Guidelines for the Use of Adjuvants in Research

Special Emphasis on Freund's Adjuvant

The use of adjuvants in animal research requires careful consideration. While relatively nonspecific inflammation may promote robust immunity, the investigator needs to evaluate the effect of associated local and/or systemic pain and distress of the research animal with the scientific benefit that may be gained from the experiment. The use of potent inflammatory agents, particularly Complete Freund's Adjuvant (CFA), can result in severe side effects. Although it is expected that alternatives to CFA should be used whenever possible,[1,8] the use of CFA may be scientifically justified for the induction of autoimmune disease models for which currently no comparable alternatives are known to exist.[1-5, 9]

When consistent with the scientific objectives, e.g. routine antibody production, adjuvants known to produce less intense inflammatory responses should be considered as alternatives to CFA. These may include currently licensed adjuvants such as aluminum compounds (e.g. Alum), squalene-in-water emulsions (MF59 and AS03), monophosphoryl lipid A (MPL), Ribi adjuvants, combined with alum (AS04); adjuvants in pre-clinical development (e.g. Montanides), polymeric microparticles, saponins (e.g. Quil A QS-21, ISCOMS, ISCOMATRIX), immunostimulatory nucleic acids (e.g. CpG oligodeoxynucleotides, poly IC:LC); other toll-like receptor-agonists (e.g. flagellin, imidazoquinolines, small molecules), cationic liposome formulations (CAF) combined with immunestimulators such as trehalose dibehenate (TDB) virus-like particles, nanoparticles,[19-21] and oligonucleotide complexes;[22] and other procedures or emulsions such as subcutaneously- implanted chambers, TiterMax, EMULSIGENS, Syntex Adjuvant Formulation (SAF), and Specol. [10-12, 16-18] In many situations, these alternatives are capable of eliciting robust cellular and humoral local or systemic immune responses with fewer side effects than those commonly seen with CFA. Extensive information on alternative adjuvants is also available online (see references). All adjuvants used in animal research must be approved by the Institute/Center (IC) Animal Care and Use Committee (ACUC), and use of adjuvants that could induce a severe reaction must be scientifically justified.

Complete Freund's Adjuvant

CFA, a mineral oil containing a suspension of whole or pulverized heat-killed mycobacteria which is emulsified together with a solution of the antigen of interest to form a water-in-oil emulsion, is effective in potentiating cellular and humoral antibody responses to injected immunogens. Adjuvant activity is a result of sustained release of antigens from the oily deposit and stimulation of a local innate immune response, resulting in enhanced adaptive immunity. An essential component of this response is an intense inflammatory reaction at the site of antigen deposition, resulting from an influx of leukocytes and their interaction with the antigens. The use of CFA is an important biologic resource for investigators, which should be used responsibly and with care in order to avoid or minimize the adverse effects of excessive inflammation. CFA may result in local inflammation and granulomatous reactions at the site of injection, lymph node structural changes, chronic inflammation, skin ulceration, local abscess or tissue sloughing, diffuse systemic granulomas secondary to migration of the oil emulsion, adjuvant- related arthritis, and very rarely, chronic wasting disease.[4,9]

For most applications, CFA is usually only necessary for the initial immunization, while Incomplete Freund's Adjuvant (IFA), which lacks mycobacteria, is the adjuvant of choice for subsequent immunizations. CFAs containing either *M. butyricum* or *M. tuberculosis* H37Ra (an avirulent strain) are commercially available. Additional information about CFA use is available online (see references).

Guidelines for Preparation and Injection

The following guidelines have proven effective in significantly alleviating complications after immunization with adjuvants. Utilization of: a) sterile technique in the preparation of antigen-adjuvant emulsions; b) aseptic preparation of the injection site; c) appropriate injection technique; d) appropriate

routes and sites of administration; e) adequate separation of injection sites; and f) use of smaller volumes at each injection site have all proven efficacious in the elimination of post-immunization complications.

1. Antigen preparations should be sterile and, ideally, isotonic, pH neutral, and free of urea, acetic acid, and other toxic solvents.
   Antigens separated using polyacrylamide gels should be further purified whenever possible in order to minimize the amount of secondary inflammation/irritation from gel fragments. If further purification is not possible, then the amount of polyacrylamide contaminant should be minimized by careful trimming. Millipore ultrafiltration of the antigen, for example, prior to mixing it with the adjuvant, is recommended to remove extraneous microbial contamination.

2. The mycobacteria in CFA is re-suspended by vortexing or shaking the ampule or vial. The CFA is then removed from the ampule or vial using sterile technique.
   Although approaches may vary, one part or less of CFA to one part aqueous antigen solution (v/v) has been recommended.[1] The CFA/antigen emulsion should be mixed deliberately and with care in order to avoid the introduction of air bubbles.

3. Formulations of CFA containing 0.5 mg/ml of mycobacterial components are commercially available and have been successfully used by many researchers. Concentrations of <0.1 mg/ml are recommended in order to minimize the inflammation and focal necrosis observed with higher concentrations.[2] Some protocols, such as autoimmune disease induction protocols, may require the use of greater concentrations than those available commercially, and must be scientifically justified and approved by the IC ACUC.

4. The use of preparations containing disrupted mycobacterial cells rather than preparations containing whole, intact bacilli may be preferred, since it is difficult to histologically distinguish the latter from live, acid-fast cells.

5. For favorable results while minimizing undesirable side effects, use the recommended injection volumes and sites appropriate for the species, size of the animal, and experimental goal (Table 1).[3,4]

6. Some routes of injection may potentially be less disruptive to the animal than other routes (e.g., subcutaneous injection vs. footpad administration).
   Whenever possible, the least invasive methodology required to accomplish the experimental goal should be utilized. More invasive injection routes should be avoided unless scientifically justified.

7. It is necessary to separate multiple injection sites by a distance sufficient to avoid coalescence of inflammatory lesions.

8. A minimum period of 2 weeks between subsequent inoculations is recommended.

9. In addition to the route of administration, the site of injection should be chosen with care in order to avoid areas that may compromise the normal movement or handling of the animal (e.g., intradermal injections in the neck scruff of a rabbit).

Routes of Administration Presenting Special Issues:

1. **Footpad Immunization**:
   Utilizing the footpad for immunizing small rodents may be necessary in studies where it is required to isolate a draining lymph node as a primary action site. Procedures to address the well-being of the subject animals should be used, e.g. limiting the quantity of adjuvant-antigen solution injected into the footpad, the use of only one foot per experimental animal, and housing on soft bedding rather than on screens. Footpad inoculation must not be used for

routine immunization of rodents without specific scientific justification. Alternative sites with potential draining lymph node utility e.g. the hock, popliteal lymph node,[13] cervical sites, auricular lymph node,[14] and superficial cervical lymph node,[15] should be used in order to prevent the animal's locomotion from being affected. If scientific justification is provided, the recommended maximum footpad injection volumes are 0.01-0.05 ml in mice and 0.10 ml for rats.[1] Rabbits must not be immunized in their feet because they lack a true footpad.

2. **Peritoneal Exudate**:
   The production of rodent peritoneal exudate by the intraperitoneal administration of antigen and adjuvant is a recognized, valid scientific procedure for obtaining high-titer reagent. Undesirable side effects of painful abdominal distention and the resulting distress can be avoided by daily monitoring and relief of ascites pressure, or termination of the experiment. Intraperitoneal injections of CFA-antigen emulsions should normally be limited to less than 0.2 ml in mice.[6]

Post-injection Observations and Treatments

Post-inoculation monitoring of animals for pain and distress or complications at the injection sites is essential, and should be done daily for a minimum of four weeks or until all lesions have healed. Supportive therapy may include topical cleansing, application of sterile petroleum jelly and/or hydrogen peroxide, antibiotics and analgesics. If overt pain or distress is anticipated or observed, the use of narcotic agonists, mixed agonist-antagonists, or other species-appropriate agents should be considered and used under the direction of the attending veterinarian (taking into account the research objective). Steroidal or non-steroidal anti-inflammatory agents must be used with caution due to their known impacts on immunological processes.

Personnel Safety

Adjuvants that contain mycobacterial products can be an occupational hazard to laboratory personnel and should be handled with extreme care. Reports of accidental needle punctures in humans have been associated with clinical pain, inflammatory lesions, and abscess formation in tuberculin-positive individuals. Tuberculin-negative individuals have tested positive in subsequent tuberculin tests after accidental CFA exposure.[7] Safety glasses should be worn in order to avoid accidental splashing of CFA in the eyes.

Other Considerations

Scientists preparing antigens for *in vivo* administration in conjunction with adjuvants should be aware of the potential presence of contaminating substances and other characteristics of the injectate which may have additive inflammatory effects. Care should be taken to consider and eliminate additional inflammatory stimuli whenever possible, e.g. excessive vehicle pH or the presence of by-products of purification such as polyacrylamide gel fragments. The preparation should be kept sterile.

Generation of ascites fluid requires the use of a "priming" agent. Pristane is a commonly used "priming" agent, however, Incomplete Freund's Adjuvant (IFA) has also been shown to be an effective "priming" agent. Concern has been expressed about the potential for discomfort and distress that may be associated with "priming" agents, particularly pristane.[32] Due to this concern, many guidelines suggest a lower 0.1 to 0.2 ml dose of Pristane.[1, 30-33] It is also recognized, as an ILAR report states, "in some strains of mice, 0.2 ml might not be sufficient to produce ascites and that as much as 0.5 ml might be required."[32] A maximum dose of 0.3 ml is recommended for IFA.[31] Consideration for using the lowest does of "priming" agents is strongly encouraged.

Table 1. Recommended Volume of CFA-Antigen Emulsion (CFA-AE) per Site and Route of Administration

Species	**SubQ** (ml)	**Intradermal** (ml)	**Intraperitoneal** (ml)	**Footpad** (ml)	
Mouse	<0.1	*	<0.2	<0.05**	
Rat	<0.1	<0.05**	<0.5	<0.1**	
Rabbit	<0.25	<0.05**	*	*	
Non-Human Primate**	Freund's Adjuvant is not generally recommended for use in Non-Human Primates, as it may interfere with TB testing results and cause excessive inflammation. Nevertheless, it is recognized that some models may require use of CFA. If used, the recommended volumes should not exceed those used in rabbits and should be scientifically justified.[23-25]				

* Not recommended

** Only when justified

Adopted by ARAC - 08/13/86
Re-approved - 05/08/96
Revised - 03/27/02, 03/09/05, 11/14/07, 11/13/10, 04/10/2013, 08/24/2016, 4/24/2019

References:

1. Jackson, L.R., and J.G. Fox. 1995. Institutional Policies and Guidelines on Adjuvants and Antibody Production. ILAR Journal 37(3): 141-152.
2. Broderson, J. R. 1989. A Retrospective Review of Lesions Associated with the use of Freund's Adjuvant. Lab Animal Sci 39: 400-405.
3. Grumpstrup-Scott, J., and D. D. Greenhouse. 1988. NIH Intramural Recommendations for the Research use of Complete Freund's adjuvant. ILAR News 30(2): 9.
4. Stills, H. F., and M. Q. Bailey. 1991. The use of Freund's Complete Adjuvant. Lab Animal Sci 20(4): 25-31.
5. Clemons, D. J., C. Besch-Williford, E. K. Steffen, L. K. Riley, and D. H. Moore. 1992. Evaluation of Subcutaneously Implanted Chamber for Antibody Production in Rabbits. Lab Animal Sci 42(3): 307-311.
6. Toth, L. A., A. W. Dunlap, G. A. Olson, and J. R. Hessler. 1989. An Evaluation of Distress Following Intraperitoneal Immunization with Freund's Adjuvant in Mice. Lab Animal Sci 39(2): 122-126.
7. Chapel, H. M., and August, P. J. 1976. Report of Nine Cases of Accidental Injury to Man with Freund's Complete Adjuvant. Clin. Exp. Immunol. 24: 538-541.
8. Stills H.F. 2005 Adjuvants and antibody production: dispelling the myths associated with Freund's complete and other adjuvants. ILAR Journal. 46(3): 280-293.
9. Billiau, A., and P. Matthys. 2001. Modes of action of Freund's adjuvants in experimental models of autoimmune diseases. J Leukoc Biol 70(6): 849-860.
10. Vaccine Adjuvants: Preparation Methods and Research Protocols. O'Hagan, Derek, T. Humana Press, 2000. DOI: 10.1226/0896037355
11. Schmidt, C.S., W. J. W. Morrow, and N. A. Sheikh. 2007. Smart Adjuvants. Expert Rev. Vaccines 6(3): 391-400.
12. Aguilar, J. C., and E. G. Rodriguez. 2007. Vaccine adjuvants revisited. Vaccine 25: 3752-3762.
13. Kamala, T. 2007. Hock Immunization: A humane alternative to mouse footpad injections. J. Immunol. Methods 328: 204-214.
14. Nierkens, S. *et al.* 2004. Evaluation of the Use of Reporter Antigens in an Auricular Lymph Node Assay to Assess the Immunosensitizing Potential of Drugs. Toxicol Sci 79: 90-97.
15. Weaver, J. L. *et al.* 2005. Evaluation of a Lymph Node Proliferation Assay for its Ability to Detect Pharmaceuticals with Potential to Cause Immune-Mediated Drug Reactions. J Immunotoxicol 2(1): 11-20.
16. Mbow, M Lamine, *et al.* 2010. New adjuvants for human vaccines. Current Opinion in Immunology 22: 411-416.
17. Spickler, Anna R. and Roth, James A. 2003. Adjuvants in Veterinary Vaccines: Modes of Action and Adverse Effects. J.Vet. Intern. Med. 17: 273-281.
18. Coler, R.N. *et al.* 2010. A Synthetic Adjuvant to Enhance and Expand Immune Responses to Influenza Vaccines PLoS One. 5(10): e13677.
19. Coffman *et al*. 2010. Vaccine Adjuvants: Putting Innate Immunity to Work. Immunity, 33 (4): 492-503.
20. Mastelic *et al*. 2010. Mode of action of adjuvants: Implications for vaccine safety and design Biologicals 38 594-601.
21. Tritto *et al*. 2009. Mechanism of action of licensed vaccine adjuvants. Vaccine, 27 (25-26): 3331-3334.
22. Lee, E.S. *et al*. 2017. Cationic Liposome-Oligonucleotide Complex as an Alternative Adjuvant for Polyclonal Antibody Production in New Zealand White Rabbits (*Oryctolagus cuniculus*). CM 67(6): 498-503.
23. LeHoan, P. *et al.* 2008. Primate Model of Uveoretinitis and Vasculitis/ Experimental Autoimmune Uveoretinitis Inducedin Cynomolgus Monkeys by Retinal S Antigen. Ophthalmic Res 40:181-188.
24. Nussenblatt, R.B. *et al.* 1981. S-Antigen Uveitis in Primates a New Model for Human Disease. Arch Ophthalmol 99: 1090-1092.
25. Guex-Crosier, Y. *et al*. 1997. Humanized Antibodies Against the a-Chain of the IL-2Receptor and Against the P-Chain Shared by the IL-2 andIL-15 Receptors in a Monkey Uveitis Model of Autoimmune Diseases. J Immunol, 158: 452-458.
26. Marcq, C *et al*. 2015. Improving adjuvant systems for polyclonal egg yolk antibody (IgY) production in laying hens in terms of productivity and animal welfare. J Vet Immunol Immunopathol. 165(1-2):54-63
27. Kolstad, A.M. *et al*. 2012. Effect of pain management on immunization efficacy in mice. J Am Assoc Lab Anim Sci. 51(4):448-57
28. Fishback, A. E. *et al*. 2016. Antibody production in rabbits administered Freund's complete adjuvant and carprofen concurrently. Lab Anim (NY). 45 (2):63-6.
29. Sanchez, P. 2014. Generating a battery of monoclonal antibodies against native green fluorescent protein for immunostaining, FACS, IP, and ChIP using a unique adjuvant. Monoclon Antib Immunodiagn Immunother. 33(2):80-8.
30. Peterson, N.C. 2000. Behavioral, Clinical, and Physiological Analysis of Mice Used for Ascites Monoclonal Antibody Production. CM 50(5): 516-526.

31. Canadian Council on Animal Care Guidelines on: Antibody Production. 2002.
32. A Report of the Committee on Methods of Producing Monoclonal Antibodies Institute for Laboratory Animal Research National Research Council. 1999.
33. Immunization Procedures and Adjuvant Products. 2005. ILAR Journal 46(3): 227-320.
34. Del Giudice, G. *et al*. 2018. Correlates of adjuvanticity: A review on adjuvants in licensed vaccines. Sem Immunol. 39: 14-21.
35. Burakova, Y. *et al*. 2018. Adjuvants for Animal Vaccines. Viral Immunol. 31(1): 11-22.
36. Sayer, S., et al. "Vaxjo: A Web-based Vaccine Adjuvant Database and Its Application for Analysis of Vaccine Adjuvants and Their Uses in Vaccine Development." J Biomed Biotechnol, 2012. Web 10 Apr 2013. http://www.violinet.org/vaxjo/index.php.

-