



Recommendations for Use and Alternatives to Freund's Complete Adjuvant

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The consideration of alternatives to potentially painful procedures is required. Freund's Complete Adjuvant has been the mainstay of immunological adjuvant in research for decades. While usually effective, may induce undesirable side effects, it causes local inflammatory lesions which can be quite severe and result in chronic granulomas, abscesses, and tissue sloughs. Injected into the footpad, it can cause chronic lameness and arthritis; injected intraperitoneally, it can cause peritonitis.. To promote proper consideration of alternatives to Freund's Complete Adjuvant (FCA) the following list was compiled.

- **Ribi Adjuvant System (RAS)** - Ribi markets a wide variety of immunologic products. The primary product marketed as an alternative to Freund's complete adjuvant is a oil-in-water emulsion containing detoxified endotoxin (MPL) and mycobacterial cell wall components (TDW, CWS) in 2% squalene. It is as convenient to use as Freund's complete adjuvant (or more so because of its lower viscosity) and it has very low toxicity, having been used in humans. In some circumstances, especially in mice, it compares well with Freund's complete adjuvant. Preparation of the adjuvant-antigen emulsion in a tissue grinder with a teflon pestle (Potter-Elvehjem type) is encouraged for optimal results but it is not essential. No longer being produced. Limited quantities are available from: Sigman M6536
- **TiterMax** - A product marketed specifically as a replacement for Freund's complete adjuvant, TiterMax is a stable, metabolizable water-in-oil adjuvant, a single injection of which the manufacturer has shown to induce antibody titers greater than 2 injections of Freund's (Freund's complete adjuvant followed by Freund's incomplete adjuvant). Available from: CytRx Corporation 150 Technology Parkway Technology Park/Atlanta Norcross, Georgia 30092 (800)-345-2987
- **Syntex Adjuvant Formulation (SAF)³** - Has been developed as an alternative to Freund's complete adjuvant. It is a performed oil-in-water emulsion stabilized by Tween 80 and pluronic polyoxyethylene/polyoxypropylene block copolymer L121. SAF activates complement by the alternate pathway and is said to bias the humoral immune response to IgG2a in the mouse. Like other oil-in-water adjuvants, it works better with proteins that have some hydrophobic aspect to promote their adherence to oil droplets. Available from Chiron Corporation, Emeryville, California.

- **Freund's Incomplete Adjuvant (FIA)** - the most inflammatory component of FCA, killed mycobacteria, is not included in FIA. FIA is routinely used for boosting immunizations subsequent to FCA. It can also be used for the initial immunization, particularly when a strong antigen is used or moderate antibody levels are sufficient. As with FCA, efficacy is dependent upon vigorous mixing of the adjuvant and antigen until a stable emulsion has formed. Available from: Numerous research product companies.
- **ALUM - aluminum hydroxide;** $Al(OH)_3$. Aluminum hydroxide is a widely used adjuvant, especially in commercial products such as vaccines. It is very well suited for strong antigens. Many sources of aluminum hydroxide are available. Commercially available as Alhydrogel, Accurate Chemical & Scientific Co, Westbury, New York.
- **SuperCarrier** - this and some similar products are convenient kits for coupling haptens, such as peptides and small proteins, to larger carrier molecules to enhance immunogenicity. The coupled proteins can be combined with other adjuvants. Syntex Research 3401 Hillview Ave. P.O. Box 10850 Palo Alto, CA 94303
- **Elvax 40W1,2** - this is an ethylene-vinyl acetate copolymer. While the production process is somewhat involved, the resulting immune response can surpass that induced by Freund's complete adjuvant. Sold in 25 gram amounts, however small gratis amounts are available. Available from: DuPont Chemical Co. Wilmington, DE (800)-628-6208; (request Elvax customer representative)
- **L-tyrosine** - a co-precipitate of the amino acid and antigen has been shown to have excellent adjuvant properties, even surpassing Freund's complete adjuvant in some circumstances. While the co-precipitation procedure is not difficult, it requires some manipulation. The cost is negligible. Available from numerous chemical companies.
- **Montanide** - a manide-oleate compound that has been shown to produce antibody levels equivalent to Freund's complete adjuvant. Small aliquots are available upon request from the producer. Available from: ISA Seppic Fairfield, NJ
- **AdjuPrime** - this is a carbohydrate polymer marketed as an alternative to Freund's complete adjuvant. Independent studies comparing its efficacy to other adjuvants do not appear to be available. The carbohydrate is thought to create both a "depot" effect and an enhancement of the interaction between the antigen and antigen-presenting cells.
- **Nitrocellulose-absorbed protein**⁸- Nitrocellulose-absorbed protein will give a desirable slow release of antigen over a period of 2 weeks to 2 months. The nitrocellulose is inert and causes minimal inflammatory response.
- **Gerbu adjuvant** - This is an aqueous phase adjuvant that does not have a depot effect. It therefore requires frequent boosting to achieve a high-titer response, but produces minimal inflammatory response. Gerbu adjuvant is available from C-C Biotech, Poway, California.
- **Immune-stimulating complexes (ISCOMS)** - ISCOMS are Ag-modified saponin/cholesterol micelles that form stable cage-like structures. ISCOM-associated antigen molecules do not form a depot site but are transported to the draining lymph nodes. Quantities of antigen as low as 1 ug have elicited a significant immune response. ISCOMS can be successfully prepared in the laboratory⁹.

Guidelines for use of Freund's Complete Adjuvant (FCA) in Laboratory Animals

Many of the undesirable side effects of FCA can be reduced or eliminated through refinements in its use. Subcutaneous and intramuscular routes with separation between inoculation sites adequate to avoid coalescence are preferred. The inoculation should be kept to the minimum volume practical and should not exceed the following guidelines.

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- Before using FCA, consider the use of the incomplete Freund's adjuvant or a different adjuvant.
- If FCA must be used, it should be limited to the initial immunizing dose. Use of two or more doses of FCA is rarely warranted and must be strongly justified with objective data. Two to three weeks should be the minimum period after initial immunization with CFA, before reimmunization with a different antigen-adjuvant combination.
- Proper preparation of the FCA-antigen emulsion will limit inflammation. The mycobacterial component of FCA should be resuspended before use by vortexing or shaking. One part or less of FCA to one part antigen (v/v) is recommended. An emulsion is properly prepared when it becomes thick, will not separate on standing and will not disperse when a droplet is placed in saline. Emulsification is enhanced by use of cold (4C) adjuvant. It has been suggested that formulations of FCA not exceeding 0.1 mg dry mycobacterial cell mass/ml should be used to limit inflammation⁷.
- Routes of administration:

Rabbits: Routes other than subcutaneous or intramuscular are unacceptable. Intradermal injections often result in skin sloughing. This route should be discouraged unless strongly justified. If the intradermal route is unavoidable, a maximum of 0.05 ml should be administered per site. For subcutaneous injections, the inoculum should be divided into fractions of no more than 0.1 ml per site and not exceed a total volume of 1 ml. Injection sites should be separated from each other widely enough to ensure continued blood supply to adjacent areas of skin and subcutis. Prior to injection the site should be clipped and aseptically prepared. Anatomic sites used for grasping, handling, or restraint such as the dorsal cervical/scapular areas should be avoided. Intramuscular inoculum should be of a volume not greater than 0.5 ml for rabbits, administered by a single injection into a deep muscle mass. The area where the needle penetrates the skin should be prepared with appropriate antiseptics. Foot pad immunization is not acceptable in rabbits.

Mice: Intraperitoneal administration has been used to obtain higher titered polyclonal or monoclonal antibodies. The volume should not exceed 0.2 ml. Peritonitis can be minimized by limiting the use of FCA to the initial immunizing dose. Subcutaneous injections should be limited to a total volume of 0.2 ml with no more than 0.1 ml administered per site.

Where scientific justification is provided (i.e. isolation of draining lymph nodes is required), foot pad immunization may be performed. The quantity of FCA administered should be minimal and only one foot per experimental animal should be injected. Also, the animal should be housed on soft bedding rather than screens to prevent painful ambulation. The maximum volume allowed is 0.05 ml for mice and 0.10 ml for rats.

- The use of sterile needles and syringes is mandatory.
- Precautions should be taken to avoid the inadvertent exposure (especially injection) of laboratory personnel to FCA. Use of gloves, safety glasses, and laboratory coats is prudent. To avoid inadvertent injection, luer lock syringes should be used, needles should not re-capped, anesthesia or sedation should be utilized, and the technician should be familiar with proper animal restraint techniques.
- Injection sites should be clean and free of debris and contamination which could result in local infection.
- Additive inflammatory effects due to use of nonsterile preparations, excessive vehicle pH, or the presence of byproducts of purification such as polyacrylamide gel, SDS, urea, acetic acid, or other solvents or potentially toxic agents should be avoided. Special precautions may be necessary if the antigen itself is a viable microbe.
- Animals should be observed at least 3 times per week for a minimum of 4 weeks post-injection. Excessive swelling, abscess or fistula formation, and infection or ulceration at immunization sites should be brought to the attention of the veterinary staff.
- Approximately 1% of a rabbits body weight in blood can be removed every two weeks for antibody harvest. Proposals requiring phlebotomies more frequent than every two weeks will require additional monitoring procedures such as measurement of hematocrit and total protein.

References:

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