PEG fusions routinely produce one viable hybridoma from 10^5 starting cells. This may be below the needed efficiency. One method that is gaining more widespread use is fusing cells by applying high-voltage electrical gradients across cell populations—a sequence of short bursts of electric current fuses adjacent membranes, increases membrane permeability, and yields hybrid cells. Electrofusion is accomplished in three steps: Pre-alignment of the cells (convergence and cell contact), membrane fusion, and post-alignment (rounding off the fused cells). This method has been applied successfully to hybridoma production with higher fusion efficiency, allowing production of more hybrid cells. In general, this has not been important for most fusions, because hybridoma production is normally limited by the screening method rather than by the frequency of hybridoma production. As more rapid screening procedures are developed, this fusion method will become more important. In addition, as techniques are developed that allow the selection of the desired antibody-secreting cell before fusion, this and other high-efficiency methods will become increasingly valuable (see Protocol 21).

PLATING STRATEGIES

Several plating strategies have been used successfully to identify hybridomas that are secreting the appropriate monoclonal antibody. The strength of the immune response (titer) can be used as a guideline to predict the frequency of positive clones. A higher titer correlates well with the number of B cells that are producing circulating antibody, which also correlates with a greater number of positive clones resulting from the fusion. This suggests that cells from high-titer animals should be plated in an increased number of plates so that the cells will be at lower cell densities. The difficulty of the screening assay also needs to be taken into account. If the screening assay is going to be very labor intensive, plating the fusion out in few plates may be preferable. Bulk cultures may be useful here as well. The fusion can also be plated in soft agar, making individual hybridoma colonies easier to isolate. This may reduce subcloning time later on. There are no set rules to govern which strategy is correct, but a few of the more common considerations are described below.

Single Cell per Well (96-Well or 384-Well Plates)

The single-cell-per-well (96-well or 384-well plates) plating strategy is illustrated in Figure 14. If the number of B cells to be fused is small and/or the screening assay is very high throughput, it may be preferable to plate the fusion out in a large number of plates so that the resulting hybridomas will be a single colony per well. Counting the cells and calculating the volume necessary to deliver 0.5 cell per well will accomplish this. (Here the goal is to have single cells per well. If the cell suspension is diluted to 0.5 cell/well, many wells will not have any cells in them, but none of the wells will have more than 1 cell/well.) This option is advantageous regardless of how high the titer is. The time it saves at subsequent stages in the hybridoma selection process, single-cell cloning, makes it worthwhile. There is also a greater chance that unique clones will not be lost by overgrowth of more rapidly dividing,
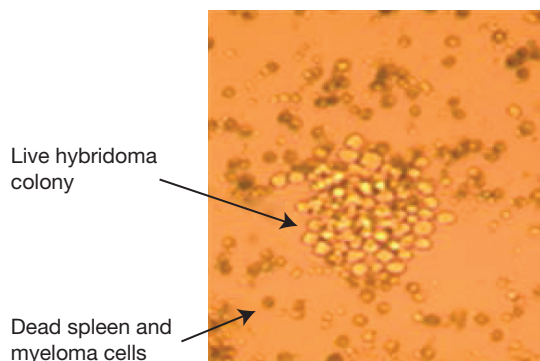

FIGURE 14. **A monoclone hybridoma colony growing out 7 d postfusion.** (Photo courtesy of Mohan Brahmandam, Dana-Farber Cancer Institute.)

Live hybridoma colony
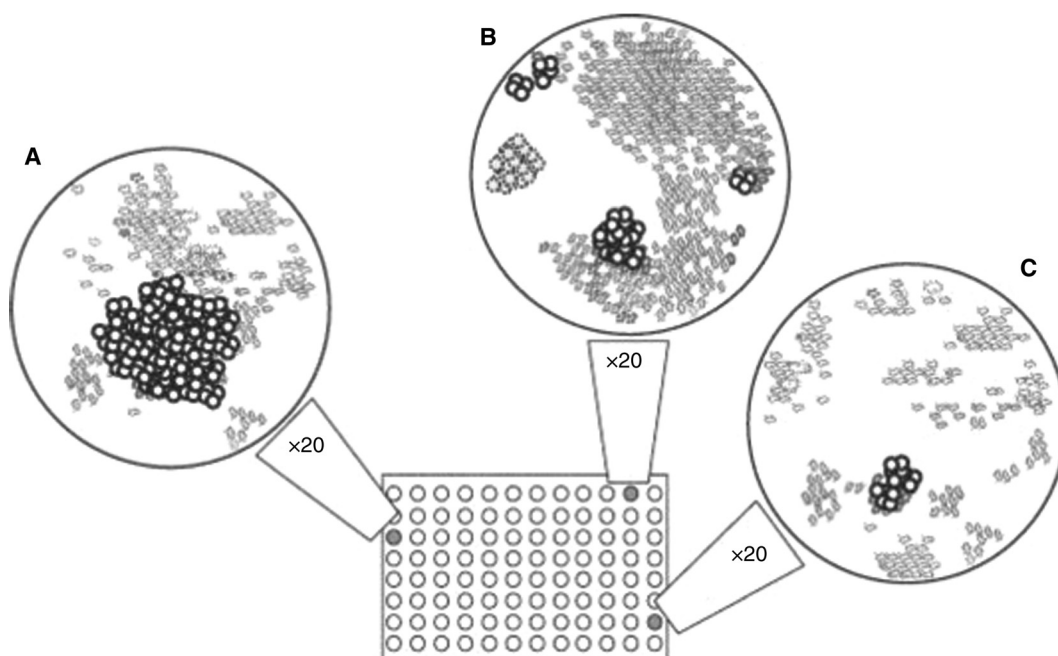
Dead spleen and myeloma cells

FIGURE 15. **Multiple hybridoma colonies in 96-well plate.** After 10–14 d postfusion, hybridoma colonies can be observed in the 96-well plates. Wells may contain a single hybridoma colony (*A*) or multiple hybridoma colonies derived from different, independently fused B-lymphocyte/myeloma cells (*B*). Some hybridomas grow more rapidly (*A*) than others (*C*), even though they came from the same fusion. (Reprinted, with permission, from Rodriguez-Calvillo et al. 2004, © Elsevier.)

nonproductive clones before the positive hybridoma can be cloned out. The main disadvantage is the much larger number of wells that will require screening.

Multiple Cells per Well (96-Well or 384-Well Plates)

The multiple-cells-per-well (96-well or 384-well plates) plating strategy is illustrated in Figure 15. It is often simpler to plate a fusion out in a small number of plates (10–20) and not be overly concerned over the number of cells per well. Although it is not advisable to have more than three to five hybridoma colonies per well, the number of plates can be based on the total number of B cells obtained from the animal. The fusion will need to be screened more quickly and subcloning should be performed as soon as the positive well is identified to ensure clonality and stability. An average immunized spleen contains 0.5×10^8 to 2.0×10^8 lymphocytes. Plating out eight to 12 96-well fusion plates should result in three to five hybridoma colonies per well, allowing the fusion to be screened in a few days. Substituting 384-well plates for 96-well plates makes it easier to obtain single-colony wells with fewer plates to screen and a smaller footprint in the incubator. The disadvantage with 384-well plates is that they are very difficult to manipulate by hand, often requiring some level of automation.

Bulk Plating (24-Well or Six-Well Plates)

Fusions do not have to be plated out into a large number of wells. When the screening assay is difficult or requires more supernatant than is obtainable from a 96-well plate, plating the fusion out into a bulk culture is an alternative. Twenty-four-well or six-well plates or even small Petri dishes can be used. Screening will have to be accomplished sooner before the desired clones are lost. The advantages are that the number of wells to screen is greatly reduced, but each well may contain multiple positive hybridomas that will require multiple subclonings to ensure stability and clonality.

FIGURE 16. Hybridoma colonies in soft agar plate. Hybridomas may be plated out in semi-solid agar instead of 96-well plates. As shown here, each independent hybridoma colony appears as a spot growing in the agar that can easily be removed using a pipette and individually transferred to a plate. This eliminates the need for a round of limiting dilution subcloning. (Reprinted from http://www.stemcell.com/en/Products/Area-of-Interest/Semi-solid-cloning.aspx.)

Plating in Soft Agar

The plating-in-soft-agar strategy is illustrated in Figure 16. Soft agar can be an effective method of plating a fusion in a semi-solid medium that will allow the diffusion of antibodies but will hold the hybridomas in place. This is most commonly performed in soft agar (for the preparation of semi-solid medium, see Protocol 23). After the soft agar has solidified, the cells are overlaid with regular tissue culture medium. The antibodies can then diffuse into the liquid medium, and the medium can be removed for testing. If a well is scored as positive, the clones within the agar are removed, grown up individually, and retested. One additional advantage of this technique is that the division rate of cells in soft agar is normally slower than in liquid media; thus, there may be extra time for screening. One disadvantage is that even the hardiest hybridomas do not have high plating efficiencies in soft agar, and therefore the number of clones that arise in these conditions will be reduced.

FEEDING HYBRIDOMAS

Two strategies are used in deciding whether to feed hybridomas before screening. Early protocols suggested the removal of medium and then addition of fresh medium. This approach has not proven to be of much advantage but does lead to more work. The addition of fresh medium to the hybridoma cultures at approximately Day 4 or 5 after the fusion will improve the general health of the cultures and will keep them rapidly growing throughout the screening procedure. Some researchers prefer not to feed the cultures, allowing any poorly growing clones to die, and then to concentrate on the hardiest of the clones that grow up. Feeding is performed by adding 100 µL of fresh medium supplemented with 20% FBS, 1× OPI, or 3%–5% Hybridoma Cloning Factor, and 1× HT (HAT without aminopterin). The HAT selection should be completed after 5 d, and the cultures can be weaned slowly into HT medium. This removes the drug aminopterin, which has been suppressing de novo nucleotide synthesis. It will take a few days for the cells to recover the down-regulated function and then the hybridomas can be switched into regular 10% FBS medium without the HT supplement.

SUPERNATANT COLLECTION STRATEGIES FOR SCREENING

The most straightforward way to screen a fusion is to collect supernatants separately from all the wells in the fusion plates and screen them individually. This makes identification of wells containing positive hybridomas very obvious. When the number of fusion plates to be screened is large and the screening assay required is more difficult to perform, more manageable methods to screen the fusion more rapidly will need to be used. Pooling of tissue culture supernatants is an effective method to reduce the total number of tests that must be performed in 1 d. The major difficulty encountered when supernatants are screened as pools is that the positive well in the pool will normally need to be identified by immediate rescreening.

In some cases, the pool size may need to be adjusted during the screening. In addition, for some assays, the pool size will be limited by the sensitivity of the test. For example, some assays are dependent on the concentration of the positive antibody, and dilution of the antibody during pooling may reduce the signal below the detection level.

Two types of pooling strategies are in common use:

- *Simple Pools.* The most widely used method of pooling is a simple combination of several tissue culture supernatants. The most important variable is the choice in the number of wells that will form a pool. Because the goal of any screening strategy is to eliminate ∼90% of the cultures, gauge the size of the pool so that only about one in 10 of the pools are positive.

- *Matrices.* If the positive supernatants are likely to be rare, arrange the tissue culture supernatants in a two-dimensional (2D) matrix. Because pools of supernatants are prepared from each vertical column and each horizontal row, the correct wells can be identified by the location of intersecting positives.

SCREENING

Wells containing hybridomas are ready to start screening ∼7–14 d after fusions (about Day 7 for azaserine/hypoxanthine selection and Days 10–14 for others). Representative wells at different times after fusion are shown in Figure 17. For most assays, clones that are just visible by eye are about the right stage for screening.

Some examples of appropriate screening methods are discussed in Protocols 1–12.

- When the clone or clones in a well are ready to screen, mark the wells with some convenient numbering system. Carefully and aseptically remove ∼50–100 μL of tissue culture supernatant from each well without disturbing the hybridomas. This can be done conveniently by using a pipettor. After removing the supernatant, refeed the well with fresh medium.

- Transfer the supernatant to a suitable container. If all of the supernatant is going be used in the screening assay, the hybridoma tissue culture medium can be transferred directly to the assay vessel. However, it is normally more convenient to transfer the supernatants to a microtiter tray. Mark the tray to correspond to the original well number.

- For most antigens, the supernatants are ready for testing without any special treatments. In some instances, lower backgrounds can be achieved by removing any cells in the supernatants by centrifugation before testing.

- Any remaining supernatant should be stored at −20°C. This can be used for confirmations, further testing, or identifying individual wells from pooled samples.

- Although splenocytes do not grow in standard tissue culture medium, they do not die immediately and will continue to secrete antibodies. This may lead to the detection of false-positive wells in early screens. All positive cells should be rescreened before freezing or single-cell cloning. Supernatants should not be diluted more than 1:3 when used in screening assays.

EXPANDING AND FREEZING POSITIVE CLONES

The process of expanding and freezing positive clones is illustrated in Figure 18. The hybridomas that are to be expanded off the fusion plates should be adapted to grow in regular 10% FBS medium without special supplements as soon as possible to prevent them from becoming dependent on the presence of the supplements. The process of weaning the hybridomas off the drug selection is achieved by first growing the cells for several passages in complete medium with hypoxanthine and thymidine, but lacking aminopterin, azaserine, or methotrexate. Growing the cells in the presence of the base but without the drug allows all of the inhibitors to be diluted to a safe level and nucleotide biosynthesis to be
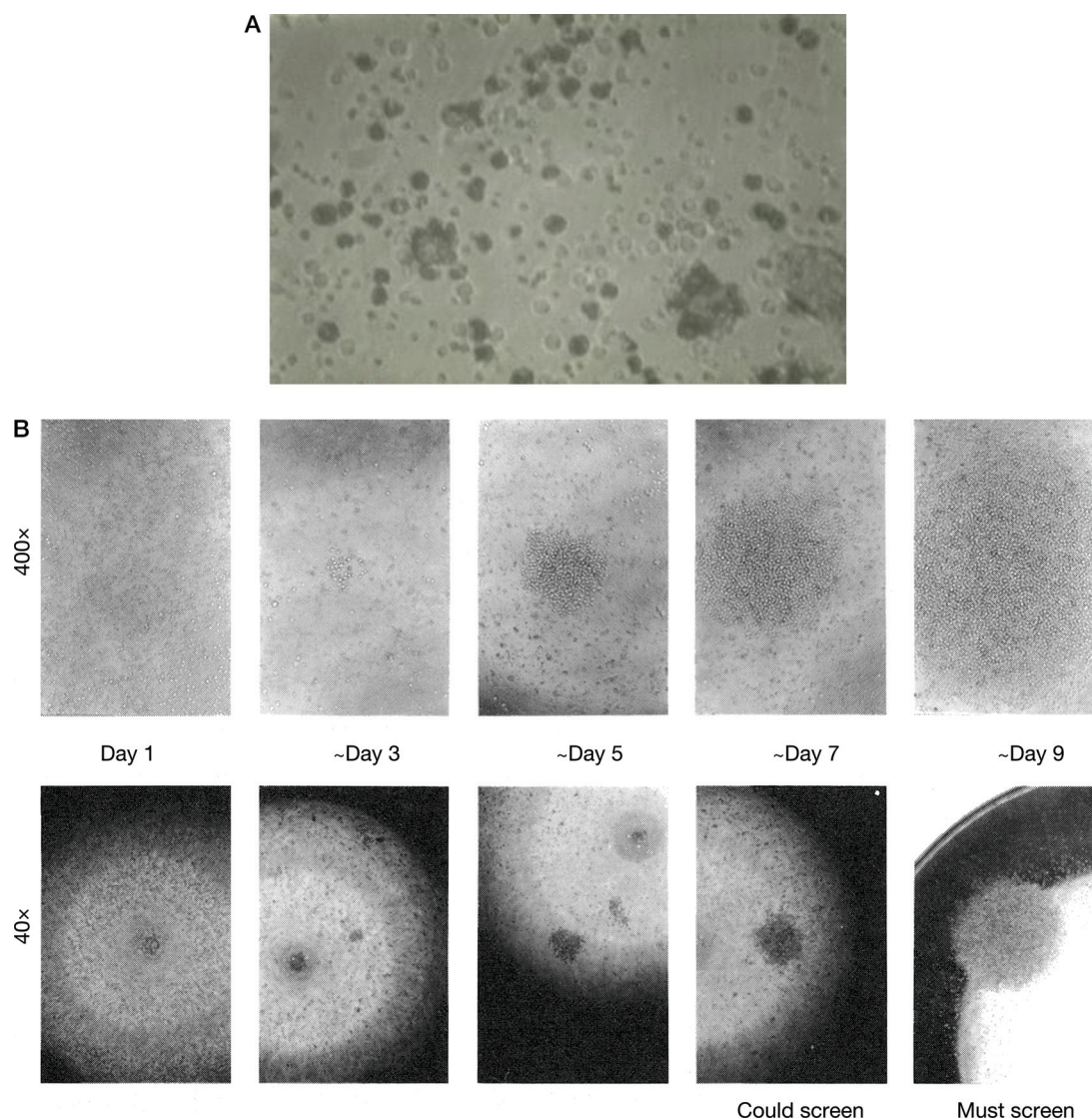
FIGURE 17. Progression of hybridoma growth postfusion. (*A*) Fusion mixture after 3 d of growth in HAT medium. Note that very few cells are visible and there is much debris. (*B*) Representative wells at various days after fusion. (*A*, Reprinted, with permission, from Liddell 2005, © Wiley.)

up-regulated before removing the bases. This process should have been started 5−7 d after the fusion was plated. By the time the hybridoma colonies need to be expanded off the fusion plates, the weaning from HT-supplemented 20% FBS medium into HT-supplemented 10% FBS medium should have been completed in the 96-well fusion plates.

After positive hybridoma wells have been identified, the cells are transferred from the 96-well fusion plate to 0.5 mL of medium supplemented with 10% FBS, 1× OPI or 3%−5% Hybridoma Cloning Factor, and 1× HT in a 24-well plate. As the 24-well cultures are fed, they are slowly adapted to regular 10% FBS medium without supplements. After the 24-well culture becomes dense, the hybridoma cells may be transferred into 5.0 mL of medium supplemented with 10% FBS in a T25 flask. After 24−28 h, the cells should have recovered from the expansion and will be growing in log phase. An additional 5 mL of medium supplemented with 10% FBS can be added to the flask, making a total of 10 mL of medium. Some cells should be frozen down as backup. This is also a convenient stage at which to collect 8−9 mL of supernatant, in case any further testing of hybridomas needs to be performed, before concentrating on particular clones. However, if the correct
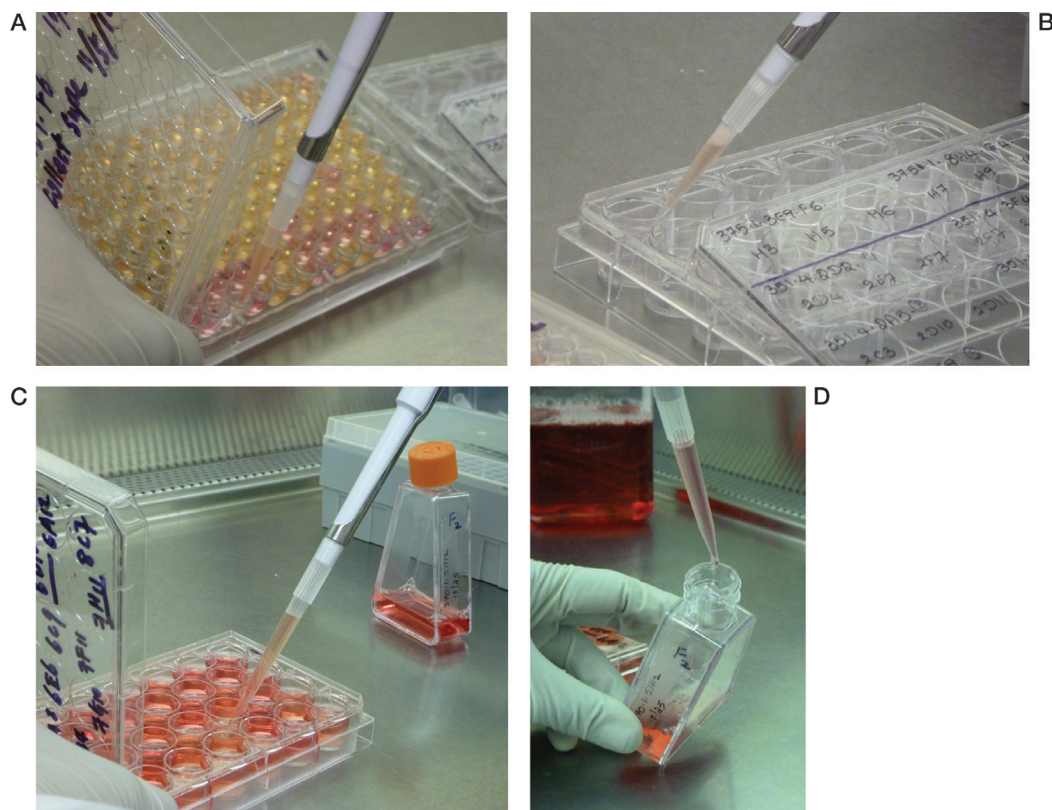
FIGURE 18. Expansion of hybridoma colonies. Transfer of hybridoma from a 96-well plate (*A*) to a 24-well plate (*B*), and from a 24-well plate (*C*) to a T25 flask (*D*).

clones have already been identified, the cells should be single-cell-cloned as early as possible. This can be performed as early as when the hybridomas are expanded off the fusion plates. Techniques for the freezing and storage of hybridoma cell lines are described in Chapter 8.

Often it is difficult to maintain cell viability when transferring hybridomas from one size culture to another. Presumably this is caused by the dilution of the growth factors in the medium and may be caused in part by overgrowth in the previous stage. If these problems exist, add a drop or two of Hybridoma Cloning Factor. Stepping the weaning process back to when the cells were growing well or using feeder cultures may help get the cells to the next stage (see Protocols 14–17 for the preparation of feeder cultures). In addition, adding a sample of the diluted culture back into the original well will serve as a good backup if any problems arise.

After the screen has been completed, the decision on the appropriate next steps will depend on the number of positives that have been identified. If no positives are found and the immunization yielded a strong response, the fusion should be repeated, but the choice of screening method should be re-evaluated. If the immune response was weak, new approaches to the immunization should be tried. If only a few positive clones were identified, these should be tested as early as possible to determine whether they will perform adequately in the appropriate assays. If a comprehensive set of immunochemical reagents is needed, additional fusions are likely to be needed. If the fusion has been very successful (greater than 50 positives), it is seldom worthwhile and often practically impossible to carry and maintain all of the clones. In these situations, many of the clones are likely to result from fusion of sibling antibody-secreting cells and therefore will not generate new antibody activities. Some sort of secondary screen should be considered to identify particularly valuable clones. This might be based on affinity or perhaps the subclass of the resultant antibodies. The antibodies should be screened in as many different assays as possible to characterize them fully. It is unlikely that every monoclonal antibody will work in every assay for which it may be required. It

is preferable to uncover which hybridomas produce antibodies that work in the assay and which do not. This will allow a panel of monoclonal antibodies to be identified that will meet all current and future needs.

Single-Cell Cloning

After a positive tissue culture supernatant has been identified, the next step is to clone the antibody-producing cell. The original positive well will often contain more than one clone of hybridoma cells, and many hybrid cells have an unstable assortment of chromosomes. Both of these problems may lead to the desired cells being outgrown by cells that are not producing the antibody of interest. Single-cell cloning ensures that cells that produce the antibody of interest are truly monoclonal and that the secretion of this antibody can be stably maintained.

Isolating a stable clone of hybridoma cells that all secrete the correct antibody is the most time-consuming step in the production of hybridomas. Depending on the chances of the original positive being derived from a single cell, the easiest and quickest methods to prepare single-cell clones will differ. If the positive well contains multiple clones or if secretion of the antibody is highly unstable, the cloning should be performed in two or more stages. In the first cloning, you should try to identify a positive well with only a few clones and then try to isolate a single-cell clone from this stage. This often can be achieved by a combination of different cloning methods. For example, quick cloning by limiting dilution could be followed by cloning with a single cell pick or single-cell sorting using a flow cytometer into a 96-well plate.

Because hybridoma cells have a very low plating efficiency, single-cell cloning is normally performed in the presence of feeder cells, growth supplements, or conditioned medium. Good feeder cells should secrete the appropriate growth factors and should have properties that allow them to be selected against during the future growth of the hybridomas. Feeder cell cultures are normally prepared from splenocytes, macrophages, thymocytes, or fibroblasts (Protocols 14–17). To ensure that a hybridoma is stable and single-cell-cloned, continue repeating the cloning until every well tested is positive. Cloning hybridoma cells by limiting dilution is the easiest of the single-cell-cloning techniques. In Protocol 22 two approaches are described, one rapid technique for generating cultures that are close to being single-cell-cloned and one for single-cell cloning directly.

Even though every attempt is made to ensure that the cells are in a single-cell suspension before plating, there is no way to guarantee that the colonies do not arise from two cells that were stuck together. Therefore, limiting dilution cloning should be done at least twice to generate a clonal population. It is better to keep re-subcloning until every well showing hybridoma growth has antibody activity and then subclone it once more to be sure. This will ensure that the expanded single-cell subclones are both stable and clonal.

It is also possible to subclone using a flow cytometer. This method separates a bulk population of hybridomas into single cells, which are then deposited individually into wells of a 96-well cell culture plate containing 100 μL of medium supplemented with growth factors and/or feeder layer cells. The bulk parental population does not require staining with fluorescent tags to be sorted into single cells. The individual hybridomas will grow out into colonies that are then screened for antibody activity. As with the limiting dilution method, the process should be repeated until all wells showing hybridoma growth test positively for the correct antibody activity.

Cloning of hybridoma cells in semi-solid medium is another commonly used method for producing single-cell clones (see Protocol 23). The technique is easy, but, because it is performed in two stages, it does take longer than other methods. Not all cells will grow in soft agar, and there may be a bias on the type of colony that appears. However, most of the commonly used myeloma fusion partners have relatively good cloning efficiencies in soft agar, and consequently, so do most hybridomas. Even though every attempt is made to ensure that the cells are in a single-cell suspension before plating, there is no way to guarantee that the colonies do not arise from two cells that were stuck together. Therefore, single-cell cloning in soft agar should be repeated at least twice before the cells are considered clonal.

Unstable Lines

If hybridomas continue to produce <100% positive wells, even after four or more single-cell-cloning steps, the lines probably have an unstable assortment of chromosomes. If the antibodies produced by these cells are particularly valuable, extra work to save these lines may be necessary. Three strategies can be used.

- The most straightforward strategy is to continue the single-cell cloning on a regular basis, trying to isolate a stable subclone. Perhaps surprisingly, this often works. The screening assays should be adjusted to screen not only for the presence of the appropriate antibody, but also for the levels of antibody produced. Wells that contain a stable subclone of the original should produce higher levels of antibodies. If the stable variant is generated early in the proliferation within a well, the differences in antibody production between the well containing the variant and those that do not will be significant. At this stage, many investigators stop screening with an antigen-specific assay and only screen for the level of mouse antibody produced (see Chapter 15). After a stable line is generated, the specificity of the antibody should be re-established.

- Repeat the fusion process and fuse (or "back-fuse") an important hybridoma line again with a myeloma, and allow the chromosomes to reassort from the beginning, hoping to isolate the stable variant from this source. The same myeloma line originally used for the fusion, a different myeloma line, or a myeloma line engineered to express survival enhancers (e.g., Bcl2) or growth factors (e.g., murine IL-6) can be used. To date, most refusions have been performed by standard techniques and extensive screening. However, the introduction of a selectable drug selection marker into a suitable myeloma cell line should make selection against the parental myelomas easier. The hybridoma would carry a functional *HPRT* gene, whereas the myeloma would carry, for example, a neomycin gene. Selection for both genes should yield only successful secondary hybridomas.

- Use molecular biology techniques to isolate and clone out the immunoglobulin heavy- and light-chain genes from a clonal hybridoma line and transduce them into a Chinese hamster ovary (CHO) or NS0 production cell line. These cells would then produce and secrete the monoclonal antibody without the stability issues inherent in the original hybridoma cells. The cDNA can easily be stored as a backup as well.

DEALING WITH CONTAMINATION

During the early stages of the fusion, contamination will mean the loss of the well or the entire fusion; however, in subsequent stages, important hybridomas can sometimes be saved. Because hybridoma generation involves obtaining tissues (spleen and lymph nodes) from animals, the potential for contamination is always present. The contamination will usually show up within 24 h of the fusion. If not dealt with immediately, the fusion will be lost. Yeast and molds are the most difficult to eradicate. Bacteria may be controllable if contamination has not reached excessive proportions (see Fig. 19).

If the fusion shows contamination, the first thing is to determine what the contamination is and then how widespread it is. If it is a yeast, mold, or heavy bacterial problem, then a large effort will need to be expended to save the fusion. If another immunized animal has a good titer, it may be more worthwhile to cut your losses and perform a new fusion. If the fusion cannot be repeated and must be saved, it will require daily attention for a few weeks. This will not guarantee that the fusion can be saved.

Contamination in the Fusion Wells—A Few Wells Only

- Contaminated wells can be identified by their unusual pH or turbidity. Confirm the presence of the contaminating organisms by observing them under the microscope. Mark the wells.
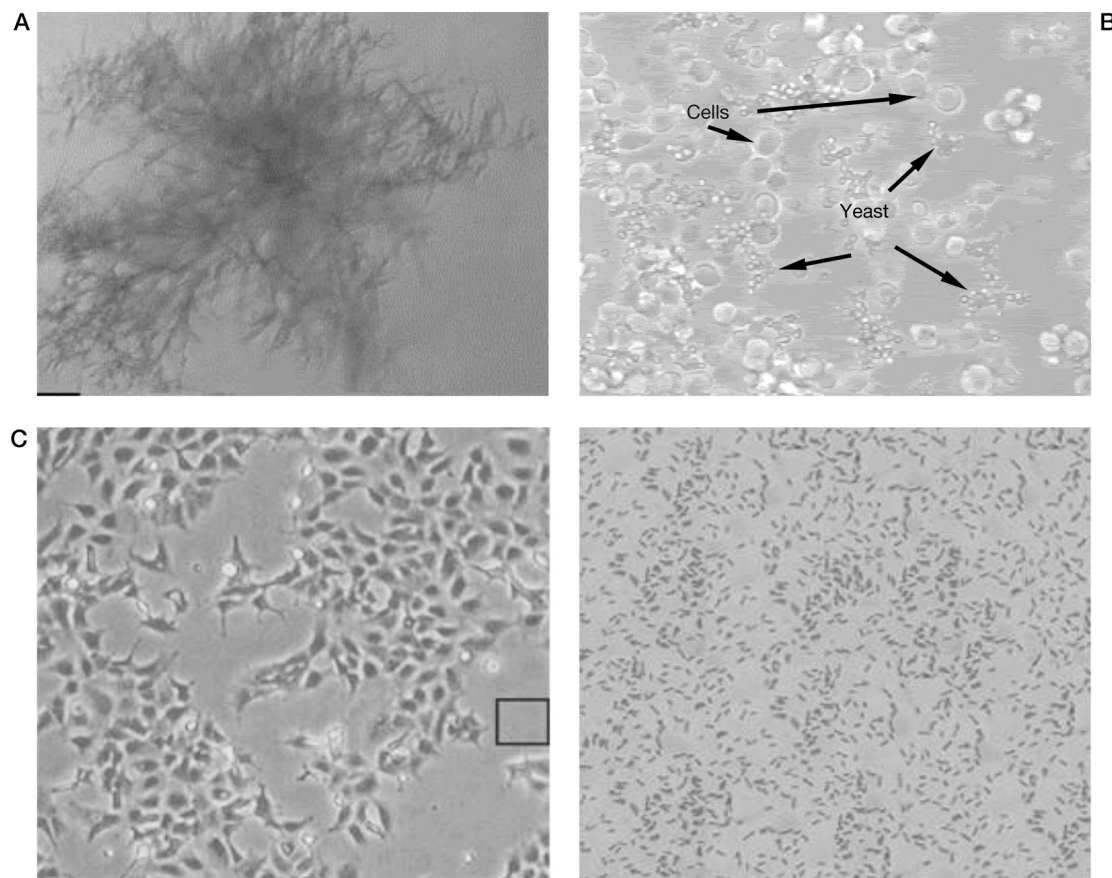
FIGURE 19. **Common forms of contamination.** (*A*) Fungi. (*B*) Yeast. (*C*) Bacteria. Adherent 293 cells contaminated with *Escherichia coli* (tiny shimmering granules). The magnified area shows the individual *E. coli* cells. (*A,B*, http://brensoft84.blogspot.com/2008/05/brennan-serial-cell-killer.html; *C*, reprinted, with permission, from Life Technologies Corporation, © 2013 [http://www.invitrogen.com/site/us/en/home/References/gibco-cell-culture-basics/biological-contamination/bacterial-contamination.html].)

- Move to the tissue culture hood after all other work has been completed for the day. Carefully remove the lid. If the underside of the lid is damp, replace with a new lid. Dry the top and edges of the plate itself by aspiration before replacing the lid. If there is contaminated medium on the lid, then autoclave the whole plate without any further work.

- Remove the medium from the contaminated well by aspiration. Check to make sure that there is no medium in between the adjacent wells. Try to avoid generating any aerosols. Slowly add enough 10% bleach to the well to bring the level right to the rim. Allow it to sit for 2 min at room temperature.

- Remove the bleach from the contaminated well by aspiration. Add enough ethanol to the well to bring the level right to the rim. Remove by aspiration and repeat.

- Dry the well by aspiration and add a new, sterile lid.

Contamination in the Fusion Wells—Gross

- Autoclave the plates.

Contamination of a Cloned Line

- If the line has been frozen, it is easiest to go back to the most recent freeze down and thaw a fresh vial of the cells.

- If the line has not been frozen, inject the cells into mice that have been primed for ascites production (see Chapter 8). The animals must be of a compatible genetic background to your hybrids (e.g., BALB/c×BALB/c into BALB/c or BALB/c×C57B1/B6 into BALB/c×C57B1/B6 F$_1$). If no mice have been primed with 0.5 mL of pristane the required 1 wk in advance, inject 0.5 mL of Freund's adjuvant into the peritoneum. Wait 4 h to 1 d and inject the hybridomas. Inject at least two mice for each contaminated culture.

- When and if ascites develop, shave and sterilize the abdomen of the animal. Tap the ascites and transfer it into a sterile centrifuge tube (see Chapter 8 for more information on ascites production).

- Centrifuge the ascites at 400g for 5 min at room temperature.

- Remove the supernatant. Resuspend the cell pellet in 10 mL of medium supplemented with 10% FBS, and transfer it to a tissue culture plate. The supernatant can be checked for production of the appropriate antibody. If positive, save for use. A subclone plate should be set up and single colony wells screened for the expected antibody to ensure stability.

- Handle as for normal hybridomas, except keep the cells separate from the other cultures until there is little chance of the contamination reappearing.

The success rate may be as high as 80%.

Animals injected with infected cultures should be kept isolated from the main animal colony and monitored closely for signs of infection.

ISOTYPING AND SUBCLASSING OF MONOCLONAL ANTIBODIES

Many techniques for using monoclonal antibodies require antibodies with specific properties. One set of these properties is unique to the individual antibody itself and includes such variables as specificity and affinity for the antigen. These properties all depend on differences in the antigen-combining domain of the antibody and can be assayed by comparing the properties of the monoclonal antibodies in tests that measure antigen-binding activity.

A second set of important properties for monoclonal antibodies is determined by the structure of the remainder of the antibody—sequences encoded by the antibody common regions. These properties include the class or subclass of the heavy chain or the light chain (Fig. 20). The different classes or subclasses will determine the affinity for important secondary reagents such as Protein A. The type of heavy and light chain can be distinguished by simple immunochemical assays that measure the presence of the individual light- and heavy-chain-specific polypeptides. This is normally achieved by raising antibodies specific for the different mouse heavy- and light-chain polypeptides. The production of these antibodies is possible because the light- and heavy-chain polypeptides from different species are sufficiently different to allow them to be recognized as foreign antigens. Most often these anti-mouse immunoglobulin antibodies are raised in rabbits as polyclonal sera, and then the antibodies specific for a particular heavy or light chain are purified on immunoaffinity and immunodepletion columns. Although these chain-specific rabbit anti-mouse immunoglobulin antibodies can be made in the laboratory, it is normally easier to purchase them from commercial sources. There are a large number of different subclassing (isotyping) assays available.

Originally, the Ouchterlony double-diffusion assays were the most common method for determining the class and subclass of a monoclonal antibody. They have been largely superseded by other techniques, but they still are useful, particularly when only a few assays will be performed. In these assays, samples of tissue culture supernatants (often concentrated 10-fold) are pipetted into a well in a bed of agar. Class- and subclass-specific antisera are placed in other wells at equal distance from the test antibody. The two groups of antibodies diffuse into the agar. As they meet, immune complexes form, yielding increasingly larger complexes as more antibodies combine. When large multimeric complexes form, the immune complexes will precipitate, forming a line of proteins that is
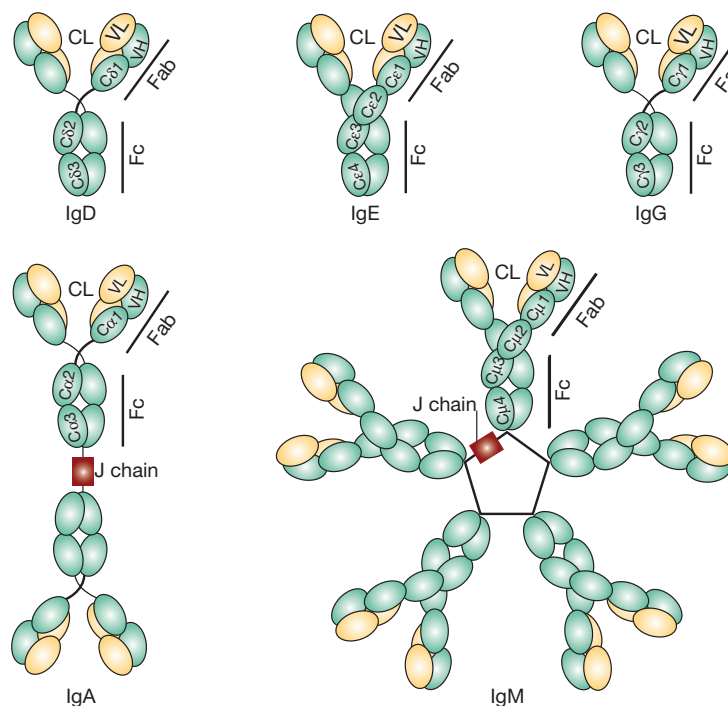
FIGURE 20. Immunoglobulin structures. All immunoglobulins are Y-shaped heteromeric protein complexes that are composed of two light chains and two heavy chains. In all immunoglobulins, the amino termini of the light and heavy chains form two arms referred to as the Fab portion, which contain the antigen-binding region of the molecule at the end. The carboxyl termini of the heavy chain form a stem referred to as the Fc domain, which has subclass-specific interactions with other molecules including complement or Fc receptors. There are five different classes of immunoglobulins: IgG, IgA, IgD, IgE, and IgM. These are differentiated by the type of heavy chain used and their ability to form multimers of Y-shaped complexes, as seen in the IgA and IgM subclasses. (Reprinted, with permission, from Rojas and Apodaca 2002, © Macmillan.)

either visible to the naked eye or that can be stained to increase the sensitivity. The precipitated proteins form what is referred to as a "precipitin line" (see Protocol 24).

Any of the assays used to screen hybridoma fusions that detect antibodies with a secondary anti-mouse immunoglobulin antibody can be adapted to screen for class or subclass. For example, if the detection method used horseradish peroxidase (HRP)–labeled rabbit anti-mouse immunoglobulin to locate antibodies bound to the antigen, then substituting anti-class- or subclass-specific antibodies will identify the type of heavy chains. This type of reaction can be used in assays in which the antigen is bound to the wells of an ELISA plate, incubated with hybridoma supernatant, and the antibody–antigen complexes are screened with HRP-labeled class- or subclass-specific secondary antibody (Protocol 25). In an alternative, each row of an ELISA plate has a capture antibody for a specific Ig class, subclass, or light chain bound to it (see Fig. 1 in Protocol 26). The monoclonal antibody being tested is then added. It will bind only to the wells coated with the specific class, subclass, or light chain antibody that recognized its heavy chain. It is then visualized using a nonspecific anti-mouse Ig antibody that is labeled with HRP (Protocol 26).

A final method for determining antibody subclass is by Cytometric Bead Array (CBA). This method, developed by Becton, Dickinson and Company (BD), allows the sensitivity of fluorescence using flow cytometry to be combined with conventional immunoassay methods. In essence, a bead is used as a capture surface in place of the individually coated wells of an ELISA plate. This method very simply allows for a rapid identification of heavy- and light-chain isotypes from a single sample. The increased sensitivity of fluorescence detection combined with multiplexing for the different subclasses requires less sample volume and less time than conventional ELISA isotyping methods (Protocol 27).

Selecting Class-Switch Variants

During the normal development of a humoral response, the predominant class of antibodies that is produced changes over time, beginning primarily with immunoglobulin Ms (IgMs) and developing into IgGs. These changes and others like them occur by genetic rearrangements that move the coding region for the antigen-binding site from just upstream of the IgM-specific region to the IgG region. These events are described in detail in Chapter 2. These rearrangements help the host animal tailor the immune response to the various types of infection. The different classes and subclasses of antibodies also have properties that make them more or less useful in various immunochemical techniques. These differences make the preparation of antibodies of certain classes or subclasses very valuable.

It has been shown that a process that appears similar to the natural class and subclass switching occurs in vitro, although at a very low frequency. Therefore, any population of hybridomas will have a small proportion of cells secreting antibodies with a different class or subclass of antibody. The antigen-binding site will be identical in these antibodies. If these cells can be identified and cloned, then antibodies with the same antigen-binding site but with different class or subclass properties can be isolated. These "shift variants" generally are useful in one of two cases, either switching from IgM to IgG or from IgG1 to IgG2a. Often these switches are used to produce antibodies that bind with higher affinity to Protein A.

When trying to identify any class or subclass switching variant, it is important to remember that the rearrangements that occur will remove and destroy the intervening sequences, so only those heavy-chain constant regions that are found further downstream can be selected for. The order of the heavy-chain constant regions is μ, δ, γ_3, γ_1, γ_{2b}, γ_{2a}, ϵ, and α. Investigators should also be certain they need these variants, because the assays are tedious. It may often be more advantageous to set up another fusion rather than try to isolate switch variants.

The most useful approach for most laboratories was developed by Scharf and his colleagues (for a summary, see Spira et al. 1985). First, a suitable assay must be developed. Because of the large number of assays that must be performed, enzyme-linked assays are generally more useful. The assay for antibody capture (Protocol 1) can be easily adopted by changing the detection reagent to an IgG- or IgG2a-specific rabbit anti-mouse immunoglobulin antibody. (Not all companies supply reagents that are sufficiently specific for these tests; one useful source is SouthernBiotech. All sources should be tested carefully before use.)

In this approach, hybridoma cells are washed by centrifugation, and the cell pellet is resuspended in medium supplemented with 20% FBS at a density of 10^4 cells/mL. Then 100 μL of the cells and medium are dispensed into the wells of 10 96-well microtiter plates. This yields approximately 1000 cells per well with about 1000 wells. Therefore, about 10^6 cells are being screened per assay. After the cells have grown, a sample of the tissue culture supernatant is removed and screened for the presence of the IgG or IgG2a antibodies. Between one and five positive wells may be seen. The strongest positive wells are chosen, and these cells are transferred to fresh medium. Passaging of the cells is continued until they are numerous enough to clone again. In the second round, the cells are plated at 100 cells per well. The procedure is repeated, and then the cells are plated at 10 cells per well. In the last round, the cells are single-cell-cloned using one of the techniques described earlier.

Another method for isolating class-switch variants uses flow cytometry. Like plasma cells, hybridomas express an Ig surface receptor of the same subclass as the antibody they produce. These can be sorted by flow cytometry using FACS. Hybridoma cells are grown out in large numbers ($>10^9$ cells), collected in 50-mL sterile centrifuge tubes, and washed in a 1% BSA/PBS buffer (pH 7.2−7.4) containing 0.02% sodium azide. The cells are resuspended in 1 mL of an FITC-labeled rabbit anti-mouse subclass-specific (IgG1 or IgG2a) antibody diluted 1:40 in 1% BSA/PBS buffer (pH 7.2−7.4) containing 0.02% sodium azide and incubated for 30 min at 4°C (on ice). The cells are then washed and resuspended (1−5 mL) in 1% BSA/PBS buffer (pH 7.2−7.4) containing 0.02% sodium azide. The hybridoma cells can then be sorted for IgG1 (or IgG2a) expression by FACS. These cells can be collected in bulk or plated out directly as single cells per well in 96-well plates containing 20% FBS medium with

growth supplements. Subcloning is recommended to ensure clonality, and the isotype should be verified after the cells have recovered.

Sometimes it is possible to induce class switching. Treatment with a mutagen, such as Acridine Orange, may push hybridomas to class-switch from IgM to IgG1 before obtaining the other IgG subclasses. Spira et al. (1996) and Kim et al. (1999) have reported success treating hybridomas with Acridine Orange and obtaining a panel of variants producing IgG1, IgG2a, IgG2b, IgE, and IgA subclasses.

Using molecular biology technology, it is possible to remove the CDR regions of an IgM antibody and graft them onto the framework of any subclass desired. Chimeric antibodies between species have been created as well, mainly as a precursor for creating human antibodies from murine hybridomas.

INTERSPECIES HYBRIDOMAS

Antibody-secreting cells isolated from one species but fused with myelomas from another species yield interspecies hybridomas. These types of fusions were common in the early years of hybridoma production when antibody-secreting cells from immunized rats (or hamsters) were often fused with mouse myeloma cells. This was a common strategy before good rat myeloma fusion partners (YB 2/0 or Y3-Ag1.2.3) were available. Such interspecies fusions yield hybridomas that secrete rat antibodies, but the hybridoma cells cannot be grown conveniently as ascites tumors. Therefore, antibody production is almost entirely limited to tissue culture sources. Today, this is not an issue. Many different forms of in vitro antibody production are available commercially or for production in the laboratory, for example, roller bottle cultures, Integra CELLine 1000 flasks, stirred tanks, and hollow fiber bioreactors.

HUMAN HYBRIDOMAS

Human monoclonal antibodies have a huge potential as therapeutic agents and new drug candidates. Although this field has been marked by exciting publications announcing breakthroughs, the actual progress in setting up the routine production of human hybridomas for laboratory use has been slow. For most research applications, producing human hybridomas still does not offer many, if any, advantages. A major obstacle has been obtaining reactive human B cells. The deliberate immunization of human subjects is not ethically feasible, and relying on collection of peripheral blood lymphocytes from patients with cancers and autoimmune diseases has not been very effective. It is clear that the time interval between the antigen boost and the collection of the immune cells is crucial for a successful fusion. Methodologies for priming human B cells in vitro have not been as robust in obtaining large numbers of immune cells as in vivo boosting of mice. The two most successful strategies that are used are standard fusions with human myeloma cells and the use of the Epstein–Barr virus (EBV) to transform antibody-secreting cells.

One of the major problems in producing human hybridomas has been the lack of a suitable myeloma partner. Isolation and the difficulty in culturing human myeloma cells have been major impediments. Several human lymphoblastoid and plasmacytoma lines have been isolated and are now in use. Most of these lines are obtained from myeloma patients in advanced stages of the disease. This could be a factor with their use, antibody-producing capability, and stability of the resulting hybridoma cells.

The use of EBV transformation to allow antibody-secreting cells to grow in standard tissue culture systems has solved some of the problems in human monoclonal antibody production. One unfortunate drawback of this approach is that the resultant transformants seldom secrete large amounts of antibodies. This has been overcome in some cases by fusing the EBV-transformed cell with a mouse myeloma cell line to allow the secretion of large amounts of antibodies. The combined use of EBV and secondary fusions points out two important aspects in hybridoma research. One is the use of other vectors, such as

oncogenes, to deliver important genetic information. Second, if a particular hybrid does not possess all of the properties that are needed for a particular use, the line may be re-fused with other hybrids to achieve these properties.

There are several publications that describe progress in the isolation of human antibody-secreting cells, and these types of references should be checked for the details of producing human hybridomas (Ritts et al. 1983; Posner et al. 1987; Kawahara et al. 1990; Buchacher et al. 1994; Lowe et al. 2009; Wang 2011; Gorny 2012).

FUTURE TRENDS

Few changes in the techniques used to produce hybridomas have been adopted since the original methods of Köhler and Milstein (1975) were reported. However, hybridoma construction is still evolving. In several areas, preliminary work has already been reported that will form the basis for more widespread use of new techniques.

Humanization Technology

Methods for humanizing murine antibodies, as well as antibodies from other species, have improved in recent years. The time and cost of these endeavors have also been greatly reduced. Developments in this area have been driven by the pharmaceutical industry's need for biotherapeutics. Not only can murine antibodies be made almost entirely human, but also they can be improved on or optimized. Designer framework structures are being developed that are not only fully human, but increase serum half-life and reduce T-cell recognition, making for improved antibody-based drug therapies.

Antibody Optimization

The process of antibody optimization can take an antibody and improve on its characteristics such as affinity, specificity, stability, potency, productivity, manufacturability, and effector functions. This is accomplished by making changes within the CDR regions of the antibody using molecular biology artificially to influence the maturation process or induce mutations. High-throughput screening technologies are advancing that have increased the screening capacity for testing huge numbers of mutated antibodies in a matter of hours, selecting for multiple different attributes simultaneously. Antibody optimization can take an antibody expressing the desired recognition but having poor affinity and make it into a winner without the need to immunize more animals or perform additional fusions.

Single-Cell PCR Technology

Advances have been made in PCR technology that enable immunoglobulin DNA from individual B cells to be isolated and grown out, then made into production cell lines that pump out large quantities of monospecific antibodies. Not only can this technology produce antibody-secreting cell lines directly from individual B cells taken from a spleen or lymph node of an immunized animal, but also it can be used to rescue existing unstable hybridomas that would otherwise have been lost. The process would take a fraction of the time and cost of developing conventional hybridomas, and it would be applicable to any species once primers became available.

Synthetic Antibodies

Scientists are already working at generating antibodies by cobbling together DNA libraries to engineer antibody molecules outside the immune system and then trying to identify the protein they bind to. Linking this technology up with advancing computer technology and three-dimensional (3D) structural biology could lead to designing and artificially synthesizing an antibody to any protein, regardless of species homology or antigenicity. There have already been reports of plastic antibodies (polymer

nanoparticles) being created through a process called molecular imprinting that form around proteins, which are then dissolved away to leave empty cavities that are capable of binding the protein in mice.

Next Generation of Mice

Köhler and Milstein originally used BALB/c mice to generate monoclonal antibodies. Since then, the need to produce better antibodies has driven scientists to create better mice. Using transgenic technology, different genes have been added to B cells in mice to make their immune systems more reactive. Mice have been developed that overexpress Bcl-2 and BAFF, anti-apoptotic agents, in the hope that rare and unique B cells that would not normally survive the fusion process could be made into viable hybridomas. Mice were also created that had their immune response genes removed and replaced with human versions so that they would react to human antigens and generate human antibodies. Companies like Abgenix, Medarex, and Kirin Brewery's Pharmaceutical Division were the first to generate these mice. Then Regeneron produced the VelociMouse, using improved genetic engineering to incorporate more of the human immune repertoire into the mouse. The latest entries in this field are the Kymouse from Kymab (http://www.kymab.com), which uses stem cell technology to capture the entire diversity of the B-lymphocyte component of the human immune system, and the IMG-AbS platform mice from ImmunoGenes (http://www.immunogenes.com). The latter company has engineered a transgenic mouse that overexpresses the neonatal Fc receptor (FcRn). In vivo, the FcRn plays a role in protecting IgG from degradation. ImmunoGenes has found that it also plays a role in antigen presentation, aiding the generation of serum titers and antibody generation.

Hybridoma technology has changed the way research has been done. The need for new and better antibodies has simply exploded in recent years and will likely continue to do so for some time yet. Diagnostic and research applications have become very reliant on antibody reagents. New targets are discovered every day, feeding the need for continued antibody generation. As technologies advance, the way we generate and produce antibodies will evolve, but the need for new and better antibodies will continue for some time to come.

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