

Polyclonal and Monoclonal Antibodies

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1. Introduction

The breadth of repertoire yet beautiful specificity of the antibody response is the key to its physiological efficacy *in vivo*; it also underpins the attractiveness of antibodies as laboratory and clinical reagents. One aspect of the body's reaction to invasion by a microorganism is the activation and clonal expansion of antigen-reactive B lymphocytes. Once these have matured into plasma cells, each clone of cells will secrete its own unique specificity of antibody—thus, the invading pathogen will be met by a barrage of antibody molecules capable of binding to many different sites on its surface. Such a polyclonal response, whose range of specificities and affinities can shift with time, is ideal for combatting infection, and indeed for certain laboratory applications (such as secondary reagents for immunoassay); however, in many experimental and clinical situations the ability to have an unlimited supply of a single antibody that is clearly defined and of reproducible specificity and affinity is of greater value. To produce such a reagent it is necessary to isolate and culture a single clone of B lymphocytes secreting antibody of the appropriate characteristics—that is, to produce a monoclonal antibody (mAb).

2. Generation of an Immune Response

2.1. Selection of Animal for Immunization

The generation of an immune response to the antigen of interest is a necessary prerequisite to the production of both polyclonal and monoclonal antibodies; the major difference between the two systems lies mainly in the size of the animal to be immunized. Since polyclonal antibodies are collected from the serum of the immunized individual it is advisable to use as large an animal as

possible; for commercial reagents rabbit, goat, and sheep are the usual choices, although pig, donkey, horse, and kangaroo antibodies are also available. For a phylogenetically more distant view of a mammalian immunogen, the chicken can be very useful.

Two further factors affect the choice of animal. First, the greater the genetic disparity between donor antigen and recipient to be immunized, the greater the number of distinct epitopes to which the immune response can be directed. Thus, unless the target antigen is a defined alloantigen, the recipient should be as phylogenetically unrelated to the donor as possible. For polyclonal antibodies this is not a problem since the choice of recipient is a wide one. However, for mAbs, for which mouse, rat, and hamster are the best source of immune cells (*see Subheading 4.2.*), this can cause a problem for those working with rodent antigens.

Second, it is better to use female recipients since they, in general, mount a more effective immune response than their male counterparts—a characteristic that has as its downside an increased incidence of autoimmune disease. Additionally, the use of outbred or F1 hybrid animals will bring in a wider range of Major Histocompatibility Complex (MHC) molecules and hence potentially will increase the range of epitopes presented to T lymphocytes, thus enhancing the generation of T-cell help (*see Subheading 2.2.1.*).

2.2. Selection and Preparation of the Immunogen

2.2.1. Immunogenicity

Immunologists distinguish between the terms antigen and immunogen. Although at first sight this may seem a prime example of unnecessary jargon, there is a very sound scientific basis for the distinction. Whereas an immunogen is any substance that can generate an immune response (such as the production of specific antibodies), an antigen is one that can be recognized by an ongoing response (e.g., by antibodies) but may be incapable of generating a response *de novo*. This difference reflects the requirement for T lymphocyte activation in the generation of most antibody responses—the so-called “T-dependent” antigens, in which T-cell–derived signals are needed for full B-cell activation and expansion (only molecules that are directly mitogenic or have a high crosslinking capacity can activate B cells in the absence of this T-cell “help”). For T-cell help to be effective, the epitope seen by the T cell and the epitope seen by the B cell that it is helping must be present on the same antigen (termed “linked recognition”), although these epitopes need not be identical (*I*). Thus, when the antigen is a whole microorganism or complex macromolecule there is plenty of scope for the provision of both T- and B-cell epitopes and the antigen will be an effective immunogen. In contrast, where

the antigen is a small chemical group, such as the “hapten” di- or trinitrophenyl (DNP, TNP) that cannot be presented to T cells, the antigen will be unable to generate an immune response unless coupled to a larger “carrier” protein macromolecule (2). Similarly, a peptide antigen, unless it can by chance bind to one of the recipient’s MHC molecules, will not be presented to T cells and so will require conjugation to a larger carrier molecule. In addition, carbohydrate antigens, although they may be large in size, cannot be presented to T cells via classical MHC molecules and so again will require a carrier if a good high-affinity antibody response is to be generated.

The practical implications of this are that if the antigen of interest is nonprotein (e.g., bacterial capsular polysaccharide) or is a small peptide, it should be coupled to a large protein molecule of known immunogenicity, such as bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH) (3,4).

2.2.2. Choice of Immunogen Preparation

The preparation used for immunization is very much “project specific” and depends mainly on the purpose for which the antibody is being generated. Hence the immunogen could be: whole, killed, bacteria, or virus; a tissue homogenate; live whole cells in suspension (although after injection in vivo these will be neither alive nor whole for very long!); purified protein; or, as discussed in **Subheading 2.2.1.**, a hapten-carrier conjugate. In general, for polyclonal antibody production the immunogen should be as pure as possible so that only relevant B-cell clones are generated; for mAb generation purity at the immunogen stage is less crucial since a stringent screening procedure (*see Subheading 4.5.*) later in the technique will ensure that only the specific clones are selected.

2.2.3. Adjuvants

The immunogenicity of an immunogen can be enhanced by the coadministration of an adjuvant. These have two main modes of action: the provision of an in vivo depot from which antigen is slowly released, and the induction of an inflammatory response to enhance the overall immune responsiveness of the host animal in the vicinity of the injected immunogen. The most frequently used adjuvants are Freund’s complete (FCA) and incomplete adjuvant (FIA). These comprise mineral oil, emulsifying agent, and, in the case of FCA, killed mycobacteria. Several commercial adjuvants with similar properties are also available.

Adjuvants should never be used with the iv route of immunization. FCA should be used at only the first immunization, with FIA used thereafter. The use of adjuvants is strictly regulated, and guidelines must be adhered to.

2.3. Immunization Schedule

Immunization schedules are based on both logic and empiricism. The former requires a basic knowledge of how an immune response is generated. For the latter, it is sufficient to say—if you have a system that is successful, do not change it!

The main points to consider are the isotype and the affinity of antibody that you require. IgM antibodies are produced predominantly during the early phase of an immune response, and if it is this isotype that you require you should immunize the recipient either once, or at the most twice. Conversely, if IgG antibodies are wanted, a minimum of three or four immunizations is advisable. The likelihood of producing high-affinity antibodies is increased by repeated immunization (affinity maturation occurs in germinal centers during development of memory B cells), and is also theoretically enhanced by increasing the time between immunizations to ≥ 4 wk, since as immunogen is cleared from the body only those B cells with high affinity receptors will be triggered by the low levels of immunogen remaining (5).

2.4. Route of Immunization

The route by which an immunogen is administered is governed both by the species of recipient and by the immunogen itself. Soluble molecules and single cell suspensions can be injected intravenously, but all other preparations should be injected via a nonvascular route (intraperitoneal, intramuscular, intradermal, subcutaneous). Intraperitoneal immunization is good for small animals, such as mice and rats, but for larger recipients the intramuscular, subcutaneous, and intradermal methods are more appropriate.

For mAb production, for which a source of activated B lymphocytes is required, it is important to consider the route of immunization, since the site of antigen entry will affect the anatomical location of the immune response; in general iv entry will lead to an immune response in the spleen, whereas ip, id, im, and sc entry will focus immunity in the draining lymph node. The degree of such compartmentalization varies according to the species; thus for rats, ip immunization may give little response in the spleen and it is wise to use lymphocytes from the draining lymph nodes in addition to the spleen when performing the fusion. In contrast, in mice ip injection gives a good splenic response, which is fortunate given the small size of murine lymph nodes! A good compromise may be to use a non-intravenous site for all but the last immunization, and then to give a final boost, without adjuvant, intravenously to drive the immune response to the spleen (6). For polyclonal antibodies the site of entry is less important since all will eventually give rise to antibodies in the circulation.

2.5. Assessment of Immunity

Before collecting blood for serum (polyclonal antibodies) or spleen/lymph node cells (mAbs), it is useful to take a small sample of blood from the immunized animal to test for antibody production. Different recipients may vary in both the quantity and speed of their immune response, and the number of immunizations can be adjusted accordingly.

3. Polyclonal Antibodies

Once the recipient is fully immunized and antibodies specific for the immunogen can be detected in the serum (e.g., effective in immunohistochemistry at <1:100), a larger sample of blood can be collected (volume determined by the species of the recipient). A good polyclonal antiserum should contain approx 1 mg/mL specific antibody, although the actual concentration will depend on both the purity and immunogenicity of the immunogen used. Where the immunogen is a purified molecule, it can then be used to affinity-purify the specific antibody from the serum (e.g., secondary, antimouse Ig antibodies for use in immunoassays). If this is not possible, the serum should be used with caution since it will contain many specificities of antibody other than those directed against the chosen target.

4. Monoclonal Antibodies

4.1. Overview

The technique of mAb production was first devised by Köhler and Milstein, as part of an investigation of antibody diversity and affinity maturation (7,8). The technique has found an enormous range of laboratory, clinical, and industrial applications, and provides an excellent example of the crucial value of pure basic research to the more applied areas of science. The importance of their discovery was recognized by the award of the Nobel Prize in Medicine in 1984 (9).

In theory, the production of a mAb is very simple; it requires the growth of a clone of B lymphocytes and collection of the antibody produced by these genetically identical cells. In practice, however, it is much more difficult since B lymphocytes are mortal and cannot live for long *ex vivo*. The major purpose of the technology that we use is therefore to confer immortality on these important cells. This is achieved by fusion of an antigen-specific B cell with a myeloma cell, such that the resulting hybridoma inherits the ability to secrete specific antibody from its B lymphocyte parent and the property of immortality from the myeloma parent. The fusion itself is both rapid and simple, but a considerable amount of labor-intensive work is subsequently required to isolate individual antigen-specific clones from the multitude of fused and

nonfused cells that emerge from this initial step in the technique (for technical details *see* Freysdóttir; Chapter 17).

4.2. Myeloma Cells

Successful B cell hybridomas have been produced using myeloma cells of mouse and rat origin; several of these are available commercially. The two crucial features of these lines are that they have been genetically selected for an inability to produce their own immunoglobulin heavy and light chains, thus ensuring that the only antibody produced after fusion with a B cell is that encoded by the B cell's genome; and failure to survive in HAT (hypoxanthine, aminopterin, thymidine) selection medium (**Subheading 4.4.**), thus providing a means by which nonfused myeloma cells can be removed from hybridoma cell cultures.

The choice of myeloma depends to some extent on the ease with which they can be cultured. The nonadherent mouse lines are easier to handle and divide more rapidly than the adherent rat cells lines, and although successful mAbs have been produced using the rat system, the majority have been generated using murine myeloma cells.

A second consideration in the selection of the myeloma line is the species in which the immunized B lymphocytes will be generated. For optimal hybridoma stability the B cells and the myeloma cells should be from the same species since "mouse \times mouse" and "rat \times rat" hybridomas are genetically more stable, losing fewer chromosomes during the early stages after fusion when compared to species-mismatched hybrids. However, fusions using cells from closely related species, such as "rat \times mouse" and "hamster \times mouse," are also relatively stable and have been very successfully used to generate a wide range of rat and hamster mAb. It is only with more phylogenetically distant fusions that the problems of chromosome instability pose a serious problem, as for example with "rabbit \times mouse" or "sheep \times mouse" (6).

4.3. The Fusion

The original fusogen used by Köhler and Milstein was Sendai virus (7); however the technology was soon simplified, with polyethylene glycol replacing the virus. The effect of the fusogen is to cause fusion of adjacent cells; initially only the outer cell membrane fuses, leading to the formation of large "cells" that contain two different nuclei, one derived from the myeloma and the other from the B-cell parent. After the first mitotic division the nuclear contents are also pooled and two daughter hybridoma cells are produced. It is at this and subsequent early cell divisions that chromosome loss is likely to occur.

4.4. Selection of Fused Cells

Once the fusion has been performed, two major problems must be addressed: first, not all cells will have fused with a partner cell (discussed in this section); second, of those cells that did enter into a fusion, many will have fused with an inappropriate cell type, such as a T lymphocyte or a macrophage. Others will have fused with a B lymphocyte of an irrelevant specificity (*see Subheadings 4.5. and 4.6.*).

The first problem to be dealt with is the presence of unfused cells in the cultures. Spleen cells that have not entered into a fusion are in fact very easy to remove, since their mortality ensures that they will die within 1–2 wk in vitro. Unfused myeloma cells pose a much greater threat since they are immortal and if allowed to remain in the cultures will, with time, completely overgrow the slower-growing hybridoma cells.

The problem is overcome by the use of the selective medium HAT (Hypoxanthine, Aminopterin, Thymidine). Aminopterin blocks the main biosynthetic pathways for DNA and RNA synthesis. Cells containing a normal genome (unfused spleen cells and “myeloma × spleen cell” hybrids) can switch to alternative “salvage” pathways providing they are also given a source of hypoxanthine and thymidine for RNA and DNA synthesis, respectively. Utilization of these molecules for the salvage pathway requires the enzymes hypoxanthinephosphoribosyl transferase (HGPRT) and thymidine kinase; these are present in all normal cells, but the myeloma cells have been selected for loss of expression of HGPRT, and thus cannot survive in HAT medium. Thus, after 2–3 wk in selective medium, all unfused myeloma cells will have died resulting from their lack of HGPRT and all unfused spleen cells will have died because of their lack of immortality.

4.5. Tracking the Antigen-Specific B-Cell Hybridomas

After HAT selection, the cultures will contain only hybrids; the next step is to locate those hybrids that are secreting antibody specific for the target antigen.

To track the relevant hybrids, a small sample of supernatant medium is removed from each tissue culture well and is tested for specific antibody activity. The assay selected for this testing will depend very much on the final use for which the antibody is being prepared, and should be as close to this final use as possible since antibodies will perform with differing efficiencies in different assay systems. Nevertheless, certain characteristics are required of all assay systems: sensitivity (~1 µg/mL), reliability, speed, and ability to deal with multiple samples (you may have to test >100 samples/d). Suitable techniques include immunohistochemistry, flow cytometry, Western blotting, and

enzyme-linked immunosorbent assay (ELISA). This is one of the most crucial steps in mAb production since an mAb is only as specific as the tests that you have put it through.

4.6. Ensuring Monoclonality

The next problem that must be solved is that of clonality. At the start of culture a large number of different cells is placed in each well, and even after HAT selection and antibody screening more than a single clone of cells will exist in each well. Although after screening you know which wells contain cells that are secreting antibody with specificity for the immunogen, other hybrids in the same well may be secreting antibody to an irrelevant antigen (e.g., pathogens previously encountered by the immunized animal), or that may produce no antibody at all (e.g., “T cell \times myeloma”).

The most popular method for establishing monoclonality of specific antibody-secreting cells is that of limiting dilution. Hybridomas from wells that contain specific antibody activity are transferred to 96-well tissue culture plates at very high dilution such that the average plating density is one cell per three wells, although the actual cell plating follows a Poisson distribution. Feeder cells are also added (e.g., peritoneal macrophages) to provide cell contact and cytokines. Once the plated hybridomas have grown, their supernatant medium is again tested for antibody activity, and the cells from positive wells are again cloned by limiting dilution. The antibody screening and recloning is then repeated once more to ensure monoclonality. In addition, if NSO murine myeloma cells were used for the initial fusion, the distinct compact colonies formed can readily be recognized, providing visual confirmation of the presence of a single clone. Alternatively, hybridomas can be cloned by flow cytometry or in semisolid agar.

4.7. Bulk Production

At this stage of the technique it is likely that the precious, specific antibody-secreting hybridomas comprise a maximum of one million cells in a volume of 200 μ L of antibody. The next task is therefore to grow the cells up in bulk so that a large amount of antibody can be produced and cell samples can be frozen and stored for future use (*see Subheading 4.8.*). Hybridoma cells dislike sudden alterations in their lifestyle, so expansion of cell numbers should be carried out by gradually increasing the size of the container in which they are grown. Thus, they are transferred from small (200 μ L) wells to larger (2 mL) wells and from there to small, medium, and then large flasks. After this, the hybridomas can be grown in a variety of in vitro culture systems designed for bulk production of mAb, ranging from large, but simple, culture flasks and bottles to the purpose-designed complex hollow fiber and other specialized

equipment. Culture under standard conditions provides an antibody yield of 2–50 $\mu\text{g/mL}$, but much higher concentrations can be obtained with the specialized systems.

An alternative method that is now rarely used is the production of ascite mAb. Hybridoma cells can be grown as an ascitic tumor in genetically compatible, or immunoincompetent, hosts, and the antibody-containing ascitic fluid collected. The advantage of the method is that high concentrations of antibody can be produced (approx 10 mg/mL); the major disadvantage is that it requires the use of experimental animals. Given the efficiency with which mAb can be produced by *in vitro* methods, use of the ascites method cannot now be justified.

4.8. Paranoia

The production of a mAb is highly labor-intensive and will, on average, take approx 6 mo from initial immunization to the point at which you have sufficient mAb for use. Moreover, at many points along this route disasters can occur and annihilate all your hard work. For this reason it is entirely acceptable to indulge in a little paranoia!

A major threat is contamination of the cell cultures with bacteria, yeast, or fungi. All are serious, but by far the most devastating is mycoplasma since it is not visible by eye and its presence is frequently only appreciated when the infected cells exhibit poor growth and low yields of antibody. All cell lines should therefore be tested regularly for mycoplasma contamination, using one of the many kits that are available commercially. The most frequent source of mycoplasma contamination is via the introduction of a new cell line into your laboratory. The only effective policy is to test all new lines prior to admitting them into your main tissue-culture facility (keep them in “quarantine”). This may cause friction with the donor, who will state categorically that his or her cells are clean, but it is wise to be absolutely firm on this! Wherever possible, contaminated cultures should be rapidly disposed of. If the infected culture is very valuable, it can be treated with antibiotics or an antifungal agent, as appropriate, although the latter frequently causes death of the hybridoma cells as well.

As soon as you have sufficient cells, aliquots should be frozen and stored in liquid nitrogen. Batches should be frozen on different days, to provide insurance against contamination, and should be stored in more than one liquid nitrogen tank. This will protect you against the accidental drying out of the liquid nitrogen tank and the loss of your irreplaceable cells.

Finally, do treat your antibodies with respect. Individual antibodies have very different properties; some are stable for months and even years at +4°C, whereas others can survive at this temperature for only a few days. The majority are stable for long periods of time if stored at –20 or –80°C, preferably the latter. The preparation of the antibody also affects its storage properties. Tis-

sue-culture supernatants store well, since the serum proteins present in the medium improve the stability of the low concentration of mAb ($\sim 10 \mu\text{g/mL}$). Purified antibodies should be stored in neutral isotonic buffer (phosphate-buffered saline, Tris-buffered saline) at a concentration $>500 \mu\text{g/mL}$. Lower concentrations should be stored with a carrier protein (e.g., 0.1% BSA). Antibody activity is progressively destroyed by repeated freezing and thawing, so mAb should be stored in appropriately sized aliquots.

5. Polyclonal vs Monoclonal Antibodies: The Choice

Which is better, a monoclonal or a polyclonal antibody? The answer to this question depends on the use to which the reagent is to be put.

Polyclonal antibodies have been particularly useful in two situations: first, where it is beneficial for a reagent to recognize more than one epitope on a target molecule, and second, where the molecule of interest is highly conserved.

One of the most frequent and important applications of polyclonal antibodies for the detection of multiple epitopes is as secondary, conjugated, reagents for indirect immunoassays (e.g., ELISA, Western blotting, immunohistochemistry, flow cytometry; *see* Chapters 30, 32–35), where polyclonal binding to the primary layer antibody leads to considerable amplification of the signal.

Highly conserved molecules are in general poorly immunogenic, since they will closely resemble the recipient's own equivalent molecule, to which it will be tolerant. It has therefore proved useful to move away from mouse, rat, and hamster to genetically more distant mammals, such as rabbit and sheep, or even to birds, such as the chicken (*10,11*); however, in these species polyclonal antibodies are the only feasible conventional option.

Despite these advantages, polyclonal antibodies have several disadvantages. A major problem is that of quantity, since a single animal is unlikely to produce sufficient reagent. This leads to a second, related problem: no two animals will produce an identical response to the same immunogen. Indeed, the same animal will respond differently to each dose of immunogen, as its immune response evolves. Polyclonal antibodies are therefore limited in the amount that can be produced and, once exhausted, can never be exactly reproduced since subsequent batches will contain a different range of specificities and affinities.

Monoclonal antibodies, in contrast, provide an unlimited source of antibody that is homogeneous and, once characterized, predictable in its behavior. mAbs have been invaluable in providing excellent primary reagents to molecules previously defined by less reliable polyclonal antibodies. However, their major impact has been in their use to discover and characterize the structure and function of novel molecules. For example, almost every molecule that we now know

to be important in an immune response, with the exception of CD4 and CD8, owes its identification to the generation of specific mAb (**12**). Once fully characterized, mAbs provide highly reproducible reagents for clinical diagnostic assays and, with modification to reduce their immunogenicity, they have potential for clinical therapy (*see* Chapters 3, 5–12, and 14).

The disadvantage of mAbs is that they cannot provide signal amplification (unless an artificial “polyclonal” antibody is made by mixing several mAbs); and since only rodent B cells have been really successful, there are epitopes to which these reagents cannot be generated. An important recent approach to this latter problem has been the use of phage display for selection of recombinant monoclonal antibodies, a technique that avoids the *in vivo* deletion of self-reactive specificities (**13,14**); (Chapters 4 and 37).

Thus, each type of reagent has distinct advantages and disadvantages, and there is a clear need for both in the generation of antibodies for use in laboratory and clinic.

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