PRODUCTION OF ANTIBODIES

Antibodies are serum immunoglobulins with binding specificity for particular antigens. Although antibodies can be identified in the serum of individuals or patients that have been exposed to particular pathogens, the usual methods for eliciting antibodies involve immunization with purified or partially purified antigen preparations. Antigens used are most commonly proteins or peptides, but carbohydrates, nucleic acids, small organic molecules (haptens) conjugated to appropriate protein carriers, cells, and cell and tissue extracts can also be employed.

The first consideration is usually whether polyclonal or monoclonal antibodies are needed. Polyclonal antibodies are particularly valuable for immunoprecipitation (*UNIT 8.3*) and immunoblotting (*UNIT 8.10*), whereas monoclonal antibodies can have exquisite specificity and can be derived for almost any purpose. Choice of the species of animal to be used for immunization is based in part on whether antibodies of great specificity are required—in which case genetically defined strains can be very helpful—or antibodies of wide cross-reactivity are needed.

The amount of antibody needed must also be evaluated. Clones of hybridomas (somatic cell hybrids of B cells from an immunized animal's spleen and myeloma tumors permissive for the production of monoclonal immunoglobulins) provide an essentially limitless supply of a constant reagent. Nevertheless, the initial investment in producing a monoclonal antibody is quite large, whereas relatively large amounts of a polyclonal antiserum can be obtained from a single rabbit or from several genetically identical rats or mice.

The following unit describes the production of polyclonal antisera by immunization with antigen emulsified in adjuvant (*UNIT 2.4*). The method described is applicable to rabbits, rats, mice, and hamsters, and can also be used with larger animals such as sheep or goats. Another adjuvant method employing immune stimulating complexes (ISCOMs) is presented in *UNIT 2.11*. Detailed protocols for producing monoclonal antibodies are provided in *UNIT 2.5*. With minor variations, these are applicable to mice, rats, and hamsters. Once a stable hybridoma line is established, the need for producing large amounts of the monoclonal antibody must be confronted. This can be achieved either by producing tissue culture supernatants from large numbers of cells or by producing ascites fluid in the appropriate animal (*UNIT 2.6*).

Production of Polyclonal Antisera

STRATEGIC PLANNING

Production of good antisera depends in large part upon the quality, purity, and amount of available antigen as well as on the specificity and sensitivity of the assay. For protein antigens, if possible, the material should be biochemically homogeneous and, depending on the intended use, should be in either a native or denatured conformation. Be aware that minor contaminants are often (unfortunately) more antigenic than the immunogen of interest, and antisera resulting from immunization may have more activity against the contaminants than against the protein of interest. Antisera to be used for screening bacterial expression cDNA libraries or for immunoblots are best made against denatured protein, whereas those to be used for screening cDNAs expressed in eukaryotic transfection systems or for immunoprecipitation of native-cell-synthesized structures might best be made against native protein.

Contributed by Helen M. Cooper and Yvonne Paterson
Current Protocols in Immunology (1995) 2.4.1-2.4.9

Although the advances offered by the development of monoclonal antibody techniques have revolutionized the specificity, uniformity, and quantity of antibodies, there remain many circumstances in which polyclonal antibodies are more desirable than monoclonal antibodies. Production of polyclonal antisera takes less time and effort than production of monoclonal antibodies, requires relatively simple and readily available equipment, and produces reagents that can be used for immunoprecipitation, immunoblotting, and enzyme-linked immunosorbent assays (ELISAs). The basic and alternate protocols describe the production of polyclonal antisera specific for protein antigens in rabbits, rats, mice, and hamsters. The support protocol presents a method for preparing serum from blood. Polyclonal antipeptide antisera can be produced by substituting carrier-conjugated peptides (*UNIT 9.4*) for the purified protein antigens.

Choice of animal for the production of antibodies depends upon the amount of antiserum desired, the evolutionary distance between the species from which the protein of interest has been derived and the species of the animal to be immunized, and prior experience with the immunogens. Rabbits are the usual animal of choice because they are genetically divergent from the human and mouse sources of the proteins most often studied. Rabbits provide as much as 25 ml of serum from each bleed without significant harmful effects. For smaller-scale experiments, or for those that rely on precisely defined antibody specificities, inbred mouse strains may be the system of choice. Because mice are smaller, the volume of antigen suspension used for immunization is significantly less and the amount of serum that can be obtained from a single bleed does not exceed 0.5 ml. Rats and hamsters may be used when larger amounts of serum are needed, or when the greater evolutionary distance is advantageous. With repeated bleeding, as much as 5 ml of serum can be obtained from these species. Additional discussion of the choice of species for the production of monoclonal antibodies is given in *UNIT 2.5*.

The choice of adjuvant for in vivo animal use has become problematic in recent years. Freunds adjuvant has been reliably and widely used for over fifty years (Freund et al., 1937). However, there is a degree of distress and discomfort to the animal associated with its use which requires that the responsible investigator explore alternatives (McWilliam and Niemi, 1988). In most studies that have compared available alternatives to Freunds adjuvant, the basic protocol given here, which uses a CFA/IFA immunization regimen, gives far superior antibody titers to the commonly used commercially available adjuvants (Johnston et al., 1991; Deeb et al., 1992; Smith et al., 1992). However, in some instances TiterMax (Bennett et al., 1992) and Ribi Adjuvant Systems (Mallon et al., 1991) have performed as well as Freunds adjuvant. The use of these adjuvant systems as alternatives to Freunds adjuvant is described in the alternate protocol.

BASIC PROTOCOL

IMMUNIZATION TO PRODUCE POLYCLONAL ANTIBODIES USING FREUNDS ADJUVANT

In the presence of adjuvant, the protein antigen is injected intramuscularly, intradermally, or subcutaneously into an animal of the chosen species. Booster immunizations are started 4 to 8 weeks after the priming immunization and continued at 2- to 3-week intervals. Prior to the priming immunization, following the primary and each booster immunization, the animal is bled and serum prepared from whole blood (see Support Protocol).

Instructions on the different strategies for immunization (intramuscular, intradermal, or subcutaneous) can be found in *UNIT 1.6* and for bleeding (from marginal vein or artery of ear for rabbit; various other sites for mouse, rat, or hamster) in *UNIT 1.7*. Factors important in preparing specific high-titer antisera, procedures for modifying protein antigens to enhance their immunogenicity, and choice of host animal are discussed in the Commen-

tary. Protocols for immunization prior to production of monoclonal antibodies (*UNIT 2.5*) and T cell hybridomas (*UNIT 3.14*) should be reviewed for these purposes.

Materials

Rabbit, rat, mouse, or hamster of appropriate strain
Complete Freunds adjuvant (CFA; Sigma)
1 to 2 mg/ml purified protein antigen in PBS (*APPENDIX 2*)
Incomplete Freunds adjuvant (IFA; Sigma)

50-ml disposable polypropylene centrifuge tubes
3-ml glass syringes with 19-, 21-, and 22-G needles
Double-ended locking hub connector (Luer-Lok, Becton Dickinson) *or* plastic 3-way stopcock

Additional reagents and equipment for animal restraint (*UNIT 1.3*), parenteral injection (*UNIT 1.6*), and blood collection (*UNIT 1.7*)

CAUTION: CFA is an extremely potent inflammatory agent, particularly if introduced intradermally or into the eyes and may cause profound sloughing of skin or loss of sight. Self-injection can cause a positive TB test and lead to a granulomatous reaction. Use gloves and protective eyewear when handling CFA.

1. Bleed the animal prior to immunization and collect blood sample in a 50-ml centrifuge tube. Prepare serum from blood and assay and store (see Support Protocol).

   This preimmune bleed is critical as a control to ensure that the antibody activity detected in later bleeds is due to the immunization.

2. Shake CFA to disperse insoluble *Mycobacterium tuberculosis* bacilli. Add 2 ml CFA to 2 ml of 0.25 to 0.5 mg/ml purified protein antigen in PBS at 4°C.

   These volumes produce immunogen sufficient to immunize 4 rabbits or up to 80 mice. Do not use Tris-based buffers for generating the emulsion.

   An effective and simple method for preparing purified protein antigen is by preparative SDS-PAGE (UNIT 8.4). If a standard-size 1.5-mm slab gel is used with a large-toothed comb, as much as 2 mg of a homogeneous protein can be loaded across the entire gel. Following electrophoresis, an edge can be sliced off with a razor blade, fixed and stained, and used to identify the region containing the protein band (UNIT 8.9). The gel slice containing the protein may then be directly added to several milliliters of PBS (APPENDIX 2) and emulsified as described below with an equal volume of CFA. The acrylamide serves as an additional component for the protein depot provided by the adjuvant.

3. Draw up the CFA/antigen mixture into a 3-ml glass syringe with a 19-G needle. Remove needle, expel as much air as possible, and attach syringe to the double-ended locking hub connector or the plastic 3-way stopcock (see Fig. 2.4.1). Attach an empty 3-ml glass syringe at the other end and force the mixture back and forth from one syringe to the other repeatedly. When the mixture is homogeneous and white, disconnect the connector or stopcock, attach a 21-G needle, and test whether the emulsion is stable by extruding a small drop onto the surface of 50 ml cold water in a 100-ml beaker. A good oil-in-water emulsion should hold together as a droplet on the surface of the water. If the drop disperses, mix the antigen using the hub-connected syringes until it forms an emulsion.

   Heat will be generated by this procedure. Chill on a bed of ice from time to time to keep the mixture as close to 4°C as possible.

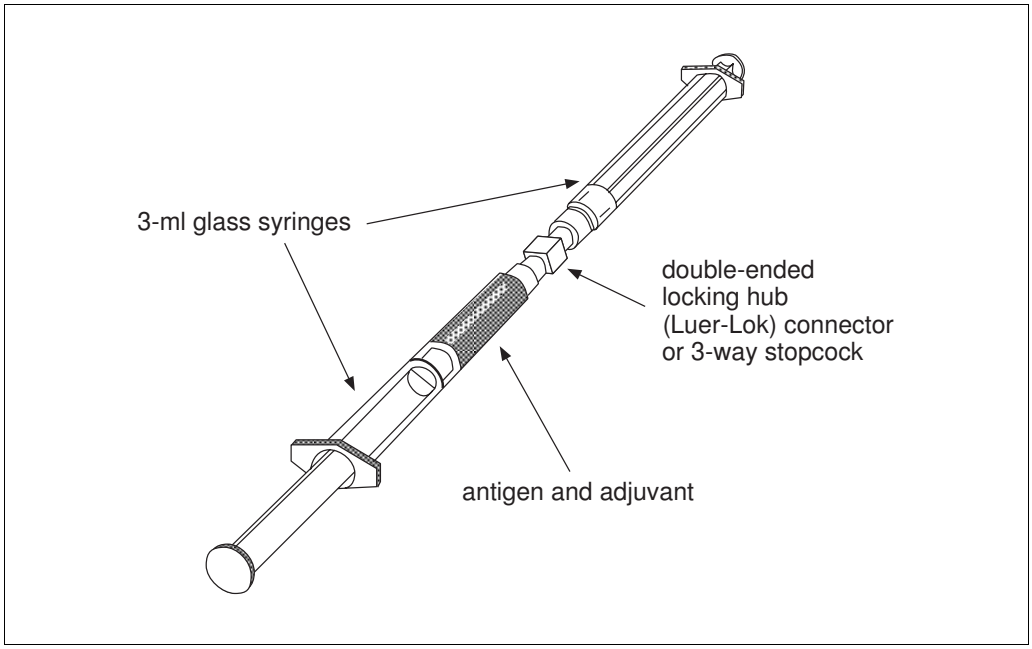

Figure 2.4.1 Double-syringe device for preparation of antigen-adjuvant emulsions.

4. Transfer all of the adjuvant-antigen emulsion to one syringe and remove the connector or stopcock. Attach a 22-G needle to the syringe and remove air bubbles.

5. Restrain the animal and inject the adjuvant/antigen emulsion into multiple intramuscular (i.m.), intradermal (i.d.), or subcutaneous (s.c.) sites.

   Discard the unused immunogen. For extremely valuable antigens, the emulsion may be stored at 4°C for several weeks and reemmulsified before use. However, denaturation of protein antigens may take place under these conditions. For immunization of small rodents (e.g., mice), it is often better to carry out injections intraperitoneally (i.p.; UNITS 1.6 & 2.5*).*

6. Bleed the animal 10 to 14 days following the priming immunization and collect blood sample. Prepare serum from blood (see Support Protocol).

7. Prepare antigen for booster immunizations, following steps 2 to 4. When CFA is the primary adjuvant, use IFA as the adjuvant for all subsequent immunizations.

8. Administer the first booster immunization 4 to 8 weeks after the priming immunization, bleed the animal 7 to 14 days later, and collect blood sample. Prepare serum from blood (see Support Protocol).

   Some investigators will administer the first booster immunization as early as 2 weeks after the primary immunization.

9. Administer further booster immunizations at 2- to 3-week intervals. Bleed animal 10 to 14 days after each boost and collect blood sample. Prepare serum from blood (see Support Protocol).

   Repeated intradermal immunization should be avoided as it can cause skin ulceration. Following primary intradermal or subcutaneous immunization, it is preferable to boost with intramuscular injections for the rabbit. Some investigators prefer primary intramuscular injections with boosters at other sites.

IMMUNIZATION TO PRODUCE POLYCLONAL ANTISERUM USING OTHER ADJUVANTS

ALTERNATE PROTOCOL

For highly immunogenic antigens the use of Freunds adjuvant can certainly be avoided. For other immunogens it may be necessary to test a number of adjuvant systems. The use of two commercially available adjuvants is described in this protocol.

Additional Materials

TiterMax #R-1 (CytRx; store <24 months at 4°C) *or* Ribi Adjuvant System (RAS; Ribi ImmunoChem; store at 2 to 8°C and do not freeze)
1-ml plastic syringes

1a. *Using TiterMax:* Emulsify aqueous antigen with TiterMax adjuvant (see Basic Protocol; follow steps 1 through 5, except use 0.5 ml antigen and 0.5-ml vial TiterMax in step 2 and plastic syringe in step 3).

TiterMax contains microparticulate silica coated with block copolymer CRL-8941, sorbitan mono-oleate, and squalene.

Although glass syringes are recommended for Freunds adjuvant emulsions, all-plastic syringes should be used with TiterMax.

Each reconstituted 0.5-ml vial will immunize 20 mice, 10 rabbits, or 1 goat. Unused antigen/adjuvant emulsion can be stored at 4°C, −20°C, or −70°C for as long as the antigen is stable. It may be necessary to re-emulsify before using.

1b. *Using Ribi Adjuvant System:* Warm the vial of RAS 5 to 10 min at 40° to 45°C. Add 2 ml antigen in PBS directly through the rubber stopper using a syringe with a 21-G needle. Vigorously vortex the vial 3 min at room temperature with the cap seal in place. The final volume of adjuvant/antigen is 2 ml containing 2% squalene oil.

Each vial of RAS contains 0.5 mg each of monophosphoryl Lipid A (MPL), synthetic trehalose dicorynomycolate (TDM), and cell wall skeleton (CWS) in 44 μl squalene and Tween 80.

Each reconstituted vial will immunize 10 mice or 2 rabbits or goats. Unused adjuvant/antigen emulsion can be stored several months at 4°C. However, if the entire vial will not be used initially, it is better to reconstitute to 1.0 ml with saline alone, store at 4°C, and mix aliquots 1:1 with antigen in saline as needed.

2. Transfer the antigen emulsion to a 1-ml syringe, attach a 22-G needle to the syringe, and remove air bubbles.

3. Restrain the animal and inject the adjuvant/antigen emulsion.

Rabbits should receive 40 μl TiterMax/antigen emulsion i.m. in each thigh. High antibody titers have been obtained with 30 to 50 μg of antigen per rabbit. Rabbits should be immunized with 1.0 ml RAS containing 50 to 250 μg of antigen in multiple sites: 0.05 ml i.d. at six sites, 0.3 ml i.m. in each thigh and 0.1 ml s.c. in the neck.

4. Bleed the animal and prepare antigen for booster immunization (see Basic Protocol, steps 6 and 7).

Serum antibody responses have been reported to be slower for both RAS and TiterMax than for Freunds adjuvant (Smith et al., 1992).

5. Administer booster immunizations at 4, 8, and 12 weeks. Bleed the animal 10 to 14 days after each booster immunization. Prepare serum from blood (see Support Protocol) and cease immunization when high antigen-specific titers have been achieved.

Boosting with TiterMax may not be necessary for all antigens. If a second immunization is necessary, use soluble antigen in place of antigen/adjuvant at 4 weeks. If titers are still low after 10 to 14 days, a booster dose of antigen/TiterMax adjuvant can be given immediately. Increasing the amount of antigen may also help.

Ribi ImmunoChem strongly recommends that booster injections of RAS adjuvant/antigen be repeated no more frequently than every four weeks.

SUPPORT PROTOCOL

PREPARATION OF SERUM FROM BLOOD

Each blood sample is allowed to stand 4 hr at room temperature and overnight at 4°C until a clot forms. After removal of the clot and debris, the serum is assayed and stored at –20°C.

Additional Materials (also see Basic Protocol)

Blood samples (see Basic Protocol)
Sorvall H-1000B rotor or equivalent

Additional reagents and equipment for immunoblotting (*UNIT 8.3*), immunoprecipitation (*UNIT 8.10*), ELISA (*UNIT 2.1*), or double-immunodiffusion assay in agar (*UNIT 2.3*)

1. Allow blood to stand in the 50-ml centrifuge tube 4 hr at room temperature to allow clot to form, then place overnight at 4°C to allow clot to retract.
2. Gently loosen the clot from the sides of the tube with a wooden applicator stick (do not break up the clot), then remove the clot from tube with the applicator.

   If a clot has not formed, initiate clotting by placing a wooden applicator stick into the tube containing the collected blood, then begin again at step 1.
3. Transfer serum to a 50-ml centrifuge tube. Pellet any remaining blood cells and debris by centrifuging 10 min in H-1000B rotor at 4000 rpm (2700 × *g*), 4°C, and save supernatant.
4. Assay antibody titer by the appropriate method: immunoprecipitation, immunoblotting, ELISA, or double-immunodiffusion assay in agar.
5. Store serum in aliquots in screw-top tubes at –20°C.

   Some sera lose activity on repeated freezing/thawing; others aren't stable at 4°C.

COMMENTARY

Background Information

The kinetics of development of a specific antibody response upon immunization of a rabbit with antigen are illustrated in Figure 2.4.2. After the primary immunization, naive B cells are stimulated to differentiate into antibody-secreting plasma cells. For most soluble protein antigens, specific antibody begins to appear in the serum 5 to 7 days after the animal is injected. The antibody concentration (titer) continues to rise and peaks around day 12, after which it decreases. Similar kinetics are observed with mice, rats, hamsters, and rabbits.

In addition to differentiating into antibody-forming cells, the antigen-stimulated B cells proliferate to form a large population of memory B cells, which quickly become activated after the booster injection is administered. Thus, the lag period before the appearance of the specific antibody is much shorter after a booster injection than that observed for the initial immunization. In addition, a significantly higher titer of specific antibody is achieved and sustained for a longer period of time. The peak of antibody production occurs 7 to 14 days after boosting. As a consequence of the existence of the memory B cells, less antigen is required to stimulate a strong secondary response. Memory B cells are long-lived; therefore, a specific antibody response can be elicited as much as 6 months to a year after the last booster. Finally, the average affinity and degree of specificity of the antibody population for the antigen increase with re-

peated immunizations (Klinman and Press, 1975).

Adjuvants greatly enhance the specific antibody titer, as they allow the antigen to be released slowly, thus ensuring the continual presence of antigen to stimulate the immune system. Freunds adjuvant has been used extensively in the preparation of antigen because it induces a high, long-lasting antibody titer that is often still measurable 25 weeks or more after boosting. The presence of mycobacteria in complete Freunds adjuvant (CFA) activates the T cell population, providing necessary lymphokines for B cell stimulation and maturation. CFA may cause granuloma and subsequent necrotic abscesses, so it should be used only for primary immunization. Incomplete Freunds adjuvant (IFA) is adequate for booster injections. In the past decade, adjuvant research has concentrated on the production of effective adjuvants that minimize animal trauma. Wherever possible, to reduce animal discomfort, less noxious adjuvants should be used as described in the alternate protocol. In addition some countries are restricting the use of CFA in laboratory animals. Published comparisons of commercial adjuvants with the basic CFA/IFA protocol vary widely in their conclusions. In most cases, however, CFA/IFA produces higher titers of higher-affinity antibodies in a shorter time period (Johnston et al., 1991; Deeb et al., 1992; Smith et al., 1992). Conflicting results (Mallon et al., 1991; Bennett et al., 1992) may reflect differences in the immunogenicity of the antigens used.

Critical Parameters

New Zealand red or white rabbits are generally the best source of specific antisera because 30 to 50 ml of whole blood can be obtained at each bleed. The life span of a rabbit is 5 to 6 years, so a continual source of specific antiserum can be provided over a period of time by one rabbit after booster injections. In this regard, the recommended times between booster injections are not critical; the animal may be rested for several months between subsequent boosters, after the primary and secondary booster injections. Blood collection, however, must take place 7 to 14 days after each booster to ensure a high titer.

Preimmune serum from the same animal is the preferred negative control. If additional control serum is required, either immune serum from animals immunized with totally unrelated antigens or pooled serum from naive animals will be adequate. Occasionally, spurious antibody activities from nonimmunized animals may mimic the activity of the immune serum.

Antibody specificity may vary widely between individual animals with respect to the dominant antigenic epitopes recognized on a given protein antigen. Therefore, antiserum from a single animal should be used throughout a study. If more than one animal must be used for particular antisera, the antisera should be pooled. Large-scale production of antisera can be carried out in goats, sheep, and horses with appropriate veterinary guidance. If serum is taken from inbred animal strains, the variability in antibody specificity, as observed in outbred rabbits, is less of a problem.

The most important factor in producing a highly specific polyclonal antiserum is the pu-

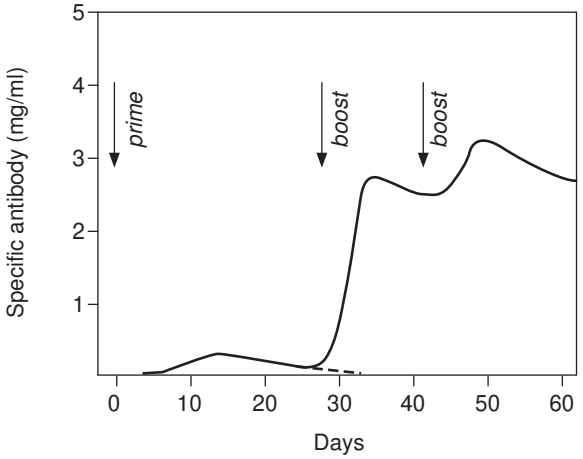

Figure 2.4.2 Kinetics of development of the specific antibody response. Arrows indicate when priming and boosting immunizations were administered. Actual amounts of specific antibody produced will vary considerably depending on immunogenicity of the protein.

rity of the antigen preparation used for immunization. The immune system is very sensitive to the presence of foreign proteins. Any contaminating proteins in the antigen preparation can potentially induce a strong immune response when injected in the presence of adjuvant. When antisera are employed in sensitive techniques such as immunoblotting or the screening of cDNA or genomic libraries, significant antibody titers to protein contaminants can be a major problem. Thus, the antigen preparation should contain no significant contaminating proteins. Ideally, there should be no visible contaminating bands when 10 to 20 μg are analyzed on an SDS-polyacrylamide gel stained with Coomassie brilliant blue (*UNITS 8.4 & 8.9*).

If the antiserum is to be used in functional assays, extra care must be taken to ensure that the immunizing antigen is in its native form, because antibodies directed to denatured forms of the protein antigen will interact weakly, if at all, with the antigen in its native conformation. On the other hand, antibodies used in immunoblots, immunoprecipitation of primary in vitro translation products, and immunoscreening of λgt11 expression libraries may be most effective if generated against a denatured protein with reduced and carboxymethylated disulfide bonds.

Troubleshooting

Inability to attain high-titer antiserum after several booster injections may be due to a variety of factors as described below.

Use of inappropriate adjuvant. Some experimentation may be necessary to optimize the antigen/adjuvant ratio for different antigens. If the alternate protocol still fails to produce a good antibody titer after three immunizations, switch to the basic protocol.

Inadequate antigen emulsification. If the emulsion fails the drop-on-water test described in the basic protocol (step 3), repeat the emulsification process. Be sure to use phosphate-buffered saline. Avoid plastic syringes and Tris-based buffers with CFA and IFA.

The antigen is a poor immunogen. In general, the immunogenicity of a protein is related to the degree to which it differs from "self" proteins (Benjamin et al., 1984). Large bacterial or viral proteins such as hemagglutinin or bacterial-coat proteins are highly immunogenic, whereas proteins from mammalian sources, such as polypeptide hormones or cell-surface receptors, may be poorly immunogenic due to tolerance. Protein antigens can be made more immunogenic in two ways. First, they can be chemically linked to a carrier protein that is known to be a good immunogen. Common carrier proteins include keyhole limpet hemocyanin (KLH), fowl immunoglobulin, and bovine serum albumin (BSA). Coupling peptides to carrier proteins is described in *UNIT 9.4*; the same protocols can be used to couple the protein antigen of interest to the desired carrier. Second, the immunogenicity of an antigen may be enhanced by its polymerization into large aggregates via a cross-linking agent such as glutaraldehyde. The protocol in *UNIT 9.4* for the coupling of peptide antigens to a carrier protein with glutaraldehyde can also be used to polymerize any protein antigen. With both the coupling and polymerization procedures, any insoluble antigen complexes formed should be removed prior to immunization by centrifuging 10 min at 15,000 × *g*, 4°C.

Host animal's immune system may be compromised by bacterial or viral infection. Refer to *UNITS 1.1 & 1.2* for discussion of the consequences of poor animal husbandry. Utilize animals from reliable, pathogen-free sources and maintain them in appropriate infection-free facilities.

Only a few animals have been immunized. Because of the vagaries of immune-response genes in outbred animals such as rabbits, some antigens may not induce a good antibody response in a significant proportion of randomly selected animals. Thus, it is best to immunize several different animals and to screen the sera for the best responder. Obviously, this is less of a problem in homozygous inbred strains, but with a new antigen it is wise to test several strains for their antibody response.

An insufficient amount of antigen was used. Although recommended concentrations of antigen for rabbits are 0.25 to 0.5 mg/ml injected into multiple sites, for a total of 1 to 2 ml in the same animal, good results can be obtained with $\frac{1}{10}$ to $\frac{1}{20}$ of the concentration in the same volume. It is always tempting to use less of a precious antigen, but often too low a dose leads to too low a response.

Anticipated Results

For large or nonevolutionarily related proteins, a titer of 5 to 10 mg/ml of serum can be expected after repeated boosts (hyperimmunization). When immunizing with small or highly conserved protein species, a titer of 1 to 2 mg/ml of specific antibodies is more likely. Antibody titers and affinity for the antigen will be low after primary immunization and the first booster immunization, but both titer and affin-

ity will increase with subsequent immunizations.

Time Considerations

Preparation of the immunogen and immunization will take ~3 hr on each occasion. Collection of antisera will take 1 to 2 hr, depending on the number and species of animals.

Collection of antiserum after the primary immunization will be at 10 to 14 days. This will be a low-titer, low-affinity serum. The first booster normally is given 4 to 8 weeks after the primary immunization but can be given as early as 2 weeks after the primary if time is critical. Ideally, there should be at least 19 days between the primary and the secondary bleeds. A second booster is given at 6 weeks with a bleed on day 52 to 59. This will usually be the first high-titer bleed. If a titer of <1 mg/ml of specific antibody is obtained, subsequent boosting immunization will be necessary.

Literature Cited

Benjamin, D.C., Berzofsky, J.A., East, I.J., Gurd, F.R.N., Hannum, C., Leach, S.J., Margoliash, E., Michael, J.G., Miller, A., Prager, E.M., Reichlin, M., Sercarz, E.E., Smith-Gill, S.J., Todd, P.E., and Wilson, A.C. 1984. The antigenic structure of proteins: A reappraisal. *Annu. Rev. Immunol.* 2:67-101.

Bennett, B., Check, I.J., Olsen, M.R., and Hunter, R.L. 1992. A comparison of commercially available adjuvants for use in research. *J. Immunol. Methods* 153:31-40.

Deeb, B.J., DiGiacomo, R.F., Kunz, L.L., and Stewart, J.L. 1992. Comparison of Freund's and Ribi adjuvants for inducing antibodies to the synthetic antigen (TG)-AL in rabbits. *J. Immunol. Methods* 152:105-113.

Freund, J., Casals, J., and Hismer, E.P. 1937. Sensitization and antibody formation after injection of tubercle bacilli and paraffin oil. *Proc. Soc. Exp. Biol. Med.* 37:509.

Johnston, B.A., Eisen, H., and Fry, D. 1991. An evaluation of several adjuvant emulsion regimens for the production of polyclonal antisera in rabbits. *Lab Anim. Sci.* 41:15-21.

Klinman, N.R. and Press, J. 1975. The B cell specificity repertoire: Its relationship to definable subpopulations. *Transplant. Rev.* 24:41-83.

Mallon, F.M., Graichen, M.E., Conway, B.R., Landi, M.S., Leonard, T.B., and Hughes, H.C. 1991. A comparison of antibody response using a synthetic adjuvant system and Freund's complete adjuvant in rabbits. *Am. J. Vet. Res.* 52:1503-1506.

McWilliam, A. and Niemi, S.M. 1988. Freund's adjuvant. Canadian Council on Animal Care Resource. 12:1.

Smith, D.E., O'Brien, M.E., Palmer, V.J., and Sadowski, J.A. 1992. The selection of an adjuvant emulsion for polyclonal antibody production using a low-molecular-weight antigen in rabbits. *Lab. Anim. Sci.* 42:599-601.

Key Reference

Harlow, E. and Lane, D. 1988. Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

A comprehensive methods book with many modern techniques.

Contributed by Helen M. Cooper
Ludwig Institute for Cancer Research
Melbourne, Australia

Yvonne Paterson
University of Pennsylvania
Philadelphia, Pennsylvania