UBS AWERB 3Rs Guidelines:

The use of Freund's Complete Adjuvant (FCA or CFA) or Freund's Incomplete Adjuvant (IFA or FIA)

Introduction- Animal Welfare and Refinement-The use of FCA has been associated with a variety of lesions, including localized injection site granulomas. The potential for pain and distress have resulted in numerous regulatory guidelines (CCAC 2002 ; Grumstrup-Scott and Greenhouse 1988 ; Leenaars et al. 1999). The purpose of these guidelines are to ensure consideration of the nature of the antigen and the host immune responsiveness for research is balanced against the extent of inflammation, necrosis, and potential pain and distress to the animal.

- **Preparation**- changes in the paraffin oil used in commercially available IFA meant thick emulsion using the syringe-to-syringe technique is not possible. Follow a method published by Brand et al (paper attached) to make your own IFA/CFA and use a homogeniser, taking care not to generate heat to inactivate the collagen that had previously been dissolved in acetic acid.

- **The volume** – 50µl total volume per mouse

- **The site** – injected subcutaneously into the skin just above the base of the tail (not the tail itself)

- **Injection technique**- keep the needle in place for a few seconds, for a bleb to appear, before withdrawing the needle.

- **Injection**-use a small grey needle (27G) connected to a 1 ml leur lock syringe

- **Site preparation**-clip the fur from the skin first and clean the area with an alcohol swab before injecting and mop up any leakage afterwards.

- **Anaesthesia**-use of isoflurane anaesthesia is advised to keep the mouse as still as possible

- **The composition** – consistency of the CFA or IFA emulsion is important
    - Commercially made it is less viscous, so needs wiping away after needle withdrawal from the skin, as the leakage causes ulceration.

- **The passing on of skills** – SOPs, training and competency records and any other procedural documentation should be regularly reviewed. To ensure that these skills and learning points are transferred through the team across the years.

AWERB Statement- The use of adjuvants such as Complete Freund's requires special care and consideration of their inflammatory properties. Volumes and frequencies are normally specified in a PPL, but as an example, in mice, <0.05 ml at each of 2 sites will be used. Injections of Complete Freund's should be confined to the priming step and the s/c route to prevent granuloma formation.

Collagen-induced arthritis - Brand et a

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APPENDIX A- COMMON ADVUVANTS

https://ccac.ca/Documents/Standards/Guidelines/Antibody_production.pdf

This is a list of the more commonly-used adjuvants and their basic properties. It is not an endorsement of any specific product.

Freund's Complete Adjuvant (FCA) is a water-in-oil emulsion of mineral oil, mannide mono-oleate, and heat-killed *Mycobacterium tuberculosis* (or *M. butyricum*) or components of the organism. FCA is a potent adjuvant that stimulates both humoral and cell-mediated immunity. FCA is often strongly reactive as the mineral oil cannot be metabolized and the mycobacterial components can elicit severe granulomatous (inflammatory) reactions. The concentration of mycobacteria varies greatly among commercial preparations of FCA. In this regard, it should be noted that if the concentration of mycobacteria is less than 0.5mg/ml (0.1mg/ml in Broderson [1989]), less severe inflammatory reactions result.

Freund's Incomplete Adjuvant (FIA) is the same as FCA minus the mycobacterial cells or cellular components. FIA is less effective than FCA for inducing high antibody titres and enhancing cell-mediated immunity.

Ribi™ Adjuvants are oil-in-water emulsions in which the antigen is blended with a minimal volume of oil and then emulsified in a saline solution containing the surfactant Tween 80 (Ribi, *et al.*, 1975). Although oil-in-water emulsions are less viscous and easier to inject than water-in-oil emulsions, they are poor adjuvants alone, requiring immunostimulants to increase the immunogenicity. The immunostimulants used in the Ribi™ system include two refined mycobacterial products (trehalose 6,6'-dimycolate and cell wall skeleton) and a purified gramnegative bacterial product (monophosphoryl lipid A). For a further explanation of the biological activities of Ribi™ adjuvants see Jennings (1995). Ribi™ adjuvants are usually less potent than FCA, but they are also less toxic and have provided satisfactory adjuvant activity for many purposes.

Monophosphoryl lipid A (one of the components of the Ribi™system) is by itself a potential adjuvant option. As with aluminum hydroxide, the safety profile of MPL is well documented and provides excellent adjuvant activity for some immunogens.

TiterMax™ Adjuvant relies on a microparticulate water-in-oil emulsion formed with a non-ionic block copolymer (CRL-8941) and squalene, a metabolizable oil. CRL- 8941 is coated with silica particles which stabilize the emulsion. Stability is a key property of TiterMax™ as it enables the emulsion to contain a wide variety of antigens without the use of large amounts of toxic emulsifying agents. The copolymer enables more antigen to be carried on its surface than if the antigen were in solution. It also activates complement, assisting in retention of the antigen in the lymphoid tissues and activation of immunoreactive cells. TiterMax™ induces increased expression of class II (Ia) major histocompatibility complex molecules (MHC) on macrophages, and increases the presentation of Ag to T cells. When TiterMax™ works well, it can induce antibody titers as high or higher than FCA and exhibits less toxicity. However, adjuvantation with TiterMax™ is not always successful. In addition, TiterMax™ contains small quantities of an emulsifying agent and silica particles which have been implicated in delayed-type vaccine reactions.

Quil A is a partially purified form of saponin or triterpene glycoside derived from the bark of the *Quila saponara* tree and purified to reduce the presence of components which cause adverse local reactions. In general, saponins should not be injected intraperitoneally or intravenously due to their hemolytic activity. As Quil A is only semi-purified, it can still induce significant pyrogenic and local reactions. Therefore, prior to using a formulation containing Quil A, it should be evaluated *in vitro* (i.e., for cell culture toxicity). The adjuvant properties of Quil A rely on the formation of iscoms (immune stimulating complexes) which are especially useful in inducing Abs to membrane antigens. The iscoms are 35nm clathrate bodies like micelles made of Quil A, cholesterol, phosphatidyl choline and antigen. The purified saponin component which has the highest adjuvant/toxicity ratio is called QS21. Saponin containing formulations should never be administered via mucosal surfaces, such as intranasal when induction of IgA is desired.

Mineral-Based Adjuvants. Three mineral compounds are generally used in adjuvants:

aluminum hydroxide; aluminum phosphate; and calcium phosphate. Aluminium salts are claimed to be superior to all other adjuvants in their ability to increase immune responses against weak immunogens, including those for which FCA does not work. Alhydrogels are sterile aluminium hydroxide gels that are pyrogen-free and stable, they have a high adsorptive capacity. At pH <9 alhydrogels have a positive charge so they readily adsorb negatively charged molecules (e.g., proteins at neutral pH).

CpG DNA is a non-specific immune activator that can be used alone or in combination with other adjuvant components to augment immune responses. Commercial preparations of CpG DNA comprise short pieces of DNA (oligonucleotides) that contain unmethylated cytosine-guanine dinucleotides within a certain base contact. The mammalian immune system has evolved to recognize these sequences, which are found naturally in bacterial DNA, as a sign of infection. Different CpG DNA sequences activate the immune systems of different species, and the commercial preparations of this adjuvant are therefore species specific.