technicians (Category B) and animal welfare officers/laboratory animal specialists (Category D). For

instance, a curriculum for animal technicians should include the basic principles of immune response, injection and bleeding techniques, and the development of skills for observation, anaesthesia and euthanasia of the animals.

Another important aspect is animal housing. It is recommended that, whenever possible, animals should be housed under conditions that encourage their natural behaviour. The requirements of the Council of Europe (11) can be used as a point of reference. However, it was also noted that, in most laboratories, animals are housed individually without environmental enrichment, for example, rabbits. In general, the group housing of animals is recommended, although in specific situations, individual housing might be justified. There might also be microbiological considerations in favour of individual housing, because the more-intensive direct contact between individuals housed in groups encourages the transmission of pathogens (for example, coccidia). Some participants reported the successful group housing of castrated bucks. However, the ethical implications should be balanced against the advantages of group housing, and in some countries castration is not permitted, because it is stressful to the animals and is considered to be an unethical interference with the integrity of the animal.

The principles of Good Animal Experimentation Practice, as laid down in European Union legislation, *Council Directive 86/609/EEC* (12), must be followed in all aspects of the immunisation process. A number of crucial steps were identified: the preparation of the antigen, from its initial purification to its eventual mixing with adjuvant, the injection of antigen/adjuvant, the bleeding of the animal, and the processing of antiserum. It is recommended that particular attention should be paid to the quality of the antigen preparation (for example, removal of endotoxin, formaldehyde or sodium axide), the storage of the reagents, and the sterility of the instruments used for injection and bleeding (13).

Choice of Laboratory Animal

The selection of the animal species for the production of pAbs depends, at least in part, on the amount of antiserum needed and the ease of obtaining blood samples. The intended use of the pAbs can also play a role. In an ELISA, the antibody which binds to the antigen (the primary antibody) should be from a different species to the conjugated (secondary) antibody used in the next step of the assay. When there is no need for a specific species, the animals from which samples of blood are relatively easy to obtain (rabbits and mice) should be preferred over those which are difficult to bleed, for example, guinea-pigs and hamsters. The selection might also be related to the purpose of the experiment, as each species, strain, stock or breed of animal might differ in its immune response. An example is the difference between Balb/c mice and C57BL/6 mice, the first strain generally being a Th2-like responder, and the latter a Th1-like responder, although these may not always be hard and fast rules. The choice of a strain of mice or rats might also be influenced by the availability of inbred strains or outbred stocks. When an inbred strain is used, genetic variation between animals is restricted, such that hyporesponsiveness or hyper-responsiveness to a particular antigen might occur quite uniformly among members of the strain (uniform failure or uniform success). When an outbred stock is used, genetic variation among the animals may lead to a range of responses among members of the group. Although many environmental factors and a number of genetic factors can influence an animal's immune response, the major histocompatibility complex genotype plays a major role, in that it defines an individual's potential for antigen presentation and thus the individual's antibody response potential to a particular antigen. Chickens could be used as an alternative to mammals for the production of pAbs. The production of avian antibodies (IgY) in chickens is considered to be a refinement, since the collection of blood in the production of IgY has been replaced by the extraction of antibodies from egg yolk. In addition, chickens might be preferred for scientific reasons, for example, their phylogenetic distance from mammals. Unfortunately, the production of IgY in chickens is not widespread. This is probably due to a number of factors, such as traditions among researchers, the infrequent use of the chicken as a laboratory animal in general, the limited availability of conjugated antibodies, specific requirements for housing, and lack of experience with chickens and

chicken antibodies. Also, chicken antibodies are more difficult to purify than are mammalian antibodies. Further information on IgY production is given in ECVAM workshop 21 (14).

Traditionally, female animals are most frequently used in pAb production. Female animals are generally more docile for handling purposes, and are less aggressive in social interactions, and can therefore be group-housed more successfully. Although there is some evidence that androgens can slightly dampen the antibody response, there are no overriding scientific reasons for not using male animals.

The immune status of the immunised individual may also determine the outcome of an immunisation procedure. Young adults should be used for pAb production, as the immune response is immature at an early age and drops with age after the period of young adulthood. When animals are re-used after other procedures (only one use for pAb production is appropriate), it is important to keep the age of the animals in mind. When chickens are immunised, they should be of egg-laying age by the time antibody is to be harvested. Recommendations as to the age at which animals should first be immunised are given in the Swiss guidelines (15) and by Hanly *et al*. (13; Table I). Some guidelines recommend the use of animals at an earlier age (16). In addition, it is important to consider the weight of the animals.

Immunisation Protocol

The nature of the antigen, as well as the intended purpose of the antiserum produced, should be reflected in the choice of the immunisation protocol. Before proceeding with the immunisation, the investigator should consider the toxicity of the antigen preparation due to, for example, contaminating lipopolysaccharide (also called endotoxin), or chemical residues used to inactivate micro-organisms (for example, sodium azide, formaldehyde, or β-propiolactone), or an extreme pH, and should make adjustments, if appropriate. Essential factors to be considered in the preparation of an immunisation, including the selection of an adjuvant, the route and volume of injection, and the immunisation schedule, are described below.

Selection of immunological adjuvants

Adjuvants are used to enhance the immune response. The ideal adjuvant can be characterised as a substance which stimulates high and sustainable antibody titres (even with small quantities of antigen), is efficient in a variety of species, applicable to a broad range of antigens, is easily and reproducibly prepared in an injection mixture, is easily injectable, is effective in a small number of injections, has low toxicity for the immunised subject, and is not harmful to the investigator. Unfortunately, the adjuvant that meets all these criteria still does not seem to exist.

There are more than 100 known adjuvants, but many of them would not be routinely used for the production of pAbs, due to cost or difficulty in the preparation of the injection mixture. The different adjuvant categories that can be used for pAb production are briefly described in Appendix 2. These categories include oil emulsions, mineral salts, saponins, microbial products, synthetic products, and adjuvant formulations containing mixtures of products. Commercially available adjuvants that are used for routine pAb production include Freund's complete adjuvant (FCA), Freund's incomplete adjuvant, adjuvants from the Montanide® ISA series, Gerbu™, Quil A, aluminum salts, TiterMax™, and RIBI™. More-detailed information on adjuvants can be obtained from several review articles and books (17–20).

Table I: Recommended age after which animals should be used for polyclonal antibody production

Animal	Age
Mice	6 weeks
Rats	6 weeks
Rabbits	3 months
Guinea-pigs	3 months
Chickens	3–5 months
Goats	6–7 months
Sheep	7–9 months

Data from references 13 and 15.

The choice of adjuvant is, in principle, left to the investigator, but the workshop participants agreed that the overall welfare of the laboratory animal to be immunised should be of primary importance when selecting an adjuvant. It is recommended that animal ethics committees should be involved in the evaluation of immunisation protocols with regard to animal welfare aspects.

The (antigen/adjuvant) inoculation mixture should be prepared aseptically to minimise the risk of possible contamination. It is also essential to check whether the antigen to be used requires the presence of an adjuvant or possesses innate adjuvanticity. Adjuvants are usually not necessary when whole bacteria, whole cells or other particulate antigens (for example, cell fractions and bacterial cell walls) are used, but they are often necessary in the case of soluble antigens (proteins, peptides, polysaccharides). An adjuvant might also be necessary when only a very limited amount of antigen is available, when native antigens are used, or when a specific type of response is required.

When an oil emulsion is used in an immunisation experiment, the stability and quality of the emulsion should be checked. It is not difficult for an investigator to test whether a water-in-oil emulsion has been formed, because a drop of the mixture placed on the surface of water in a dish will retain its shape and not disperse. On the other hand, dispersion of the emulsion droplet over the surface of the water is indicative of an oil-in-water emulsion.

Selection of route of injection

Suggested injection routes for antigens with or without adjuvants in experimental mixtures are given in Table II.

The intramuscular route of injection was a major point of discussion. Some participants argued that the intramuscular route is frequently used without problems, while others considered this route neither acceptable nor necessary for injections with adjuvant, especially for small rodents such as mice. In animals with large muscles, large volumes of material can be accommodated. Antigen can be absorbed by lymphatics in this region, but antigen and adjuvant can spread and reside along interfacial planes between muscle bundles (because of leakage from the muscle bundle or because of misplacement of the injection mixture) and establish contact with nerve bundles, where serious pathology consequent to inflammatory processes can occur (13, 21). This is especially true for small ani-

Table II: Suggested routes of injection with or without adjuvant

Primary injection Day 0		Booster injection(s) Day 28 and/or later	
With adjuvant	**Without adjuvant**	**With adjuvant**	**Without adjuvant**
s.c.	i.v.	s.c.	s.c.
i.m.	s.c.	i.m.	i.m.
i.d.[a]	i.m.	i.d.[a]	i.v.[b]
	i.p.		i.p.[b]
	i.d.[a]		i.d.[a]

s.c. = subcutaneous, i.m. = intramuscular, i.p. = intraperitoneal, i.d. = intradermal, i.v. = intravenous.

[a]*For i.d. injection at multiple sites, it was the opinion of the participants that this route should be allowed for certain purposes in the rabbit and in large animals, to stimulate the required immune response.*

[b]*With i.v. or i.p. booster injections, there is a risk of inducing anaphylactic shock in the animals.*

mals such as mice, for which the intramuscular route should only be used by experienced researchers and biotechnicians. Furthermore, local reactions after intramuscular injections can easily be overlooked (22).

The intraperitoneal injection of adjuvant mixtures is not recommended, since it is known to induce inflammation (macroscopically evident), peritonitis (with the risk of ascites formation), and behavioural changes (for example, decreased activity and weight loss).

Herbert (23) considered intravenous administration to be the route of choice for small particulate antigens such as viruses, bacteria or cells (where danger of anaphylaxis is low), because the antigen distribution is broad and capture by lymphoid tissues is high. However, due to the risk of embolism, it is inappropriate for oil adjuvants (oil emulsions), viscous gel adjuvants or large particulate antigens (for example, bacterial aggregates, heterologous lymphocytes when raising anti-lymphocyte sera, or other heterologous whole mammalian cells [23]).

The workshop participants agreed that alternative injection sites, such as the footpad, are not to be recommended for pAb production. With precious antigens in very small quantities or with protein bands from electrophoretic separating gels (for example, precipitating bands from immunoelectrophoresis experiments), there could be scientific reasons for the use of the intra-lymph node injection procedure with an ocular grade needle, as described by Goudie *et al.* (24). Some authors have recommended intra-splenic injection for similar reasons (25). The participants agreed that investigators should provide scientific justification to ethical committees for such protocols (for example, the need to use extremely valuable or unique and irreplaceable antigens, or extremely small quantities of antigen).

Determination of volume of injection

To ensure that animals experience minimal discomfort at the antigen injection site, the injection volume should be as small as possible. Agreed maximum volumes per site of injection are shown in Table III. These volumes are based on the use of an injection mixture that forms a depot at the site of injection, for example, an immunostimulatory oil emulsion or a viscous gel. If the inoculum contains an immuno-potentiator, for example, mycobacteria or a muramyl dipeptide derivative, the amount of antigen in the injection mixture should generally not exceed 25μg for a mouse or 200μg for guinea-pigs, rats or rabbits. The inoculum should be spread among multiple injection sites in larger animals.

If the intraperitoneal route of injection is required for adjuvants such as oil emulsions and viscous gels, the maximum volume injected should not exceed 0.2ml. If the intradermal route is required (use should be limited

Table III: Maximum volumes for injection of antigen/depot-forming adjuvant mixtures per site of injection for different animal species

Species	Maximum volume per site	Primary injection	Subsequent injections
Mice, hamsters	100μl	s.c.	s.c.
Mice, hamsters	50μl	i.m.[a]	i.m.
Guinea-pigs, rats	200μl	s.c., i.m.	s.c., i.m.
Rabbits	250μl	s.c., i.m.	s.c., i.m.
Sheep, goats, donkeys, pigs	500μl (if in multiple sites 250μl/site)	s.c., i.m.	s.c., i.m.
Chickens	500μl	s.c., i.m.	s.c., i.m.

s.c. = subcutaneous, i.m. = intramuscular.

[a]*One hind limb.*

to rabbits and large animals), the maximum dose of adjuvant/antigen mixture would be 25μl per site at no more than four sites.

Maximum volumes that are allowed for injections of antigen solutions without adjuvant have been described by Iwarsson *et al.* (26) and van Zutphen *et al.* (27) and are given in Table IV.

Design of boosting protocol/immunisation schedule

The boosting protocol can have a decisive influence on the result of the immunisation. The time between two immunisation steps can influence both the induction of B memory cells and the class switch of B cells (from IgM to other antibody classes and subclasses). Specific recommendations for the interval between primary and booster immunisations are usually not cited. In general, a booster can be considered after the antibody titre has plateaued or begun to decline. If the first immunisation is performed without a depot-forming adjuvant, the antibody titre will usually peak 2–3 weeks after immunisation. When a depot-forming adjuvant is used, a booster injection can follow at least 4 weeks after the first immunisation. For booster immunisations, an adjuvant is not always necessary.

The participants agreed that, in most cases, the endpoint of pAb production should be judged when the antibody titre has reached an acceptable level. This should usually occur after a maximum of two boosters.

Table IV: Maximum volume of injection and needle size used for injection of antigen without adjuvant per route of injection for different animal species

	s.c.		i.m.		i.d.		i.p.		i.v.	
Species	**Vol.[a]**	**Needle[b]**	**Vol.**	**Needle**	**Vol.**	**Needle**	**Vol.**	**Needle**	**Vol.**	**Needle**
Mice	0.5	≤ 25G	0.05	25–27G	0.05	26G	1	≤ 23G	0.2	≤ 25G
Hamsters	1	25G	0.1	≤ 25G		26G	2–3	≤ 23G	0.3	≤ 25G
Guinea-pigs	1	≤ 20G	0.1	25G	0.05	26G	10	23G	0.5	≤ 27G
Rats	1	23–25G	0.1	25G	0.05	26G	5	23G	0.5	25–27G
Rabbits	1.5	20G	0.5	23G	0.05	26G	20	21G	1–5	20–21G
Sheep or goats	5	20G			0.05	26G	10	21G	30	
Pigs (> 50kg)	3	19–21G	2	20G	0.1	26G	250	20G	20	21–23G
Chickens	4	23G	0.5	21–23G		26G	10	21G	0.5[c]	23G

s.c. = subcutaneous, i.m. = intramuscular, i.p. = intraperitoneal, i.d. = intradermal, i.v. = intravenous.

[a]*Maximum injection volume in ml.*

[b]*Needle size in G (gauge: 27G = 0.40mm, 26G = 0.45mm, 25G = 0.50mm, 24G = 0.55mm, 23G = 0.60mm, 22G = 0.70, 21G = 0.80mm, 20G = 0.90mm, 19G = 1.00mm).*

[c]*MI/kg body weight.*

Data from references 26 and 27.

If the antibody response is still insufficient for laboratory purposes at this time, the experiment should, in general, be terminated. In the case of antigens of interest with a low molecular weight, such as peptides or steroids, injections might have to be repeated several times before the antiserum reaches the titre and specificity required for the application. However, long immunisation schemes, with repeated boosting, not only result in the production of antibodies with increased affinity for the antigen of interest, but also in the production of more antibodies specific for contaminants in the antigen preparation. Such multispecific antisera require absorption before they are monospecific, a process with some inherent difficulties.

Animals can be rested for long intervals between boosting. Even when serum antibody titres have dropped to relatively low levels, a booster injection into an animal that has previously established a memory response will usually re-establish a high serum antibody titre (13). Intermittent bleeding of a hyper-immunised animal appears to help maintain a high serum antibody level. Thus, regular interval blood collections after a sufficient serum antibody titre has been reached could facilitate the collection of adequate amounts of pAbs to an antigen that is in very limited supply. However, animals must not be kept in antibody production programmes unnecessarily (as already discussed).

Primary injections with very low amounts of antigen (picograms) are not recommended, since this does not stimulate the immunologic memory sufficiently, and might induce tolerance to the antigen. Frequent immunisations with relatively low amounts of antigen can be counterproductive. In addition, animal welfare argues against such schedules. However, low amounts of antigen for booster immunisation may help raise the average affinity of the antibodies subsequently produced (28).

In general, booster injection sites should be distant from previous injection sites. Booster antigen mixtures should never be inoculated into granulomas or swellings induced by earlier immunisations. Booster immunisations do not need to be administered by the same route used for the primary immunisation (29). Adjuvants that contain mycobacteria or their components (for example, cell walls) should only be used once per animal, because severe hypersensitivity reactions may result following re-exposure of the host to mycobacteria (30, 31). Booster immunisations applied intravenously or intraperitoneally with aqueous soluble antigens might result in systemic anaphylaxis, caused by the rapid release of histamine and other mediators from basophils and perivascular mast cells.

Blood Collection

Blood samples should be taken with minimum stress for the animal. Animals to be immunised should be conditioned to be confident with the animal care staff. This is not only important for animal well-being, but also ensures that the animals do not exhibit stress-mediated vasoconstriction, which would make blood sampling difficult.

Blood collection should be performed under conditions where it is possible to keep the animals warm, a prerequisite for ensuring good blood supply to the periphery. It is also important that the animals are not subjected to sudden noises or other environmental stress-inducers during blood collection, or, for that matter, during their routine housing.

The application of organic solvents to induce vasodilation is not recommended, because of the toxic and carcinogenic potential of the solvents for laboratory animals and humans.

When blood is collected for antibody production, it can be advantageous to prepare plasma (use of an anticoagulant such as heparin, citrate, or EDTA) instead of serum since the fluid yield can be increased significantly.

Blood should be collected only from the sites recommended by the BVA/FRAME/RSPCA/UFAW Joint Working Group on Refinement (32; see Table V).

When possible, a collection method not requiring anaesthesia should be preferred. This may favour the choice of a species in which blood sampling in conscious animals is easy for the operator and unstressful for the animal. Small ruminants and rabbits are thus advantageous compared to small rodents.

If an animal is not stressed during bleeding, the use of a sedative to facilitate blood

Table V: Vessels appropriate for blood collection

	Animal								
Vessel	**Mouse**	**Rat**	**Ham-sters**	**Guinea-pig**	**Rabbit**	**Dog, cat**	**Goat, sheep**	**Horse**	**Chicken**
Vena jugularis				E	E	B	B & E	B	B
Aorta	E	E	E	E	E				
Vena saphena	B	B	B	B		B			
Vena cephalica antebrachii						B			
Vena cava	E	E	E	E	E				
Tail vein/artery	B	B							
Lateral ear vein					B				
Central ear artery					B				
Heart puncture	E	E	E	B & E	E				B & E
Periorbital vein plexus[a]	B & E	B & E	B & E						

B = blood collection, E = exsanguination.

[a]*Sampling from the periorbital vein plexus is aesthetically questionable, requires both skill and general anaesthesia, and is not permitted in some countries.*

Data from reference 32.

sampling is usually unnecessary. However, in large-scale production systems, operators may find it advantageous to use sedatives to maintain low stress levels and to achieve rapid blood collection.

The needle used should be matched to the vessel size and should preferably be of relatively large bore to facilitate rapid collection. Vacutainers may be used, provided that the vein does not collapse. The bleeding of rabbits through an ear vein should be performed with a needle rather than with a scalpel, because of the difficulties inherent in stopping bleeding of a cut vessel. Butterfly needles are recommended, as they allow the animal to move its head during the procedure.

The volume to be removed per bleeding should not exceed 15% of the total blood volume; in practice, an amount up to 1% of the total body weight can safely be removed (33).

International guidelines differ with respect to frequency of blood collection (1–4 weeks), irrespective of the bleeding interval. The maximum volume allowed should not be removed more frequently than once a fortnight.

Exsanguination should be performed under general anaesthesia and is best carried out by heart puncture. After exsanguination is completed, small rodents should be subjected to cervical dislocation and larger animals should be euthanised by an overdose of an appropriate anaesthetic agent.

Assessment of Side-effects

In order for investigators to minimise pain and distress in immunisation procedures, the side-effects induced by immunisation have to be assessed. Some assessment procedures for quantifying discomfort in animals have been developed; these include the protocol proposed by Morton & Griffiths (34), and systems which measure changes in activity and behavioural patterns for a given period (35–37).

Animals should be checked daily and, in addition to the routine check-up which includes observance of the animal's general appearance and food and water intake, the

site of injection should be inspected. However, it should be noted that checking the food and water intake of individual animals may not be possible when animals are group-housed. When comparative studies or new experiments are performed (for example, with new combinations of antigen and adjuvant), the assessment of side-effects should also include studies of pathological changes at the end of the experiment; for this purpose, animals should be necropsied and multiple tissues should be examined. Pathological changes may not always be evident during clinical observation, depending on the route of injection (for example, the injection site cannot be examined after intraperitoneal injection). Pathological changes might not be confined to the site of injection.

Blood collection can also cause side-effects, especially when the animals are anaesthetised. Although blood samples should preferably be taken from unanaesthetised animals, in some cases anaesthesia might be needed (for example, for exsanguination, bleeding from the orbital sinus and heart puncture). When Hypnorm™ is used as an anaesthetic in blood sampling, the animals should be checked afterwards, since bleeding may continue. After a blood sampling procedure, the puncture site should be monitored for closing and healing of the wound. Specific attention is necessary when the ear artery of a rabbit is used. The artery must be compressed for a sufficiently long period of time (sometimes up to 5 minutes) for reliable closure and to avoid leakage.

Advanced Techniques and Alternatives

The traditional ways of adjuvanting protein have sometimes failed to result in polyclonal antiserum, for example, against small peptides and carbohydrate antigens. Therefore, it has been necessary to develop alternative approaches for antigen preparation. The conjugation technology and the multiple antigen peptides (MAP) procedure are described below. By coupling synthetic peptides or carbohydrates to protein carriers, it is possible to promote the T-cell helper function that supports a vigorous antibody response. In general, proteins (for example, keyhole limpet haemocyanin and bovine serum albumin) which are highly immunogenic themselves, are used as carriers. Therefore, an antibody response is also elicited against the carrier molecule (38), and the antiserum will need to be absorbed to render it monospecific. The MAP procedure overcomes some of the shortcomings of the classical conjugation approach described above for eliciting an antibody response against peptides. In place of a large protein carrier (which in itself would be immunogenic), a relatively non-immunogenic core matrix, consisting of trifunctional amino acids (such as lysine), is used as the "carrier". Lysine, with its extra amino group, can be used to form a lysine "tree" (core matrix) to which a number of peptides can be attached (39). The entire construct, including the peptides, can be generated with a peptide synthesiser. The number of peptides to be incorporated will be proportional to the number of lysine residues in the core matrix. The molecular weight of the resulting MAP will also reflect this. Accordingly, the density of B-cell epitopes is significantly higher with the MAP approach than with traditional protein carriers. The MAP approach has been successfully used with aluminum adjuvant (Alhydrogel), as well as with oil emulsion adjuvants (40) to raise antibodies to peptides. However, for the MAP construct to be immunogenic, it must contain both T-cell receptor epitopes and B-cell epitopes within the peptide of interest, because the lysine matrix itself does not provide the T-cell receptor epitopes necessary for eliciting the all-important T-cell help. A variant of the MAP procedure has been designed to allow synthesis of a multiple antigen construct which contains two different peptides, one of which is the antigen of interest for eliciting antibodies, while the other is a peptide with a strong T-cell receptor epitope (41).

Recently, Glenn *et al.* (42) demonstrated that it is possible to induce a systemic humoral immune response by transcutaneous immunisation. Cholera toxin was used as adjuvant, and bovine serum albumin, tetanus toxoid and diphtheria toxoid were used as antigens. No redness, swelling or other signs of inflammation were seen. Taking into account the large area of the skin, its accessibility, and the presence therein of potent immune cells (Langerhans cells), one could presume that this route could be a practical alternative to the most common injection routes, at least in some species. The

immunising solution could be applied for a certain period with plaster or with patches similar to those developed for transcutaneous drug delivery. However, with regard to the technique, further research should be undertaken involving other types of antigen and other adjuvants, and on the development of memory, species variations, and classes of antibodies elicited.

Oral immunisation aimed at inducing a simultaneous peripheral and mucosal immune response may be an attractive alternative to subcutaneous or intramuscular immunisation for animal welfare reasons. Oral immunisation has been conducted successfully in studies of new vaccine delivery systems in which antigens have been administered orally in biodegradable biospheres or associated with carrier proteins derived from cholera toxin or *Escherichia coli* heat-labile protein.

The development of novel DNA-based technologies, such as phage display libraries, direct cloning of antibody genes into plasmids, and molecular engineering, has introduced some very interesting possibilities for the production of antibodies which combine a high specificity with certain functional characteristics that are important for their further use (43–45). Although these possibilities are primarily seen as an alternative to the *in vivo* and *in vitro* methods currently used for the production of mAbs, under certain conditions these techniques could also be used as an alternative for the production of pAbs. At present, only a small number of laboratories have phage display libraries and the expertise needed to make use of them. However, it is evident that the availability and accessibility of these phage display libraries are growing.

Existing Guidelines on the Production of Polyclonal Antibodies

In the last decade, many sets of guidelines on the production of pAbs have been established by various national control authorities, organisations, and institutions. The increasing number of guidelines demonstrates the increased awareness of undesirable side-effects caused by some adjuvants and injection routes, but also that efforts should be made to harmonise these guidelines. It should be borne in mind that many of the immunisation protocols used for pAb production were (or are still) based on habit and tradition, rather than on scientific principles. The common aims of the established guidelines are to ensure appropriate procedures for the production of pAbs which give satisfactory results, and minimise discomfort, distress and pain in the animals involved. The guidelines are considered to be a tool for scientists, animal technicians, animal welfare officers and ethical review committees.

In 1988, the US National Institutes of Health issued their intramural recommendations for the research use of FCA (46). This was followed by Canadian Council on Animal Care guidelines on acceptable immunological procedures (30). These two sets of guidelines have had a major impact on the establishment of guidelines or codes of practice in other countries.

Several European countries have issued national guidelines on the production of pAbs, i.e. Switzerland (15), Denmark (16), The Netherlands (31), the UK (47) and Sweden (48). Variations in the legal status of the guidelines are evident between these countries. The Dutch and Swedish guidelines are not mandatory in the strict sense of the word; however, they are followed by the ethical review committees, and, in the case of The Netherlands, by the Veterinary Inspectorate. Thus, Dutch scientists have to justify explicitly when their immunisation protocol deviates from the Code of Practice for the Immunisation of Laboratory Animals (31). In the UK, immunisation protocols are reviewed and authorised by the Home Office Inspectorate, and experts in the production of pAbs are consulted when necessary. The situation in Denmark is comparable. In Switzerland, scientists are obliged to set up their immunisation protocols in accordance with the guidelines. Modification of the protocols can be required by the Swiss Competent Authority, the Bundesamt für Veterinärwesen.

Guidelines on the production of pAbs have been published by various organisations, for example the Scientists Centre for Animal Welfare (49), the Australian and New Zealand Council for the Care of Animals in Research and Teaching (50), the Tierärztliche Vereinigung für Tierschutz (22) and the Arbeitsgemeinschaft der Tierschutzbeauftragten in Baden-Württemberg

(51) and are included in several handbooks on immunological procedures (23, 38, 52, 53). In the USA in particular, but also in Europe, Australia and New Zealand, research institutes, and especially universities, have established their own guidelines, many of which are available on the Internet. Most of these institutional guidelines are not mandatory. In the USA, the Institutional Animal Care and Use Committees (IACUCs) provide guidelines that are not intended to dictate procedures, but are intended to assure proper treatment of animals that are used for pAb production. The IACUCs can request that investigators modify immunisation protocols and allow deviations from their guidelines, provided that these are for scientific reasons. This has clearly brought about some changes, which have improved attention to the welfare of the animals concerned. However, there is still some disagreement about the best immunisation protocols for the production of pAbs.

The recommendations given in some of the guidelines mentioned above were compared for the purposes of this report. Several guidelines include a lot of background information and a list of references. All of them cover, to different extents, the following important aspects of pAb production: choice of species, antigen preparation, injection route, injection volume, choice and use of adjuvants, injection technique, immunisation schedules, blood sampling and post-injection observation (Table VI). All put special emphasis on the limitations involved in the use of FCA, and similar recommendations are evident with respect to the permitted maximum volume of an FCA antigen preparation (Table VII). Some of the guidelines state explicitly which injection routes should be preferred, discouraged or not allowed (Tables VI and VII). There is a general consensus that footpad injection is not necessary for pAb production.

There is no doubt that these guidelines have had a positive impact on pAb production and have increased the attention given to animal welfare. In The Netherlands, the effect of the guidelines was evaluated in 1996 (54). It became evident that the guidelines had initiated discussions within institutes, which led in turn to the modification of immunisation protocols. However, this evaluation (and also a symposium) showed that the guidelines have to be amended in some specific areas. In Sweden, the set of guidelines have proved to be a valuable and practicable tool for both ethical committees and scientists.

Concluding Remarks

Current immunisation procedures for the production of pAbs are often based on habit and tradition. To ensure that appropriate procedures are used, many sets of guidelines have been established. The harmonisation of these guidelines is needed. In the workshop, various aspects of immunisation protocols and existing guidelines were discussed. In Appendix 1, draft guidelines for the production of pAbs are given, based on all available information. These guidelines should be regarded as a tool for use by scientists and ethical committees to improve immunisation procedures in order to minimise pain and distress to the animals involved. The guidelines should initiate discussions, which should lead to the further modification of immunisation protocols.

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Table VI: Aspects that are included in a number of existing guidelines

Guideline	Use of FCA	Use of other adjuvants	Different routes	Preferred routes	Immunisation protocols	Preferred interval prime-boost	Observation after injection	Background information and references
ANZCCART	+	+	+		+		+	+
ATBW	+	+	+	s.c.		≥ 4 weeks		
BVET	+	+	+	s.c.	+	≥ 4 weeks	+	+
CCAC	+		+				+	
HO	+		+	s.c.			+	
NIH	+		+					
TVT	+	+	+	+?	+	≥ 4 weeks	+	+
VPHI	+	+	+	s.c.	+	≥ 4 weeks	+	+
Danish guidelines[a]	+	+	+	s.c. or deep i.m.	+		+	+

s.c. = subcutaneous, i.m. = intramuscular.

ANZCCART = Australian and New Zealand Council for the Care of Animals in Research and Teaching (Australia/New Zealand; 50), ATBW = Arbeitsgemeinschaft der Tierschutzbeauftragten in Baden-Württemberg (Germany; 51), BVET = Bundesamt für Veterinärwesen (Switzerland; 15), CCAC = Canadian Council on Animal Care (Canada; 30), HO = Home Office (UK; 47), NIH = National Institutes of Health (USA; 46), TVT = Tierärztliche Vereinigung für Tierschutz (Germany; 22), VPHI = Veterinary Public Health Inspectorate (The Netherlands; 31).

[a]*Data from reference 16.*

Table VII: Maximal volume (ml) of Freund's complete adjuvant containing inoculum per injection site[a]

	s.c.			i.m.			i.p.			i.d.			f.p.		
Guideline	**Rabbit**	**Rat**	**Mouse**	**Rabbit**	**Rat**	**Mouse**	**Rabbit**	**Rat**	**Mouse**	**Rabbit**	**Rat**	**Mouse**	**Rabbit**	**Rat**	**Mouse**
ANZCCART	0.25			0.25	0.2	0.1	0.5	0.1	0.1	0.05	nr	nr	np	0.05	0.05
ATBW	0.1	0.1	0.1						0.02	0.05	np	np		npu	npu
BVET	0.1	0.1	0.1				0.2	0.2					np	npu	npu
CCAC	0.25	0.1	0.1	0.5	nr	nr	np	0.1	0.1	0.05	nr	np	np	npu	npu
HO	0.25	0.2	0.1												
NIH	0.1	0.1	0.1	0.5						0.05	nr			npu	npu
TVT	0.1	0.1	0.1	0.5	nr	nr		0.2	0.2	0.05			npu	npu	npu
VPHI	0.1	0.1	0.1	0.5				0.2	0.2	0.05	np	np		npu	npu

ANZCCART = Australian and New Zealand Council for the Care of Animals in Research and Teaching (Australia/New Zealand; 50), ATBW = Arbeitsgemeinschaft der Tierschutzbeauftragten in Baden-Württemberg (Germany; 51), BVET = Bundesamt für Veterinärwesen (Switzerland; 15), CCAC = Canadian Council on Animal Care (Canada; 30), HO = Home Office (UK; 47), NIH = National Institutes of Health (USA; 46), TVT = Tierärztliche Vereinigung für Tierschutz (Germany; 22), VPHI = Veterinary Public Health Inspectorate (The Netherlands; 31).

s.c. = subcutaneous, i.m. = intramuscular, i.p. = intraperitoneal, i.d. = intradermal, f.p. = footpad.

nr = not recommended, np = not permitted, npu = not permitted unless.

[a]*Maximum volumes of inoculum without an adjuvant can be found in several handbooks (23, 38, 52, 53).*

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Appendix 1

Draft Guidelines for the Production of Polyclonal Antibodies

General Considerations

Training

Qualified technical staff (Category B of the FELASA recommendations) should perform immunisation procedures in laboratory animals. The technical staff should have a basic understanding of immunological principles and be experienced in the handling, injecting, anaesthetising and bleeding of the animal species used for antibody production. In particular, aspects of asepsis and anaesthesia are very important. Furthermore, the technical staff should be capable of recognising signs of pain and distress in the animals, and must be responsible for taking action when necessary. The investigator should have biomedical training and should be qualified in laboratory animal science according to the FELASA recommendations (Category C).

Hygiene

The general principles of asepsis should be applied to immunisation protocols at all stages with regard to: the preparation of the antigen/adjuvant mixture; the administration of the mixture to laboratory animals; and the bleeding of the animals.

Housing of animals

Animals should be kept under conditions that allow important natural behaviour. Immunisation protocols are generally for medium-term to long-term experiments. Therefore, depending on the animal species used, group housing of animals is preferable.

Laboratory animals

The number of animals used, and thus the volume of antiserum produced, should be in accordance with the expected need for the polyclonal antibody (pAb). Priority should be given to a reduction of distress in the animals, rather than to a limitation of the number of animals.

Selection of animal species

The species of animals to be selected should be related to the amount of serum needed, the application of the antibodies to be produced, and the characteristics of the antigen concerned.

Microbiological status

In the case of small laboratory animals, the microbiological status should preferably be specific pathogen-free.

Immunisation protocol

Only the least harmful materials and the least severe protocols necessary should be used. To minimise the potential for induction of side-effects and to maximise the potential for successful pAb production, the investigator should check the quality of the antigen and of the antigen/adjuvant mixture prior to its injection.

Use of adjuvant

The first question is whether an adjuvant is needed at all. Selection of adjuvant should be given very high priority, since it affects the welfare of the animals to be immunised, as well as the type and level of immune response. The selection of, and need for, an adjuvant should be determined on the basis of the nature of the antigen. Mineral oil adjuvants combined with bacterial components may induce considerable side-effects. Adjuvants that contain mycobacteria or their cell wall components should be used only once per animal.

Route of injection

Suggested routes of injection are given in Table I and comments regarding injection routes are given below.

Subcutaneous injection
The subcutaneous route is preferred for the injection of oil adjuvants or viscous gel adjuvants.

Table I: Suggested routes of injection with or without adjuvant

Primary injection Day 0		Booster injection(s) Day 28 and/or later	
With adjuvant	**Without adjuvant**	**With adjuvant**	**Without adjuvant**
s.c.	i.v.	s.c.	s.c.
i.m.	s.c.	i.m.	i.m.
i.d.[a]	i.m.	i.d.[a]	i.v.[b]
	i.p.		i.p.[b]
	i.d.[a]		i.d.[a]

s.c. = subcutaneous, i.m. = intramuscular, i.p. = intraperitoneal, i.d. = intradermal, i.v. = intravenous.

[a]*For i.d. injection at multiple sites, it was the opinion of the participants that this route should be allowed for certain purposes in the rabbit and in large animals, to stimulate the required immune response.*

[b]*With i.v. or i.p. booster injections, there is a risk of inducing anaphylactic shock in the animals.*

Intramuscular injection
The intramuscular route should not be the first choice for injection of oil adjuvants or viscous gel adjuvants in small animals such as mice and rats.

Intradermal injection
The intradermal route should only be used in rabbits or large animals. If intradermal injection is carried out at multiple sites, the maximum volume administered per site should be 25μl and the maximum number of sites should be four.

Intraperitoneal injection
The intraperitoneal route is not recommended when oil adjuvants or viscous gel adjuvants are used. Its use should be scientifically justified to the ethical committee on a case-by-case basis.

Intravenous injection
The intravenous route is not recommended when oil adjuvants or viscous gel adjuvants are used, because there is a high risk of lethal complications due to embolism.

Footpad, intra-lymph node or intra-splenal injection
Footpad, intra-lymph node and intra-splenal injection are not necessary for routine pAb production. The use of these routes must be scientifically justified on a case-by-case basis. If a footpad injection is given, only one hind foot should be used, with a maximum volume of 50–100μl, and the animals should be housed on soft bedding.

Volume of injection

The volume of injection should be as small as possible. Maximum dosages of depot-forming adjuvants (for example oil adjuvants, viscous gel adjuvants) per site of injection are shown in Table II.

Immunisation schedule

Number of sites
A single site of injection is preferred and a maximum of four injection sites per immunisation per animal should be allowed. If more than one injection site is used, the distance between each site should be maximised.

Booster schedule
When an adjuvant is used, the time between primary and secondary injection should be at least 4 weeks. A maximum of two booster injections is recommended.

Table II: Maximum volumes for injection of antigen/depot-forming adjuvant mixtures per site of injection for different animal species

Species	Maximum volume per site	Primary injection	Subsequent injections
Mice, hamsters	100μl	s.c.	s.c.
Mice, hamsters	50μl	i.m.[a]	i.m.
Guinea-pigs, rats	200μl	s.c., i.m.	s.c., i.m.
Rabbits	250μl	s.c., i.m.	s.c., i.m.
Sheep, goats, donkeys, pigs	500μl (if in multiple sites 250μl/site)	s.c., i.m.	s.c., i.m.
Chickens	500μl	s.c., i.m.	s.c., i.m.

s.c. = subcutaneous, i.m. = intramuscular.

[a]*One hind limb.*

Blood collection

Blood should be collected according to the recommendations given by the BVA/FRAME/RSPCA/UFAW Joint Working Group on Refinement (1) or by McGuill & Rowan (2).

When frequent blood sampling is performed, the health status of the animals should be carefully observed. The collection of blood should not exceed more than 15% of the total blood volume, which in practice, represents an amount up to 1% of total body weight. Animals should not be bled more frequently than once every fortnight when maximum blood volumes are collected.

Assessment of side-effects

During the entire experiment immunised animals should be checked daily for general appearance, as well as for food and water intake. In addition to this routine check-up, the injection site should be monitored. When comparative studies are performed, the assessment of side-effects should include pathological studies at the end of the experiment. Pathological studies should include dissection for gross examination of organs and tissues, and histological examination of specific tissues. The results should be used to optimise the design of future experiments.

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Appendix 2

Overview of Adjuvants

An overview of adjuvant categories used for routine polyclonal antibody (pAb) production is given in Table I.

Immunostimulatory Oil Emulsions

Water-in-oil emulsions, which include Freund-type adjuvants, are the adjuvants most commonly used to produce pAbs in laboratory animals. Although most investigators and commercial vendors still refer to the Freund-type adjuvants in use today as Freund's complete adjuvant (FCA, i.e. it contains mycobacteria) or Freund's incomplete adjuvant (FIA, i.e. it does not contain mycobacteria), it would be desirable to replace these terms to reflect the differences between the components in the original formulation and those in the modern formulation, as well as the differences in reactogenicity of the different formulations. Few laboratories would be in a position to make the original FCA, because it was formulated with heat-killed *Mycobacterium tuberculosis* of high virulence, a mineral oil of low quality manufactured before 1969, and a surfactant, predominantly mannide mono-oleate, of variable purity and quality. Due to a change in the oil refining procedure in the early 1970s, the mineral oil component of the original FCA is no longer available (1). It has been replaced by a higher quality oil with less-irritant proper-

Table I: Overview of categories of adjuvants that may be used for routine polyclonal antibody production

Category	Examples (references)
Immunostimulatory oil emulsions (for example, water-in-oil, oil-in-water, water-in-oil-in-water)	Freund's incomplete adjuvant, Montanide®, Specol (18)
Mineral salts	$Al(OH)_3$, $AlPO_4$
Microbial (like) products	LPS, MDP, MPL, TDM (10)
Saponins	Quil A (19)
Synthetic products	DDA (13) ISCOMs NBP (12)
Adjuvant formulations	Oil emulsion + NBP (TiterMax™) Oil emulsion + bacterial products (Freund's complete adjuvant; RIBI™) Gerbu (11)

LPS = lipopolysaccharide; MDP = muramyl dipeptide; MPL = monophosphoryl lipid A; TDM = trehalose dimycolate; DDA = dimethyldioctadecylammonium bromide; NBP = non-ionic block polymer; ISCOMs = immune stimulating complexes.

ties. The mannide mono-oleate currently in use is also of higher quality. Today, only a few adjuvant immunologists retain the use of the old FCA, which is manufactured by the Statens Serum Institute (Copenhagen, Denmark) as a "gold standard" for comparison against a new adjuvant. The workshop participants agreed that this formulation is unsuitable as an adjuvant for use in routine pAb production, due to severe side-effects. The product that is quoted as FCA in the more recent literature can be obtained from, for example, Difco Laboratories, Sigma, ICN Biomedicals, and Pierce, and consists of a refined oil and a high quality mannide mono-oleate preparation, with heat-killed *M. butyricum* or *M. tuberculosis* H37Ra, an avirulent human strain. This FCA has less-irritant and less-inflammatory properties than the original FCA, but still induces considerable side-effects in animals (2, 3).

Currently, there are immunostimulatory oil emulsions that are acceptable or even superior to the original FIA with regard to enhancing antibody responses. Moreover, the purified components in these formulations produce fewer and less-severe adverse reactions after injection, for example, the Montanide® ISA (Incomplete Seppic Adjuvant) series (Seppic, Paris, France) and NUFCA Guildhay oil (Guildhay, Guildford, Surrey, UK).

Montanide ISA 740 adjuvant is composed of highly purified mannitol octadecenoic esters (Montanide ISA 80) as surfactant, in a mixture of a metabolisable oil and a refined non-metabolisable light mineral oil classified pharmacologically as an excipient. This mixture can form a stable emulsion (especially under nitrogen storage), in the weight ratio 70:30 Montanide ISA 740:aqueous phase antigen. When properly formulated, the emulsion will remain in a single phase for at least 2 years. This adjuvant emulsion is easy to inject and is well-tolerated by the recipient animal. When injected subcutaneously into mice or guinea-pigs in accordance with the European Pharmacopoeia, there are no serious adverse effects. The Montanide ISA series has been accepted for use in all food-producing species (4), as a pharmacologically active substance generally regarded as safe.

A non-ulcerative oil (NUFCA Guildhay oil) that can be administered by the intramuscular, subcutaneous or intradermal routes at multiple sites has been introduced by Guildhay. The intramuscular site creates a focus of stimulus with fewer adverse reactions than the classical FIA on the market today. However, as NUFCA Guildhay oil has only recently been introduced, limited information on its use is currently available.

Mineral Salts

Aluminum adjuvants in the form of aluminum hydroxide or aluminum phosphate hydrated gels can be injected subcutaneously or intramuscularly for priming an immune response in the recipient. These adjuvants are generally regarded as safe and they have been used for human vaccination for more than 50 years (5). Priming immunisations with aluminum adjuvants can be followed by boosters with or without adjuvant (6, 7). The biological function of these adjuvants is related to their ability to adsorb protein antigens, thereby ensuring that soluble proteins will be taken up as particulate antigens by antigen-presenting cells (8). Due to this adsorption/function relationship, it is strongly recommended that investigators ascertain that adsorption of the antigen to the gel has been successfully accomplished prior to its injection (9).

Microbial (like) Products

Micro-organisms such as *M. butyricum* and microbial products can exhibit strong adjuvant activity. The innate vertebrate immune system has evolved mechanisms for the recognition of, and response to, certain microbial products. Although the innate immune system itself is not highly efficient, some of its response components, once stimulated, help energise the specific antibody response. The microbial products involved (primarily cell wall components) usually induce considerable undesirable inflammatory side-effects, as well as an adjuvant effect. Investigators have identified active fractions or subunits of bacterial products, for example, trehalose dimycolate, and have in some cases modified the bacterial products, for example, threonyl-muramyl dipeptide, or monophosphoryl lipid A, to achieve a balance of immunostimulatory properties and diminished inflammatory properties (10, 11).

Saponins

Saponins are triperpene glycosides which are derived from the bark of the *Quillaja saponaria* tree and which have detergent and adjuvant properties. Saponin preparations intended for use as immunological adjuvants (for example, Quil A or QS-21) are purified to reduce the presence of components which cause adverse local reactions. Food-grade saponin preparations should not be used for immunisation schemes. In general, saponins should not be injected intraperitoneally or intravenously, but only subcutaneously or intramuscularly, due to their haemolytic activity.

Synthetic Products

Synthetic adjuvants are a rather heterogeneous group of products, because their classification has no single chemical, physical, or functional basis. This group includes nonionic block polymers (NBP), dimethyldioctadecylammonium bromide (DDA), immune stimulating complexes (ISCOMs), and liposomes. NBPs can contain different hydrophobic and hydrophilic regions, which influence their surfactant and immunopotentiating properties (12). The adjuvant effect of a given NBP also depends on the antigen used in combination with it, and, as such, different NBPs may be needed for different antigens for optimal effects. DDA is not an optimal adjuvant for antibody responses (13), but is rather better for T-cell-mediated cytotoxic responses. DDA has a lipophilic character, which might be responsible for its capacity to enhance T-cell responses. It is a representative of the quaternary amines (also classified as a cationic detergent). ISCOMs are small (40nm diameter) cage-like structures prepared from Quil A, cholesterol, and phospholipids. The antigen to be inserted into ISCOMs must be amphipathic (14). ISCOMs can be recommended as an excellent first choice for viral vaccines, based on past successes. In part, this is because ISCOMs can deliver the antigen to the cytosolic compartment of antigen-processing/antigen-presenting cells, and thus direct the immune response to a cytotoxic T-cell response, which is effective against many viruses. However, ISCOMs may also facilitate antibody responses. There is generally some resistance to the use of ISCOMs, because of the perceived, but misconceived, difficulty in their preparation; as an alternative, there are commercially available "honeycomb structures", to which the antigen can be added (15). Liposomes are unilamellar or multilamellar vesicles artificially constructed from natural products. The bilayer membranes mimic those of cells. The adjuvanticity of liposomes is influenced by charge, composition, and method of preparation. The antigen can be encapsulated in the water phase or the lipid phase, or it can be coupled to the surface (see reviews by Buiting *et al.* [16] and Alving [17]).

Adjuvant Formulations

Combining different immunostimulatory agents can increase the potency of an adjuvant. Oil emulsions are frequently combined with other agents (for example NBP in TiterMax™, or bacterial products in FCA and RIBI™ adjuvants). Immunostimulatory agents such as muramyl dipeptide can be incorporated along with antigen into liposomes. In fact, many adjuvants have more than one immunostimulatory substance and more than one mechanism of action.

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