PROTEIN IMMUNISATION WITH ALUM ADJUVANT FOR POLYCLONAL ANTIBODIES

A simple way to generate polyclonal antibodies in mice or rats. Alum is a classical adjuvant discovered in the 1920s, easy to prepare and less inflammatory than Freund's Complete Adjuvant. It preferentially stimulates Th2 and IgG1 antibody responses [1]. If immunising for the first time (eg a new recombinant protein), immunise mice of 2 strains (eg BALB/c and C57BL/6) as occasionally one strain fails to respond to an antigen. Alternatively, immunise 2 rats as responses vary - compare serum titres at the end of the protocol and select the stronger antiserum.

Reagents and Equipment

1. Protein in solution, ideally 200-500 µg in 1 ml PBS, although lower concentrations, and proteins in other buffers including 8M urea or imidazole, can be used (see *Notes*). For the first (primary) immunization aim to use 50 µg antigen/mouse or 100 µg antigen/rat. Secondary boosts can be 10-20 µg.
2. 9% Potassium alum (Sigma A7167 aluminium potassium sulphate) in sterile water)
3. 1M NaOH
4. Phenol red dye (Sigma P0290).
5. A vigorous vortex machine.
6. Bench-tip centrifuge (eg Beckman Allegra X-12) with swinging bucket rotor (eg SX4750).

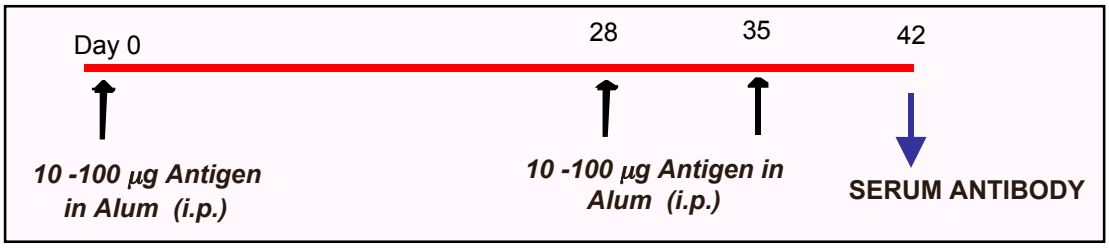
Protocol (for 2 rats or 10 mice)

1. In a 15ml falcon tube add 1 ml of 9% Potassium alum to an equal volume of protein solution.
2. Add 20 µl of phenol red indicator dye (this will go yellow).
3. Neutralise by adding 1 M NaOH dropwise, until indicator turns to pink/red as in the adjacent image (expect to add ~400 µl). Vortex regulalry, and gently, to mix well.
4. Stand for 30 mins at Room Temperature (RT°).
5. Fill tube with sterile PBS and centrifuge 10 mins, 2500 *g* at RT°. A large pellet (~0.5 ml) will be seen, the supernatant can be poured off and discarded. The pellet will require vigorous vortexing to resuspend.
6. Wash the pellet 3 x in 10 ml sterile PBS and centrifuge 10 mins, 2500 *g*, RT°.
7. Resuspend the pellet in 0.4-1.0 ml total volume. Inject 100 µl per mouse intraperitoneally, or 200 µl per rat subcutaneously.

The protocol is very flexible with respect to concentrations, solubility and buffer conditions of the protein to be used for immunisation, as any undesirable components (eg urea, imidazole) are removed in washing the pellet.

The starting volumes of protein and alum can be smaller for fewer animals or larger for lower protein concentrations, as the precipitation effectively concentrates the immunogen.

Immunization Schedule



The standard regimen is to inject 10-100 µg antigen in alum on day 0, boost with 1-10 µg at days 28 and 35, and bleed out at day 42. Timings can be varied, but be sure to rest animals for ≥28 days after primary immunization, as high affinity B cell clones mature under low antigen concentrations in the latter part of this period. Doses can be lower if antigen availability is limited.

Serum Collection

Confer with licensed animal worker for tail-bleeding, brachial artery or cardiac puncture methods. Collect blood individually for each animal, expect up to 1 ml for mice and 5 ml for rats. Allow to clot (4 hrs RT° or overnight 4°C); rim the container with a wooden toothpick to allow clot to contract (wood also binds fibrin helping the serum to separate).

To collect serum, centrifuge the fluid portion of the clotted blood, 1800 *g* for 10 mins. Expect to recover 50% of blood volume. The serum should be clear, straw-coloured; red coloration indicates extensive haemolysis - to be avodied although antibody function should not be affected.

Testing and Storage

Test each serum on ELISA plates coated with the immunizing protein antigen. Use 1/200 and 1/2000 dilutions in duplicate, and a polyvalent rabbit anti-mouse (eg Agilent DAKO P0260) or goat anti-rat (eg Abcam 97057) horseradish peroxidase-conjugated antibodies. Pool individual sera with comparable titres (typically 3-5 mice per group will be high titre).

Correct archiving of antibodies is essential. Rats are given sequential numbers, mouse are stored by experiment protocol code. Store aliquots in screw-cap tubes clearly labelled with the reference number or code in –80°C freezer tray. Update file on Maizels Lab records, currently on One Drive.

Notes

The precipitation depends on proteins absorbing to the alum. Adsorption of protein is dependent on the pI (isoelectric pH) of the protein and the pH of the medium. A protein with a lower pI adsorbs to the positively charged aluminium ion more strongly than a protein with a higher pI.

The protein solution can be in urea or detergents, as these agents will be washed out during the precipitation. Be aware, however, that if proteins are only soluble in denaturing agents, they are unlikely to folded correctly. Antibodies will then be primarily against linear epitopes, not conformational epitopes: fine for Western blots, poor for immunofluorescence.

ThermoFisher sell *Imject* (Cat No 77161) alum formulation which can be mixed with protein and directly injected without precipitation and washing which can save effort if protein is at sufficiently high concentration in a physiological buffer. However, this has been found to be far less immunogenic that traditional alum adjuvant [2}.

Alhydrogel, marketed by InvivoGen (http://www.invivogen.com/alhydrogel) uses the same principles of adhering protein to alum particles in a proprietary formula, with similar but not always as good results as traditional alum precipitation [2].

Immunisation of rats has the advantage over mice beyond the greater volumes of serum for a lower quantity of protein injected, as the antibodies can be used in immunohistochemistry of mouse tissues using rat IgG-specific secondary conjugate.

References

[1] **Marrack P, McKee AS, Munks MW.** 2009. Towards an understanding of the adjuvant action of aluminium. *Nat Rev Immunol* **9:**287-293.

[2] **Cain DW, Sanders SE, Cunningham MM, Kelsoe G**. 2013. Disparate adjuvant properties among three formulations of "alum". *Vaccine* **31**: 653-60

Version 2. Updated by Rick Maizels and Danielle Smyth 28 March 2020