gibco

24 days of stem cells

Shape the future of stem cell innovation

October 1- November 1, 2019

Join us for 24 Days of Stem Cells; a premiere virtual event featuring the latest advances in stem cell research.

This year's format will feature a new hour of cutting edge content every week day starting October 1st. Attend the sessions that are most relevant to your work - at your convenience and at your pace.

During the 24-day long event, you can:

- Access leading scientific presentations from thought leaders around the world
- Watch live training demonstrations from our stem cell experts
- Download key stem cell tools and resources
- Complete weekly challenges to earn points towards certification and prizes

Register today at
www.24daysofstemcells.com

ThermoFisher SCIENTIFIC

WILEY

Immunization of Mice

Tomás Maira-Litrán[1]

[1]Division of Infectious Diseases, Department of Medicine Brigham and Women's Hospital/Harvard Medical School, Boston, Massachusetts

The selection of an appropriate immunization strategy depends largely on the properties of an antigen, including its nature, purity, solubility, and availability. This unit describes the critical steps in the production of monoclonal and polyclonal antibodies against soluble molecules (such as proteins, peptides, polysaccharides, oligosaccharides, or hapten-conjugate vaccines), complex antigens (such as whole pathogens or outer membrane vesicles), and antigens embedded in or eluted from gel matrix following electrophoresis. © 2017 by John Wiley & Sons, Inc.

Keywords: adjuvant • antigen • monoclonal antibody

How to cite this article:
Maira-Litrán, T. 2017. Immunization of mice. *Curr. Protoc. Mol. Biol.* 117:11.4.1-11.4.11.
doi: 10.1002/cpmb.30

INTRODUCTION

Immunization schedules are generally based on logic, empiricism, and previous experience. The production of high titers of antigen-specific B-cells requires multiple immunizations of mice with the antigen under study. Yet critical aspects of an immunization scheme to carefully consider include: the mouse strain to be immunized, the type and dosage of antigen, the use of adjuvant, the mixing of antigen and adjuvant, and the choice of immunization route and immunization regimen.

Antigen is generally mixed with adjuvant (usually Freund's adjuvants) and administered into mice via intraperitoneal or subcutaneous route. Complete Freund's adjuvant, which contains heat-killed *Mycobacterium tuberculosis*, is quite irritating or toxic, engenders animal safety issues, and elicits a high level of antibodies to *M. tuberculosis* antigens. These antibodies can alter the specificity of immune sera because they will react to similar antigens shared among microbial species. The initial immunization is followed by several booster immunizations at 14 to 28 days intervals until optimal levels of reactive antibodies are reached. Mice receive a final booster immunization of antigen without adjuvant 3 to 5 days before they are euthanized, at which point spleens are removed and B cells are collected and fused with partner myeloma cells.

This unit describes immunization protocols for the production of monoclonal antibodies against three major types of antigens: Basic Protocol 1 describes immunization with soluble molecules (e.g., proteins, polysaccharides, peptides, oligosaccharides, or hapten-conjugate vaccines); Basic Protocol 2 describes immunization with highly complex antigens (e.g., whole pathogens or outer membrane vesicles); and Basic Protocol 3 and Alternate Protocol 1 describe immunization with antigens either embedded in a polyacrylamide gel slice or electroeluted from a gel slice following isolation by electrophoresis.

Current Protocols in Molecular Biology 11.4.1-11.4.11, January 2017
Published online January 2017 in Wiley Online Library (wileyonlinelibrary.com).
doi: 10.1002/cpmb.30

NOTE: All protocols involving experimental animals must be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must be conducted in strict accordance to governmental regulations regarding the care and use of laboratory animals.

BASIC PROTOCOL 1

PRODUCTION OF IMMUNE SPLEEN CELLS: IMMUNIZATION WITH SOLUBLE ANTIGENS

This protocol describes the use of soluble macromolecules, including proteins, polysaccharides, peptides, oligosaccharides, hapten-conjugate vaccines, or lipopolysaccharides (LPS) as antigens for immunization and subsequent monoclonal antibody development. Antigens are generally dissolved in PBS, formulated with an oil-based such as Freund's adjuvant and used for multiple rounds of immunization.

Materials

Mice (BALB/c is the most widely used strain)
Phosphate-buffered saline (PBS), pH 7.2 to 7.4
Antigen
Complete Freund's adjuvant (Sigma or Colorado Serum Co.)
Incomplete Freund's adjuvant (Sigma or Colorado Serum Co.)

Sterile scalpel blades
Microtainer serum separator tubes (BD Biosciences, cat. no. 365967)
Heat lamp
1- to 3-ml Luer-Lok syringes (BD Biosciences)
Female-to-female Luer-Lok connector (BD Biosciences, cat. no. 513110)
20-G and 22-G needles
Webglue (Patterson Veterinary, cat. no. 07-856-6128)
Microcentrifuge

Additional reagents and equipment for enzyme-linked immunosorbent assay (ELISA; *UNIT 11.2*; Hornbeck et al., 1991) and fusion of myeloma cells with immune spleen cells (*UNIT 11.7*; Fuller et al., 1988)

Obtain pre-immunization blood sample

1. Draw pre-immune blood from 3 to 4 mice (e.g., BALB/c; 4- to 6-weeks old, females preferred), and determine antibody titers to immunization antigen in serum by ELISA (*UNIT 11.2*; Hornbeck et al., 1991) to establish a baseline pre-immune level.

   Starting each monoclonal antibody project with more than one animal, generally 3 to 4, is always recommended. The antigenic response will likely vary between animals. Using multiple animals will likely increase immune diversity and translate into antibodies of different quantity, specificity, and affinity.

   Female mice are generally chosen for immunization since they typically develop higher antibody responses than males.

   The most common method for drawing blood is to hold a scalpel blade perpendicular to the tail, nick blood vessels in the superficial tail vein, and collect the blood into clot-activator, microtainer serum-separating tubes (Diehl et al., 2001). Anesthesia is not essential, but effective restraint is. Warm the tail with a heat lamp to increase blood flow and the volume of blood that can be collected: ~100 to 200 µl per mouse. After drawing pre-immune blood gently press the nick for ~30 sec and apply a drop of tissue glue (e.g., Webglue) to ensure hemostasis.

   *Alternatively, blood can be drawn from the mandibular vein or artery of mice and rats (Golde et al., 2005). Videos that demonstrate the technique can be readily accessed online (e.g., **YouTube**—mandibular artery/vein bleeding of mice). Stretch the skin and pierce the*

cheek skin over the mandibular vein using a specially designed lancet or needle. Siphon the accumulating blood droplet using a capillary tube or similar device. Bleeding may be done without anesthesia, but appropriate restraint is essential. After collecting blood, apply gentle pressure for 30 sec to ensure hemostasis.

2. Centrifuge the microtainer tubes in a microcentrifuge for 30 sec at 16,000 × *g*, 4°C, to separate serum from blood. Transfer serum samples to a 1.5-ml microcentrifuge tube, and store at −20°C for later use.

Prepare antigen solution

3. Prepare an appropriate volume of PBS containing antigen: 25 to 100 µg proteins, polysaccharides, and lipopolysaccharide (LPS) per mouse, or 1 to 10 µg peptide or oligosaccharide conjugate per mouse.

Prepare emulsion using two syringes and a Luer-Lok connector

4a. Decide on the total volume of antigen and adjuvant to be prepared, and select two syringes that each hold twice the antigen-adjuvant volume. For example, for a final volume of 1.5 ml of antigen-adjuvant emulsion, use two 3-ml syringes for emulsification.

5a. Load the required volume of antigen in PBS into one syringe and the adjuvant into the other syringe, expel any air from the syringes (air will hinder the formation of a stable emulsion), and securely fasten the syringes to a female-to-female Luer-Lok connector.

6a. Manually and firmly pump antigen solution through the connector into the adjuvant syringe, and then pump the entire mixture back and forth from one syringe to the other as follows: 20 cycles slow (approximately 4 sec per cycle) and 40 cycles fast (as fast as possible), and then repeat 20 cycles slow and 40 cycles fast. This may take several minutes. A cycle is defined as the process of transferring the entire contents of one syringe through the connecter into the other syringe.

When prepared correctly, a stable emulsion has the appearance of a thick, viscous, homogeneous white mixture that will not separate into oil and water phases nor diffuse when a drop is placed on the surface of water. This will be critical in the effectiveness of emulsion adjuvants.

Droplet Test: Check for correct emulsification by expelling a small drop of the emulsion onto the surface of some water in a beaker. If the droplet forms a stable bead on the surface of the water, then the emulsion is considered stable and ready for mouse immunization. If the droplet disperses across the water's surface, reconnect the syringes and carry out additional cycles of emulsification.

7a. Transfer the entire volume of emulsion into one of the syringes, remove the connector, and add a needle for injections.

Prepare emulsion using a syringe and needle

4b. Transfer the desired volume of antigen solution (aqueous phase; step 3) to a glass vial.

5b. Add an equal volume of adjuvant (oil phase).

6b. Connect a large bore needle (e.g., 20 gauge) to a 1-ml syringe, remove the rubber from the plunger of the syringe, and by gently pumping the antigen-adjuvant mixture in and out of the syringe until the mixture is fully emulsified.

The oil in the adjuvant reacts with the rubber plunger of certain plastic syringes.

7b. Check that the mixture is a stable emulsion using the droplet test described in step 6a.

Due to its viscosity the emulsion sticks to the walls of the vial making recovery difficult, and some loss of material is inevitable. Preparing a little extra emulsion should compensate for losses.

8. The antigen-adjuvant emulsion can be stored at 4°C for several days before use.

Immunize mice to prime the immune response

9. Inject the emulsion containing 25 to 100 μg of antigen per dose, per animal into mice via intraperitoneal (<0.3 ml) or subcutaneous route (<0.2 ml) using a 22-gauge needle. Inject the emulsion very slowly to avoid dislodging the needle from the syringe, and make sure that the needle penetrates several millimeters into the subcutaneous tissue or peritoneal cavity to minimize leakage. Be sure to observe mice for potential toxicity after immunization, as required by approved animal protocols, particularly when working with antigens not previously used to immunize mice.

A medium-gauge needle (e.g., 22-G) is used to inject the highly viscous emulsion that would be otherwise difficult to inject using a higher gauge needle (e.g., 25-G or 27-G). The use of a 22-G needle also reduces the risk of leakage after injection and pain associated with the use of larger bore needles (e.g., 18-G or 20-G).

Immunize mice to boost the immune response

10. After 2 to 3 weeks, immunize mice with 10 to 50 μg of antigen per animal emulsified in Incomplete Freund's adjuvant. Prepare the booster antigen-adjuvant emulsion using one of the methods described above.

Complete Freund's Adjuvant should only be used for the first (priming) immunization dose. Subsequent boosts should be given with Incomplete Freund's Adjuvant or another adjuvant and not Complete Freund's Adjuvant unless properly justified.

Alternative adjuvants can be found online.

11. After 2 to 3 weeks, immunize the mice a third time as described in step 10.

Bleed mice to assay antibody titer

12. After 2 to 3 weeks, bleed mice as outlined in step 1, and perform an ELISA to measure antibody titers to antigen. Select animal(s) with highest serum-antibody titers for the final boost and for monoclonal generation.

Perform final boost and harvest B cells

13. After 3 weeks, immunize each mouse (selected in step 12) with a final boost of 10 to 50 μg of antigen in PBS (<0.2 ml) without adjuvant via intravenous injection in the tail vein.

The main objective of the final boost is to induce a strong response and synchronize the maturation of the response with the time of B-cell collection (fusion day), 3 to 4 days after the boost. If this synchronization occurs, a large number of antigen-specific lymphocytes will be present in the local lymphoid tissue to be harvested for B-cell collection. Since spleen is generally the best tissue from which to harvest B cells, the final boost should aim to localize the response to this organ, which is best achieved via intravenous injection. If antigen cannot be injected intravenously due to, for example, solvent incompatibility with this route of immunization, an intraperitoneal injection could be used instead, and the fusion performed 5 days after the final boost.

In mice, serum titers begin to drop approximately 2 weeks after an immunization. The final boost should therefore be given at least 3 weeks after last immunization to allow most of the circulating antibodies to be cleared from the bloodstream, as they can bind to the antigen and reduce the amount of circulating antigens and in turn the strength of the boost.

14. After 3 days, harvest B cells and fuse to myeloma cells (*UNIT 11.7*; Fuller et al., 1988).

IMMUNIZATION WITH COMPLEX ANTIGENS (MEMBRANES, WHOLE CELLS, AND MICROORGANISMS)

BASIC PROTOCOL 2

Live, killed or attenuated microorganisms (e.g., bacteria, viruses, or yeast), mammalian cells, or microbial membrane vesicles (MMVs) can be used as immunogens for monoclonal antibody development. The advantages of using whole microorganisms or MMVs as vehicles for antigen delivery are two-fold. First, antigen epitopes are presented in the context of a whole organism or membranes, thus maintaining discontinuous or conformational epitopes that may be otherwise unavailable or destroyed when antigens are recombinantly expressed or extracted from their specific organism sources. Second, whole pathogens and MMVs have potent immunostimulatory capacity and provide an "adjuvant effect" that enhances the response to low-immunogenicity antigens and, in certain cases, can be formulated without the need of adjuvants.

For materials, see Basic Protocol 1.

1. Prime mice via intraperitoneal injection with 10^5 to 10^9 cells or with 5 to 25 μg of an MMV preparation emulsified with adjuvant (see Basic Protocol 1, steps 1 to 9), or directly suspended in PBS without adjuvants.

   When using whole pathogens (live or attenuated) as antigens, it is critical to ascertain that the immunization doses and protocols do not lead to infection or disease from toxic microbial components. The optimal immunization dose should be determined a priori in a pilot study by challenging mice (n=3 to 4) three times at 2 to 3 week intervals with various doses cells ranging from 10^5 to 10^9 cells per mouse and testing antibody responses to specific antigens by ELISA (see Basic Protocol 1, step 12). The optimal immunization dose should induce the highest titer of antibodies to desired target with minimal animal mortality. This dose will depend on the specific pathogen's virulence or level of attenuation.

2. Boost mice two more times at 2 to 3 week intervals with the same dose of antigen that was used to prime the immune response in step 1.

3. Bleed mice and measure the serum titers of antigen-specific antibodies by ELISA (see Basic Protocol 1, step 12).

4. Choose the mice that show the highest antibody titers in step 3. At least 3 weeks after last immunization, perform a final boost by intravenous injection with the same dose of antigen that was used to prime and boost (steps 1 and 2) in PBS but omit adjuvant.

5. After 3 days, harvest B cells and fuse to myeloma cells (*UNIT 11.7*; Fuller et al., 1988).

IMMUNIZATION WITH ANTIGEN EMBEDDED IN POLYACRYLAMIDE GEL FOLLOWING ELECTROPHORESIS

BASIC PROTOCOL 3

Antigens purified via sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) can be used to immunize animals directly, without elution from the polyacrylamide gel. Alternatively, antigens can be eluted from the polyacrylamide gel matrix using an electrical field and captured in a buffer solution contained in a dialysis membrane with an appropriate molecular weight cut off (Alternate Protocol 1).

These methods are useful when only small quantities of antigen are available. Antigens purified by electrophoresis induce robust antibody responses. But because the antigens are denatured, the resulting antibodies are more likely to target linear epitopes (regions of antigen formed by a contiguous segment of the amino-acid sequence) than conformational epitopes (antigen segments composed of residues that are sequentially discontinuous but spatially close together in three-dimensional space). This distinction could

have significant implications, depending on the desired application of the monoclonal antibodies.

In this procedure, the gel is negatively stained with a zinc-imidazole solution after electrophoresis: the zinc-imidazole complex precipitates throughout the gel, except in zones where macromolecules (e.g., proteins, peptides, glycolipids, or LPS) are present, yielding clear unstained antigen bands against a white-opaque background. The unstained antigen band is then excised from the gel, the fixative is removed, and the gel slice is ground up and used for immunization. This staining method is reversible, simple, fast (generally taking less than 20 min to complete), highly sensitive (as low as 1 ng may be detected in the gel), and inexpensive.

Materials

Antigen mixture
Preparative SDS polyacrylamide gel
0.2 M imidazole-0.1% (w/v) SDS
0.2 M $ZnSO_4$
0.2 M glycine, 25 mM Tris (pH 8.3)

Vertical SDS-PAGE apparatus and power pack
Battery-operated pestle grinder system (Fisher Scientific)

Additional reagents and equipment for one-dimensional SDS gel electrophoresis of proteins (*UNIT 10.2A*; Gallagher, 2012)

Isolate antigen by SDS-PAGE

1. Load antigen mixture on a preparative SDS-polyacrylamide gel and perform electrophoresis using a vertical SDS-PAGE apparatus (*UNIT 10.2A;* Gallagher, 2012).
2. After electrophoresis, transfer the gel to a container containing 0.2 M imidazole-0.1% SDS, and incubate for 15 min.
3. Decant the imidazole-SDS solution, add 0.2 M $ZnSO_4$ solution, and incubate the gel for approximately 30 sec.

   During this step monitor the gel for the appearance of clear unstained bands against the semi-opaque background that forms in the gel by precipitation of zinc-dodecyl sulfate.

4. Quickly remove the $ZnSO_4$ solution, and wash the gel three times in deionized water for 2 to 3 min each wash to avoid overstaining.
5. Identify and excise the band of interest from the gel with scalpel blade and place it into a microcentrifuge tube.

   Carefully remove excess polyacrylamide from the gel fragment to minimize secondary inflammation or irritation from polyacrylamide.

6. Incubate the gel slice for 10 min in 0.2 M glycine, 25 mM Tris (pH 8.3) to remove the fixative.
7. Rinse the gel slice several times with deionized water to remove excess glycine buffer.

   During the staining step antigens interact with Zn^{2+} and become reversibly fixed in the gel. Fixation can be reversed by metal chelation before antigens are used for immunization or electroelution, as described in Alternate Protocol 1.

8. Mince the gel slice with a scalpel blade in a minimal volume of PBS (e.g., 100 μl), and then homogenize in a 1.5-ml microcentrifuge tube using a battery-operated pestle grinder system.

   Alternatively, lyophilize the gel slice and gently pulverize it into a powder using a pestle homogenizer.

9. Suspend the pulverized gel slice in a minimum volume of PBS prior to injection.

   The minimum volume of PBS needed to reconstitute the gel slice for injection should be estimated by adding 100 μl aliquots of PBS to the pulverized gel until the gel is liquid.

Immunize mice

10. Immunize each mouse intraperitoneally with 200 to 400 μl of gel slurry containing 10 to 50 μg of antigen.

    Estimate the amount of antigen in the gel slurry using a semi-quantitative method by comparing the intensity of the antigen band in an SDS-PAGE gel with bands of known concentration (Crawford and Beckerle, 1991). For example, create a standard curve with a protein (e.g., BSA) of known concentration and resolve 10 μl aliquots of each fraction as well the antigen to be quantified on a SDS-PAGE. Stain the gel with Coomassie and measure the band intensities by densitometry. Create a standard curve by plotting band intensity as a function of mass, and use this curve to estimate the concentration of the antigen.

11. Boost mice twice more, at 2 to 3 week intervals, by intraperitoneal injection with 200 to 400 μl of gel slurry containing 10 to 25 μg of antigen.

12. Bleed mice and quantify antibody titers to target antigen by ELISA (see Basic Protocol 1, step 1).

13. After 3 weeks, perform a final boost via intraperitoneal injection (as in steps 10 and 11).

14. Five days after the final boost, harvest B cells and fuse to myeloma cells (*UNIT 11.7*; Fuller et al., 1988).

IMMUNIZATION WITH ANTIGENS AFTER ELECTROELUTION FROM POLYACRYLAMIDE GEL

ALTERNATE PROTOCOL 1

Antigens can be eluted from polyacrylamide gel slices using an electric current. This method is especially advantageous when the concentration of antigen is low, which would therefore require that mice be immunized with larger amounts of material. The extra acrylamide could cause unacceptable toxicity.

Materials *(also see Basic Protocol 3)*

D-Tube Dialyzer Mini, e.g., 6 to 8 kDa cutoff (EMD Millipore, cat. no. 71504) or 12 to 14 kDa cutoff (EMD Millipore, cat. no. 71505)

Choose a membrane with a molecular weight cutoff that is significantly smaller than the antigen of interest.

Horizontal electrophoresis equipment

Additional reagents and equipment for spectrophotometric and colorimetric determination of protein concentration (*UNIT 10.1A*; Simonian and Smith, 2006)

1. Run antigen mixture on a SDS-polyacrylamide gel according to standard methods using a vertical electrophoresis system under conditions that allow the maximum separation of the antigen of interest.

2. Stain gel using the negative Zinc-Imidazole staining method as described in Basic Protocol 3.
3. Place the excised antigen band (unfixed) in a D-Tube Dialyzer Mini (with appropriate molecular weight cutoff) filled with SDS-PAGE running buffer.
4. Place the D-Tube Dialyzer Mini horizontally in a horizontal electrophoresis tank containing SDS-PAGE running buffer and electrophorese the sample at 100 V at 4°C (to ovoid overheating) until the protein band migrates out of the gel slice.

   The duration of electroelution needs to be adjusted for each antigen. As a reference, it takes at least 1.5 hr at 100 V for bovine serum albumin to elute from a 10% SDS-polyacrylamide gel.
5. Remove the gel slice from the D-Tube Dialyzer Mini, and dialyze the tube contents overnight at 4°C against 1 L of PBS to remove SDS, salts, and other small-molecule contaminants.
6. Recover the antigen solution from the D-Tube Dialyzer Mini for downstream processing.
7. If the antigen is a protein, determine the protein concentration using a spectrophotometric or colorimetric protein concentration assay (see *UNIT 10.1 A*; Simonian and Smith, 2006).
8. Follow the mouse immunization protocol for soluble antigens (Basic Protocol 1) using antigen doses of 10 to 50 μg of antigen per immunization.
9. Three days after the final boost, harvest B cells and fuse to myeloma cells (*UNIT 11.7*; Fuller et al., 1988).

COMMENTARY

Background Information

The production of a large repertoire of antibodies targeting a given antigen is directly proportional to the overall level of antibodies to that antigen, as measured by ELISA, in mouse sera prior to fusion. Therefore, the goal of a successful animal immunization strategy is to generate the highest possible titer of antibodies to the immunizing antigen, as this will substantially increase the probability of isolating B-cells that secrete the monoclonal antibodies of interest. Mice with the highest ELISA titers to the target antigen are then euthanized and splenic B-cells are obtained for fusion with myeloma cells.

Antigens vary greatly in chemical nature, purity, availability, and immunogenicity, and thus require different immunization protocols for an optimal immune response. This unit describes three general immunization methods based on the nature of the antigen: soluble (Basic Protocol 1), highly complex (membrane antigens and whole pathogens, Basic Protocol 2), or antigens purified via SDS-PAGE electrophoresis (Basic Protocol 3 and Alternate Protocol 1). The choice of immunization approach should also take into account the final application of the monoclonal (or possibly polyclonal) antibody under development, and in turn the conformation of the molecule to which the antibody will bind. Will the antigen be in its native conformation or denatured? For example, for western blot applications typically detect denatured molecules. A monoclonal antibodies raised against a native, non-denatured antigen might not recognize the denatured antigen very well. In this case, immunization with denatured antigens or those purified by electrophoresis would be acceptable. Conversely, if a monoclonal antibody is to be used as a therapeutic agent (anti-cancer, anti-infectious, or immunomodulatory drugs), care should be taken to preserve the antigen's native conformation before immunization. In this case, immunizing an animal with whole cells (live or kill), antigen in MMVs, or purified antigens may be more appropriate.

Critical Parameters

Mouse strain and sex

For optimal hybridoma stability, fusions are generally performed with cells (myeloma

and B cells) originating from the same animal species (e.g., "mouse × mouse"). BALB/c mice are typically used for immunization—unless they respond poorly to the target antigen—because the myeloma cells that are most commonly used for fusion derive from BALB/c backgrounds (e.g., SP2/0 and X63Ag8.653). The key advantage of immunizing BALB/c mice has been that hybridomas can be grown as tumors in BALB/c mice to produce ascites without concern of rejection. Nevertheless, B cells from other mouse strains can also be successfully fused with BALB/c myelomas, and large quantities of hybridoma cells can now be easily grown *in vitro* in bioreactors.

Female mice are generally chosen for immunization, since they typically develop higher antibody responses than their male counterparts.

Adjuvants

Complete Freund's adjuvant and Incomplete Freund's adjuvant are perhaps the most widely used adjuvants for animal studies. But many equally effective but less toxic alternatives are now available (see below). Many adjuvants contain of mineral oil as an emulsifying agent and heat-killed *Mycobacterium tuberculosis* cells as an immunostimulatory agent. Complete Freund's adjuvant induces a potent inflammatory response that progresses to a painful granulomatous lesion at the injection site. Because adverse reactions are generally far more extensive with Complete Freund's adjuvant than with Incomplete Freund's adjuvant or other adjuvants, Complete Freund's adjuvant is typically only used in the initial primer immunization, and Incomplete Freund's adjuvant is used for booster immunizations.

Beyond toxicity, Complete Freund's adjuvant and other microorganism-derived adjuvants can elicit potent immune responses towards their microbial components. An immune response to microorganism-derived adjuvant components can produce a high background or false-positive signals during downstream screening steps. Fortunately, the antibody response to the immunization antigens can be enhanced by less toxic adjuvants that lack microorganism components, including emulsions (e.g., IFA, TiterMax, Montanides, EMULSIGENS, Syntex Adjuvant Formulation or SAF, MF59, and Specol), saponins (e.g., Quil A, and QS21), and aluminum compounds (e.g., alum).

Immunization Schedule

The required isotype and affinity of a monoclonal antibody will guide the immunization schedule to be used. For example, IgM antibodies are generally produced during the early stages of an immune response. So if IgM is the isotype needed, then one or two rounds of immunization should be sufficient. If on the other hand IgG is the target isotype, then three or four immunization rounds should provide additional time for the isotype switch to take place.

The overall number and spacing of antigen injections should also be carefully considered, as it will theoretically impact the likelihood of producing high-affinity antibodies. The probability of inducing high-affinity antibodies is increased by repeated immunization and extending the time between immunizations. As an antigen slowly clears from the body, the low level of antigen will preferentially trigger B cells with high affinity receptors. Therefore, a general immunization protocol of three to four injections spaced 2 to 3 weeks apart should be optimal to induce a robust, high-affinity IgG antibody response.

Immunization Route

The route of antigen administration will depend on multiple factors, including the species being immunized, the types of antigen and adjuvant, and the vehicle used for administration. In many cases, soluble antigens and single-cell suspensions can be safely administered via intravenous injections. For antigens that are not readily soluble in aqueous solutions (e.g., PBS or saline) and require additional components to maintain solubility (e.g., SDS, urea, or DMSO), extra care should be taken to investigate the limit of each component in the formulation that can be tolerated by intravenous injection. In mice and other laboratory animals, intraperitoneal injections are generally safe and easy to administer and will stimulate good B-cell responses in the spleen. Adjuvants with "slow-release" properties generally require subcutaneous or intramuscular administration. Footpad injections used to be a common immunization technique. Though effective, footpad injections are now discouraged because they can cause significant discomfort to an animal. Most institutional animal care and use committees (IACUCs) now require specific justification for immunization by footpad injection.

Another factor to consider when deciding the site of injection is the fact that the site of

antigen entry will invariably impact both the anatomical location of the immune response and the predominant antibody isotype produced. The degree of compartmentalization is also species-dependent. For example, mice respond to intravenous, intraperitoneal, or subcutaneous routes of immunization, and mount a strong IgM and IgG antibody response in the spleen, which is the most convenient site for the collection of activated B lymphocytes. IgA antibodies can be stimulated by a mucosal route of immunization such as oral gavage, and IgE antibodies by directly applying antigen to abraded skin is effective. B cells producing IgA or IgE antibodies may be more prominent in the gut mucosa/lamina propria or lymph nodes draining skin sites of immunization. However, it is now routine to clone the variable region of any monoclonal antibody gene into an expression vector for an antibody isotype of interest.

Troubleshooting

Failure to elicit a strong immune response against the immunizing antigen may be due to a number of factors. For example, immunization using MMVs or whole cells that express the desired antigen can, in certain cases, result in an inadequate antibody response toward the target antigen. This could reflect an overall low expression level of the antigen, poor accessibility to the antigen due to steric hindrance by other cell components, or a skewed response toward other more immunodominant antigens present in these highly complex preparations. This would result in low levels of relevant B-cell clones generated. In such cases, in vitro growth conditions should be optimized to maximize antigen expression or, alternatively, purified antigen should be used for immunization. In addition, changes in immunization dose, route or schedule may need to be implemented, as well.

Antigens could also be poorly immunogenic in mice. For example, polysaccharides (e.g., bacterial capsular polysaccharide), peptides, and oligosaccharides typically cannot be presented to T cells via classical MHC molecules, and generally fail to generate a strong immune response unless coupled to a carrier protein. Coupling these types of antigens to a carrier protein of known immunogenicity (e.g., tetanus or diphtheria toxoids) will ensure that a robust, high-affinity antibody response to the desired antigen.

An inadequate preparation of Freund's adjuvants or other mineral oil and antigen emulsions can also result in a poor antibody response to the target antigen. A well-prepared emulsion should not separate into oil and water phases during prolonged refrigerated storage.

Other parameters that could be varied to improved antibody response include antigen dose, administration route, and immunization frequency.

Anticipated Results

Successful isolation of monoclonal antibodies against a desired antigen target directly correlates with the ELISA titers of antigen-specific antibodies measured prior to fusion. ELISA titer is defined here as the dilution of serum that produces a reading of 0.1 OD unit above background at 405 nm. An ELISA titer of 1:1000 is considered the minimum antibody level required to proceed with B-cell–myeloma fusion. However, certain types of antigens (especially purified antigens such as hapten-conjugate vaccines formulated with adjuvants) will easily elicit much higher titers (e.g., 1:10,000 to 1:100,000 or higher). In addition, individual variations in antibody response are expected among immunized animals as a result of individual animal variations or minor differences in vaccine dosing across mice after multiple rounds of immunization.

Time Considerations

The standard mouse immunization protocols described in this unit (Basic and Alternate Protocols) take 6 weeks (assuming three immunizations, 2 weeks apart) to 12 weeks (assuming four immunizations, 3 weeks apart) from primary immunization to spleen harvesting. This timeline is antigen-dependent, and, for certain antigens, a significantly longer period (several months) may be required to obtain a robust antigen response.

Literature Cited

Crawford, A.W. and Beckerle, M.C. 1991. Purification and characterization of zyxin, an 82,000-dalton component of adherens junctions. *J. Biol. Chem.* 266:5847-5853.

Diehl, K.H., Hull, R., Morton, D., Pfister, R., Rabemampianina, Y., Smith, D., Vidal, J.M., and van de Vorstenbosch, C. 2001. A good practice guide to the administration of substances and removal of blood, including routes and volumes. *J. Appl. Toxicol.* 21:15-23. doi: 10.1002/jat.727.

Fuller, S.A., Takahashi, M., and Hurrell, J.G. 1988. Fusion of myeloma cells with immune spleen cells. *Curr. Protoc. Mol. Biol.* 1:11.7.1-11.7.4. doi: 10.1002/0471142727.mb1107s01.

Gallagher, S.R. 2012. One-dimensional SDS gel electrophoresis of proteins. *Curr. Protoc. Mol. Biol.* 97:10.2A.1-10.2A.44. doi: 10.1002/0471142727.mb1002as97.

Golde, W.T., Gollobin, P., and Rodriguez, L.L. 2005. A rapid, simple, and humane method for submandibular bleeding of mice using a lancet. *Lab Anim*. 34:39-43. doi: 10.1038/laban1005-39.

Hornbeck, P., Winston, S.E., and Fuller, S.A. 1991. Enzyme-linked immunosorbent assays (ELISA). *Curr. Protoc. Mol. Biol.* 15:11.2.1-11.2.22. doi: 10.1002/0471142727.mb1102s15

Simonian, M.H. and Smith, J.A. 2006. Spectrophotometric and colorimetric determination of protein concentration. *Curr. Protoc. Mol. Biol.* 76:10.1.1-10.1A.9. doi: 10.1002/0471142727.mb1001as76