Antibodies

A LABORATORY MANUAL

Ed Harlow
David Lane

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Ed Harlow
Cold Spring Harbor Laboratory

David Lane
Imperial Cancer Research Fund Laboratories

Cold Spring Harbor Laboratory
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Protein G

Protein G is a 30,000- to 35,000-dalton protein isolated from the cell wall of β-hemolytic streptococci of the C or G strains (Kronvall 1973). Less is known about protein G than about protein A, primarily because protein G was not purified until 1984 (Björck and Kronvall 1984). The interest in protein G and in other less well-characterized bacterial cell wall proteins is that these proteins have different affinities for antibodies compared to protein A. Many antibodies from species or subclasses that do not bind well to protein A bind very well to protein G. Tables 15.1 and 15.2 summarize the affinity of protein G for a spectrum of antibody sources.

Like protein A, the gene for protein G has been cloned recently, and protein G is now available in purified form, isolated from bacterial overexpression systems. This should allow more convenient applications and should speed the collection of information on its use in immunochemical tests. One disadvantage concerning protein G is that it contains a second binding site that will interact with albumin (Björck et al. 1987). Recently, the antibody binding domain has been separated from the albumin domain by genetic manipulations, and this should increase the usefulness of protein G.

ANTI-IMMUNOGLOBULIN ANTIBODIES

When purified antibodies from one species are injected into a distant species, the antibodies are often recognized as foreign antigens and elicit a strong humoral response. This characteristic can be used to generate specific anti-immunoglobulin reagents. Using anti-immunoglobulin antibodies solves many of the difficulties in locating and quantitating antibodies when these molecules are found in complex mixtures of serum or tissue culture proteins. The specificity of the anti-immunoglobulin antibodies can be used in many of the same ways and with many of the same advantages as anti-antigen antibodies. One of the major advantages of using these anti-immunoglobulin reagents is that with careful preparation they can be made species, class, subclass, or domain specific. Therefore, anti-immunoglobulin antibodies are prepared for two purposes, either as general reagents used to bind all antibodies or as selective reagents used to identify one type of antibody molecule.

Using antibodies as immunogens has proven to be one of the best experimental systems for studying how a region of a protein is recognized as foreign and how it elicits a humoral response. In practical terms, if a region of the injected antibody molecule is identical to or is sufficiently similar to the corresponding region of the antibodies from the host animal, this region will not be seen as foreign and will not elicit a response. Therefore, the regions that can be recognized as foreign in the generation of anti-immunoglobulin antibodies are determined by the genetic differences between the animal from which the immunogen is prepared and the immunized animal. In addition, as the antibodies become more dissimilar, the number of regions that will be recognized as foreign increases.

In general, animals from different genuses will respond to a number of epitopes on both the heavy and light chains, and these immunogens will induce a strong humoral response. The epitopes recognized in these types of immunizations are described as *xenotypic*. Immunoglobulins isolated from one strain of a species and injected into another strain will induce a response to *allotypic* epitopes. When immunoglobulins are isolated from one inbred strain and injected into the same strain, the epitopes that are recognized are known as *idiotypic* epitopes. Idiotypic epitopes are associated with the hypervariable regions that form the antigen combining site of a particular antibody (see Chapters 2 and 3). Idiotypic epitopes are a subset of the allotypic epitopes which in turn are a subset of the xenotypic epitopes.

One important aspect in the generation of an anti-immunoglobulin response that is not obvious from the above discussion is how similar antibodies are from species to species. This becomes apparent when analyzing the cross-reactions of anti-immunoglobulin antibodies. Although a region may be seen as foreign between one species and another (i.e., the region is different), this does not mean that the same region is not conserved in other species. For example, in a high-titered rabbit anti-mouse immunoglobulin serum, as many as 10% of the antibodies will cross-react with human antibodies. In many instances, these cross-reactions will not lead to any technical problems, but in some cases, such as staining tissue sections, these cross-reactions will ruin an experiment. These types of cross-reactions can be found at all levels between different subclasses, classes, light chains, and species. Eliminating these cross-reactions is one of the problems in preparing antibodies that are specific for different species, different regions of the antibody molecule, or different types of heavy or light chain. To isolate antibodies with these specificities, three steps may be taken. First, the immunization should be targeted to the desired region or polypeptide chain by only injecting this portion of the antibody. Second, the resulting antibodies must then be depleted of all the cross-reacting antibodies by adsorption on samples of the cross-reacting reagents. The last step is to purify the antibodies by binding to the region of interest and then eluting the purified specific antibodies (p. 313).

PREPARING ANTI-IMMUNOGLOBULIN ANTIBODIES

The major consideration when preparing anti-immunoglobulin antibodies is the host in which the antibodies will be raised. This decision should be made on the ease of using the resulting antibodies. In general, although antibodies can be raised in closely related species, most immunizations are done in distant species. Unless there are extenuating circumstances, larger animals yield more sera and should be used if possible. Most of these immunizations are done in rabbits, goats, or sheep. Of these, if protein A will be used in any of the assays, only rabbits produce antibodies that bind protein A with high affinity and are, consequently, the animal of choice. Rabbits are commonly used to prepare anti-mouse, -rat, and -human immunoglobulin antibodies, while goat, sheep, or pigs are used to produce anti-rabbit immunoglobulin antibodies.

Anti-immunoglobulin antibodies are available commercially, and unless large quantities are needed or special antibody couplings are planned, it is seldom worthwhile to prepare these reagents yourself. However, since they are relatively easy to prepare, a suitable protocol is outlined below.

1. Prepare a large batch of antibodies. See p. 288 for appropriate purification protocols. Dissolve the immunoglobulins at approximately 2 mg/ml in PBS.

2. At the time of each injection, make an emulsion of 500 μl of the immunoglobulin solution with 500 μl of Freund's adjuvant. The first injection should be done with complete Freund's; all subsequent injections should be done with incomplete.

   The emulsion can be prepared by taking the 500 μl of each suspension into separate 1-ml syringe barrels. These two solutions are immiscible but with mixing will form an emulsion. This is most easily done by pushing the mixture between the two 1-ml syringes via a two- or three-way valve as shown in Figure 6.5, p. 158). Remove all air from both syringes before connecting to the valve. Make sure the connections are tightened securely. Depress the plunger on the syringe containing the aqueous antigen solution first. Continue to push the mixture between the two syringes until it becomes difficult (approximately 10–20 times). Push the emulsion into one syringe. Remove the valve and the other syringe, and place a 23-gauge needle on the syringe. Displace any air that may be held in the barrel or in the needle by carefully depressing the plunger until the emulsion reaches the needle.

3. Inject the emulsion into multiple subcutaneous sites in a female New Zealand white rabbit. For most purposes, several rabbits should be injected at the same time to lessen the impact of animal-to-animal differences.

4. Take sample test bleeds from the rabbits 14 days after each injection. Compare the titers using one of the standard quantitative tests or in the tests in which they will be used.

5. Let the rabbits rest for 6 weeks between injections.

6. When the titers have increased to usable levels, each rabbit should be bled to obtain approximately 50 ml of blood. Continue to repeat the injections while collecting the serum 14 days after each injection.

7. Rabbits can continue to produce good anti-immunoglobulin antibodies for over a year. When the last boost has been made, sacrifice the rabbit and collect all the blood. The final bleed will yield approximately 100 ml of serum.

NOTES

i. For protocols in each of the steps above, consult Chapter 5.

ii. This protocol can be modified to produce antibodies that are specific for the antibodies from a particular species, class, or subclass, the Fc or Fab region, or the particular light chain. Begin the immunizations with the desired region or polypeptide chain. After a good immune response has developed, the resulting antibodies are first adsorbed against other regions or polypeptides to remove cross-reacting activities. Then the antibodies are affinity-purified on the starting immunogen (p. 313).