Monoclonal Antibody Supernatant and Ascites Fluid Production

A major advantage of using monoclonal antibodies over polyclonal antisera is the potential availability of large quantities of the specific monoclonal antibody. In general, preparations containing the monoclonal antibody include a hybridoma supernatant, ascites fluid from a mouse inoculated with the hybridoma, and purified monoclonal antibody. Hybridoma supernatants are easy to produce, especially for large numbers of different monoclonal antibodies, but are relatively low in monoclonal antibody concentration. Ascites fluid contains a high concentration of the monoclonal antibody but the fluid is not a pure monoclonal antibody preparation. To obtain a purified preparation of the monoclonal antibody, affinity chromatography (*APPENDIX 3*) of culture supernatants or ascites fluid can be performed; however, this obviously requires more effort (see Commentary).

Procedures detailing the production of monoclonal antibody supernatants (see Basic Protocol 1), including the production of larger amounts (liters; see Alternate Protocol 1) are presented here. The protocol for large-scale production of hybridomas or cells (e.g., for isolation of cellular proteins) involves a similar procedure (see Alternate Protocol 2). A method for producing and obtaining ascites fluid containing the monoclonal antibody is also presented (see Basic Protocol 2).

The unit ends with a set of recommendations developed by the Committee on Methods of Producing Monoclonal Antibodies (Institute of Laboratory Animal Research, National Research Council). The complete report is published by the National Academy Press (1999). The recommendations are designed to foster the judicious use of animals for the production of monoclonal antibodies and to strongly encourage the use of in vitro methods whenever possible.

PRODUCTION OF A MONOCLONAL ANTIBODY SUPERNATANT

BASIC PROTOCOL 1

There are a variety of methods for producing monoclonal antibody supernatants. In the easy version presented here, the hybridoma is grown and split 1:10. The cells are then overgrown until cell death occurs. The supernatant is harvested and the titer determined. If the titer is high, the hybridoma can be used for large-scale production in anticipation of purification or ascites production.

Materials

Hybridoma of interest (*UNIT 2.5*)
Complete DMEM-10 medium (*APPENDIX 2*)

175-cm^2 tissue culture flasks
50-ml conical centrifuge tubes, sterile
Beckman TH-4 rotor (or equivalent)

1. Place hybridoma in a 175-cm^2 tissue culture flask in complete DMEM-10 medium. Grow in a humidified 37°C, 5% CO_2 incubator until vigorously growing and ready to split.

   Most cell lines need to be split into new medium or new flasks when cell density reaches 1-2 × 10^6 cells/ml. Tissue culture flasks can be inspected with an inverted microscope and cell viability and density determined. In addition, the culture can be monitored for contamination. Cells should never be allowed to become so crowded that cell death occurs because this crisis phase increases the likelihood of phenotypic change. With experience,

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most investigators will be able to determine whether or not to split the cells by their macroscopic appearance and the color of the medium—i.e., cells may need to be split when the medium in the flask becomes turbid and more yellow than medium in the DMEM bottle (macroscopic turbidity in the absence of a color change is often a sign of bacterial contamination). The density can then be confirmed by microscopic examination.

2. Split cells 1:10 in a new 175-cm^2 flask. Fill the flask with complete DMEM-10 to 100 ml total and place in humidified 37°C, 5% CO_2 incubator until cells are overgrown, medium becomes acidic (yellow), and cells die (~5 days).

   Alternatively, add hybridoma cells at high density (1-2 × 10^6 cells/ml) in fresh medium to a tissue culture flask. Incubate 2 to 3 days in a CO_2 incubator, at which time the cells will die and the supernatant can be collected (step 3).

3. Transfer flask contents to sterile 50-ml conical centrifuge tubes. Centrifuge 10 min in a TH-4 rotor at 2700 rpm (1500 × *g*), room temperature. Collect the supernatant and discard the pellet.

4. Assay the titer of MAb supernatant by the appropriate method (see Commentary).

5. Store the supernatant under sterile conditions; it is generally stable at 4°C for weeks to months, at −20°C for months to years, and indefinitely at −70°C. Minimize thawing and refreezing by storing several aliquots.

ALTERNATE PROTOCOL 1

LARGE-SCALE PRODUCTION OF MONOCLONAL ANTIBODY SUPERNATANT

The first step in monoclonal antibody purification by affinity chromatography is the production of large amounts of the culture supernatant. The procedure described below utilizes readily available equipment and supplies and a special tissue culture roller apparatus. Cells are first grown in smaller flasks, then gradually expanded into large-volume roller flasks. The supernatant is harvested and stored until needed.

Additional Materials *(also see Basic Protocol 1)*

Complete DMEM-10 medium (*APPENDIX 2*) with 5 to 10 mM HEPES, pH 7.2 to 7.4
70% ethanol

850-cm^2 roller flask and roller apparatus
250-ml conical centrifuge tubes, sterile
Beckman JS-5.2 rotor (or equivalent)

1. Repeat step 1 of Basic Protocol 1 and split 1:10 in complete DMEM-10/HEPES to 100 ml total.

   Each 175-cm^2 flask will ultimately seed 2.35 to 2.5 liters of culture medium. Scale up the experiment as desired. If the supernatant will be used for MAb purification by affinity chromatography, the yield will be 1 to 10 mg MAb/liter. It is usually not necessary to adapt and grow cells in serum-free media (which frequently decreases yield); however, if the supernatant will be used for MAb purification by protein A–affinity chromatography, test the culture medium with FBS alone for contaminants (i.e., other proteins) that may co-purify with the MAb. Bovine newborn serum frequently contains significant amounts of Ig that will bind to protein A and should not be used as a medium supplement.

2. When cells are ready to split, transfer contents of the 175-cm^2 flask (100 ml) to an 850-cm^2 roller flask. Add an additional 150 ml complete DMEM-10/HEPES (250 ml total volume). Cap tightly, place on roller apparatus in 37°C room or incubator and grow 1 to 2 days.

   Timing of the split will depend on the cell line (see step 1 of Basic Protocol 1).

3. Wipe cap and neck of roller flask with a sterile gauze sponge soaked in 70% ethanol.

4. Open roller flask and add 250 ml complete DMEM-10/HEPES (500 ml total volume). Cap tightly and incubate on roller apparatus 1 to 2 days at 37°C.
5. Repeat wiping as in step 3.
6. Open roller flask and add ~2 liters complete DMEM-10/HEPES until flask is almost full (~2.5 liters total volume depending on capacity of flask). Cap tightly and incubate on roller apparatus at 37°C until the medium turns yellow (~5 days).

   Avoid foaming by first pouring in growth medium without FBS followed by FBS to 10% final.

   Avoid prolonged rolling, as cell fragmentation will occur and the debris will be difficult to pellet with the large centrifuge tubes.

7. Pour the culture into sterile 250-ml conical centrifuge tubes. Harvest the supernatant by centrifuging 20 min in JS-5.2 rotor at 1000 rpm (250 × *g*), room temperature. Collect the supernatant and discard the pellet. Freeze the supernatant in aliquots.

   If the supernatant will not be used in a bioassay, add 10% sodium azide to 0.02% final. If the supernatant will undergo affinity chromatography or salt fractionation, sterile filtration through a 0.45-μm filter is recommended to eliminate debris.

LARGE-SCALE PRODUCTION OF HYBRIDOMAS OR CELL LINES

ALTERNATE PROTOCOL 2

The following procedure is used to produce large amounts of cells which can be used to isolate cellular components such as membrane proteins (*UNIT 8.1*). Individual small flasks are grown, then each is used to inoculate a larger roller flask. The cells are gradually expanded by addition of fresh medium, and are harvested when the cells are near saturation densities.

1. Follow steps 1 to 6 for large-scale production of MAb supernatants (see Alternate Protocol 1) but harvest when the density is appropriate or if the cell growth plateaus.

   Estimate the amount of cells needed. This procedure will yield ~10^6 cells/ml. More cells can be obtained with faster-growing cell lines or lines that can tolerate higher densities, but we usually grow 80 liters (33 flasks) and obtain 10^{10} to 10^{11} cells.

   Seed (introduce cells into) the number of 175-cm^2 flasks necessary to produce the amount of cells required (one 175-cm^2 flask for every 2.4 liters of medium). Each flask should be treated as an independent culture. Thus, if there is contamination at any time after the 175-cm^2 flasks are initially seeded, the contamination should spread vertically (i.e., stay in the flask and roller bottle seeded by the contaminated flask) and not horizontally (i.e., not involve any other flasks or roller bottles).

   Estimate the time of harvesting by macroscopic inspection for medium color and turbidity and by taking daily cell counts and checking viability on several flasks. When the cell concentration reaches a plateau, harvesting is indicated. Do not allow cells to overgrow or cell viability will drop precipitously.

2. Pour cells into sterile 250-ml conical centrifuge tubes, centrifuge 15 min in JS-5.2 rotor at 1000 rpm (250 × *g*), 4°C, and discard supernatant.

   Each tube can be used for two spins. Harvesting 80 liters of cells requires at least three centrifuges to spin four to six tubes (1 to 1.5 liters) each, two or three people, and nearly one day. Conical centrifuge tubes are recommended because pellets in the flat-bottom bottles are harder to work with.

3. Place cell pellets on ice. Pool 10 cell pellets into one tube by resuspending cells in 250 ml of 4°C PBS. Centrifuge at 250 × *g*, 4°C, and discard supernatant.
4. Repeat and further consolidate tubes into one tube. After three washes, the cells are ready for further processing (i.e., cell lysis, radiolabeling, or other procedures).

   Alternatively, lyse smaller cell pellets as they become ready after three washes.

BASIC PROTOCOL 2

PRODUCTION OF ASCITES FLUID CONTAINING MONOCLONAL ANTIBODY

High-titer monoclonal antibody preparations can be obtained from the ascites fluid of mice inoculated intraperitoneally with monoclonal antibody–producing hybridoma cells. The fluid is collected several times after injection of the cells. It is heat-inactivated, titered, and stored.

For discussion of recently adopted guidelines on use of animals for production of monoclonal antibodies, see Background Information.

Materials

Nude mice, 6 to 8 weeks old and specific-pathogen free, *or* syngeneic host if mouse-mouse hybridomas are injected
Pristane (2,6,10,14-tetramethylpentadecane; Aldrich)
Hybridoma of interest (*UNIT 2.5*)
Complete DMEM-10 medium (*APPENDIX 2*) with 10 mM HEPES and 1 mM sodium pyruvate
PBS *or* HBSS (*APPENDIX 2*), sterile and without FBS

20- or 22-G needle and 18-G needle
175-cm^2 tissue culture flask
Beckman TH-4 rotor (or equivalent)
50- and 15-ml polypropylene conical centrifuge tubes, sterile
56°C water bath

Additional reagents and equipment for handling animals (*UNITS 1.1-1.3*), intraperitoneal injection (*UNIT 1.6*), euthanasia (*UNIT 1.8*), ELISA (*UNIT 2.1*), and counting cells and cryopreservation (*APPENDIX 3*)

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

1. Using a 20- or 22-G needle, inject mice intraperitoneally with 0.5 to 1 ml Pristane per mouse 1 week prior to inoculation with cells.

   Alternatively, the injections can be done at the same time, but the week interval is recommended to avoid leakage through two needle sites. Mice should be maintained in specific-pathogen free (spf) facility (see Commentary).

2. Grow hybridoma cells in a 175-cm^2 tissue culture flask in complete DMEM-10/HEPES/pyruvate under conditions that promote log-phase growth.

   Before injecting mice (step 7), test the supernatants for MAb activity by ELISA or appropriate assay, preferably before the cells are expanded.

   To minimize the risk of introducing a pathogen into the rodent colony, screen cells for pathogens by antibody-production assay (UNIT 1.1).

3. Transfer the culture to 50-ml conical centrifuge tubes. Centrifuge 5 min in TH-4 rotor at 1500 rpm (500 × *g*), room temperature, and discard supernatant.

4. Wash cells by resuspending in 50 ml sterile PBS or HBSS without FCS, then centrifuging 5 min at 500 × *g*, room temperature, and discarding supernatant. Repeat twice and resuspend cells in 5 ml PBS or HBSS.

   Avoid washing in FCS-containing medium because the mouse will produce antibodies to the FCS.

5. Count cells and determine viability by Trypan blue exclusion.

   Cells should be nearly 100% viable.

6. Adjust cell concentration to 2.5×10^6 cells/ml with PBS or HBSS without FCS.

7. Draw up cells in 10-ml sterile syringe. Using a 22-G needle, inject nude mouse intraperitoneally with 2 ml cells. Wait for ascites to form (1 to 2 weeks).

   Typically, three mice are injected at one time. In most cases, at least one and frequently all of the mice will develop ascites.

8. Harvest ascites by grasping and immobilizing the mouse in one hand in such a way as to stretch the abdominal skin taut. With the other hand, insert an 18-G needle 1 to 2 cm into the abdominal cavity. Enter either the left or right lower quadrants to avoid the vital organs in the upper quadrants and the major vessels in the midline. Allow the ascites to drip into a sterile 15-ml polypropylene conical centrifuge.

   If the mouse has a large amount of ascites and the fluid stops dripping from the 18-G needle, it may be necessary to reposition the needle tip by withdrawing it slowly and reinserting it in a different plane. If no ascites fluid accumulates, the mouse may be reinjected (see Commentary).

   Occasionally, the ascites is under such high pressure that a large amount squirts out as soon as the needle is inserted. For this reason, be sure that the hub of the needle is pointed into a tube before inserting the needle into the peritoneal cavity.

   Rather than tapping the mouse as soon as the ascites is apparent, allow the fluid to build up (3 to 7 days) to obtain the highest yield. Frequently, 5 to 10 ml (sometimes >40 ml) of ascites can be collected from each mouse.

9. Centrifuge the ascites 10 min in TH-4 rotor at 2700 rpm ($1500 \times g$), room temperature. Harvest supernatants and discard pellet. Store ascites fluid at 4°C until all collection is completed (<1 week).

   If the fluid clots, "rim" the clot by passing with a wooden applicator stick around its edge (between clot and tube) before centrifugation. The clot may adhere to the applicator stick and thus may be discarded or it will remain in the tube and become part of the cell pellet after centrifugation.

10. Allow the mouse to reaccumulate ascites (2 to 3 days) before reharvesting as in step 8. Process the ascites as in step 9. Repeat this process until no further ascites accumulates, the fluid cannot be collected, or the mouse becomes ill. The mouse should be euthanized at this point.

11. Pool ascites fluid collected on different days and heat-inactivate 45 min in a 56°C water bath. If a clot reforms, remove it by rimming and centrifuge as in step 9.

12. Assay the titer of MAb-containing ascites by the appropriate method (see Commentary).

    Saturating concentration (maximal activity) of the MAb should be apparent at 0.5% or higher dilutions. Lower titers usually are the result of unstable hybridomas that stop producing MAb or too many (>2) in vivo serial passes of the hybridomas.

13. Dilute >1:10 and filter sterilize through a 0.45-µm filter. Aliquot and freeze at −70°C, avoiding repeated freezing and thawing. Shelf life should be several years.

    Add sodium azide to 0.02% final if the ascites will not be used for bioassay.

COMMENTARY

Background Information

Production of MAb supernatant

There are three basic preparations that contain monoclonal antibodies: supernatant from a MAb-producing hybridoma, ascites from a mouse inoculated with the hybridoma, or purified MAb. In Basic Protocol 1, a MAb-containing supernatant is produced. The hybridoma continues to secrete MAb into the culture fluid until cell death occurs. Because the MAb is not metabolized, it accumulates in the culture supernatant. Hybridoma supernatants are advantageous because small amounts (<100 ml) can be easily obtained in a few days (<1 week), and from multiple hybridomas with relatively little effort. Such small quantities of the MAb can be used in preliminary experiments to determine if the MAb has the desired property before expending the effort required to produce ascites or purified MAb.

The first step in MAb purification, usually by affinity chromatography, is the production of large amounts of the MAb. For the noncommercial laboratory, several liters of culture supernatant are adequate for most MAb-purification protocols. The use of bioreactors or large spinner flasks would require expensive, somewhat fragile equipment. The procedure described here (see Alternate Protocol 1) involves the use of sealed roller bottles that can be rotated on a roller apparatus in any 37°C room, as the pH of the medium is maintained by the HEPES instead of a bicarbonate-based buffer system and a CO_2 atmosphere.

Production of ascites containing MAb

A convenient source of large concentrations of the desired MAb is the ascites fluid of mice inoculated with the appropriate hybridoma cells. These fluids often have a titer >100-fold more than culture supernatants and thus can be diluted significantly. Frequently, high-titer ascites preparations will have saturating MAb concentrations of 10^{-3} to 10^{-4} by flow cytometry analysis of cell-surface-antigen binding (*UNIT 5.3*). This high titer minimizes the nonspecific functional effects (e.g., in proliferation assays) of equivalent concentrations of MAb in spent culture supernatants. However, ascites used at concentrations >0.5% may also have significant nonspecific effects. In addition to the appropriate isotype control MAb ascites, ascites harvested from mice inoculated with the nonsecretory partner cell line (i.e., SP2/0) also provides a useful nonspecific control.

The general procedure involves the elicitation of nonspecific inflammation in the peritoneal cavity of an appropriate host mouse, usually with Pristane, and injection of the hybridoma cells. The tumor cells then grow as an ascites tumor and should continue to secrete the MAb. Eventually, the mouse develops a monoclonal gammopathy similar to the human disease, multiple myeloma, in that a monoclonal protein reaches high titer in the serum. Since the combination of the ascites tumor and Pristane results in an inflammatory exudate in the peritoneal cavity, the ascites should contain concentrations of the MAb similar to that in serum.

National Research Council Recommendations for Producing Monoclonal Antibodies in Animals

In 1998, the National Institutes of Health asked the National Research Council to form a committee to develop recommendations for producing monoclonal antibodies using animals. The following is a summary of the committee's recommendations.

1. There is a need for the scientific community to avoid or minimize pain and suffering by animals. Therefore, over the next several years, as tissue culture systems are further developed, tissue culture methods for the production of monoclonal antibodies should be adopted as the routine method unless there is a clear reason why they cannot be used or why their use would represent an unreasonable barrier to obtaining the product at a cost consistent with the realities of funding of biomedical research programs in government, academia, and industry. This could be accomplished by establishing tissue culture production facilities in institutions.

There are several reasons why the mouse method of producing monoclonal antibodies cannot be abandoned: some cell lines do not adapt well to tissue culture conditions; in applications where several different mouse monoclonal antibodies at high concentrations are required for injection into mice, the in vitro method can be inefficient; rat cell lines usually do not efficiently generate monoclonal antibodies in rats and adapt poorly to tissue culture conditions but do produce monoclonal antibodies in immunocompromised mice; downstream purification or concentration from in vitro systems can lead to protein denaturation and de-

creased antibody activity; tissue-culture methods can yield monoclonal antibodies that do not reflect the normal modification of proteins with sugars, and this abnormality might influence binding capacity and other critical biologic functions of monoclonal antibodies; contamination of valuable cell lines with fungi or bacteria requires prompt passage through a mouse to save the cell line; and inability of some cell lines that do adapt to tissue-culture conditions to maintain adequate production of monoclonal antibodies poses a serious problem. For these reasons, the committee concludes that there is a scientific necessity to permit the continuation of the mouse ascites method of producing monoclonal antibodies. However, note that over time, as in vitro methods improve, the need for the mouse ascites method will decrease.

2. The mouse ascites method of producing monoclonal antibodies should not be banned, because there is and will continue to be scientific necessity for this method.

There does not appear to be convincing evidence that significant pain or distress is associated with the injection into the mouse of Pristane (a chemical that promotes the growth of the tumor cells), but during the accumulation of ascites there is likely to be pain or distress, particularly when some cell lines that are tissue-invasive are used and in situations of significant ascites development. Therefore, after injection of hybridoma cells, mice should be evaluated at least daily, including weekends and holidays, after development of visible ascites and should be tapped before fluid accumulation becomes distressful. A limit should be placed on the number of taps and multiple taps should be allowed only if the animal does not exhibit signs of distress.

3. When the mouse ascites method for producing monoclonal antibodies is used, every reasonable effort should be made to minimize pain or distress, including frequent observation, limiting the numbers of taps, and prompt euthanasia if signs of distress appear.

Two of thirteen monoclonal antibodies approved by the Food and Drug Administration for therapeutic use cannot be produced by in vitro means, or converting to an in vitro system for their production would require (because of federal regulations) proof of bioequivalence, which would be unacceptably expensive. Furthermore, many commercially available monoclonal antibodies are routinely produced by mouse methods, particularly when the amount to be produced is <10 g, another situation where it would be prohibitively expensive to convert to tissue-culture conditions, However, with further refinement of technologies, media, and practices, production of monoclonal antibodies in tissue culture for research and therapeutic needs will probably become comparable with the costs of mouse ascites method and could replace the ascites method.

4. Monoclonal antibodies now being commercially produced by the mouse ascites method should continue to be so produced, but industry should continue to move toward the use of tissue-culture methods.

In a few circumstances, the use of the mouse ascites method for the production of monoclonal antibodies might be required. It is suggested that the following be used as examples of criteria to be used by an Institutional Animal Care and Use Committee (IACUC) in establishing guidelines for the production of monoclonal antibodies in mice by the ascites method.

a. When a supernatant of a dense hybridoma culture grown for 7 to 10 days (stationary batch method) yields a monoclonal antibody concentration of <5 µg/ml. If hollow-fiber reactors or semipermeable-membrane systems are used, 500 µg/ml and 300 mg/µl, respectively, are considered low monoclonal antibody concentrations.

b. When >5 mg of monoclonal antibody produced simultaneously by each of five or more different hybridoma cell lines is needed. It is technically difficult to produce this amount of monoclonal antibody since it requires more monitoring and processing capability than the average laboratory can achieve.

c. When analysis of monoclonal antibody produced in tissue culture reveals that a desired antibody function is diminished or lost.

d. When a hybridoma cell line grows and is productive only in mice.

e. When >50 mg of functional monoclonal antibody is needed, and previous poor performance of the cell line indicates that hollow-fiber reactors, small-volume membrane-based fermentors, or other technique cannot meet this need during optimal growth and production.

It is emphasized that the above criteria are not all-inclusive and that it is the responsibility of the IACUCs to determine whether animal use is required for scientific or regulatory reasons. Criteria have not been developed to define a cell line that is low-producing or when tissue-culture methods are no longer a useful means of producing monoclonal antibody.

In summary, the committee recommends that tissue culture methods be used routinely for monoclonal antibody production, espe-

cially for most large-scale production of monoclonal antibodies. When hybridomas fail to grow or fail to achieve a product consistent with scientific goals, the investigator is obliged to show that a good-faith effort was made to adapt the hybridoma to in vitro growth conditions before using the mouse ascites method.

Critical Parameters

Monoclonal antibody production by hybridomas is an unstable phenotype. Hybridoma cells always should be grown under log-phase growth conditions. Prolonged in vitro culture and in vivo passage should be avoided. Thus, the most critical parameter is whether the hybridoma of interest is secreting a high titer of the MAb. This should be checked before a major effort is made to grow large amounts of supernatant or to produce ascites fluid. The MAb titer can be determined by serial dilution of the culture supernatant in the assay appropriate for that MAb, such as ELISA (*UNIT 2.1*) or flow cytometry (*UNITS 5.3 & 5.4*). Titers of ≥1:10 should be saturating if spent culture supernatants are examined. If necessary, the hybridoma can be recloned by limiting dilution (*UNIT 2.5*) to find high-producing clones. If cells are known to produce MAb at high titers, aliquots frozen immediately after cloning (*APPENDIX 3*) will retain this phenotype.

The most critical parameter in the large-scale production of cell lines and hybridomas is the adaptation of the cells to roller flasks. Most hybridomas and other nonadherent cells that grow in suspension can be easily adapted. If the cells (particularly adherent cells) cannot be adapted, other methods should be tried. For example, large-scale production of cells for use in isolating cellular components can be performed using multiple 175-cm^2 flasks instead of roller flasks. Unfortunately, the relative surface area is small, and therefore the number of flasks required can become prohibitive. Adherent cell lines are less easily adaptable to growth in roller flasks. The surface area for growing adherent cells can be increased by the use of dextran beads (e.g., Cytodex beads from Pharmacia). These beads can increase the surface area of a culture flask severalfold.

If there is any suspicion that the cells may be mycoplasma contaminated, diagnosis and treatment are indicated (see *APPENDIX 3*). Mycoplasma-contaminated lines will produce a much poorer yield of final cell numbers because they do not grow to as high a cell density as normal cells.

Supernatants frequently contain 1 to 10 μg/ml of MAb, but the concentration is cell-line dependent. The supernatants could be concentrated by salt precipitation, but this is not generally recommended because large volumes of culture supernatants have to be concentrated to derive the amount of MAb in small amounts of ascites. Moreover, the FCS in the supernatant will also be concentrated. While the hybridoma could be adapted to culture in serum-free medium, this requires additional testing and yields may decrease. Affinity purification of the culture supernatants would take a similar amount of effort and produce purified MAb at high concentrations. Thus, instead of concentrating supernatants (if high concentrations of MAb are desired), it is recommended to produce purified MAb by first growing hybridomas at a larger scale (liters) or to produce ascites.

For ascites production, it is important to consider the appropriate host for the hybridoma since an injection of allogeneic or xenogeneic cells may result in rejection. For most mouse-mouse hybridomas, an F1 hybrid—between the BALB/c strain (origin of the commonly used SP2/0 fusion partner) and the strain from which the normal cells were obtained—could be used. For xenogeneic hybridomas, nude mice or low-dose irradiated normal mice are potential hosts. Outbred nude mice are somewhat more expensive than normal mice but do not require irradiation. It is not necessary to use the prohibitively expensive inbred nude mouse strains.

Because the level of normal immunoglobulin in mouse serum is in the same range as ascites fluid (mg/ml), ascites fluid can be only partially purified by salt fractionation or anti-mouse-Ig- or protein A–affinity chromatography (*UNIT 2.7*). However, it is a convenient source of raw material from which to affinity purify rat MAb with a mouse anti-rat κ MAb (e.g., MAR 18.5) column (*UNIT 2.7*).

Troubleshooting

It is possible that ascites fluid will not form. The reasons for this are unclear but are probably related to a property of the individual hybridoma. If the mice die without any ascites forming, particularly within 2 weeks of inoculation, try fewer cells. If the mice do not form detectable ascites after 2 weeks and they appear healthy, inject those mice—as well as naive, Pristane-primed mice—with more cells. If solid tumors form, tease cells into suspension and inject the tumor cells into another Pristane-primed mouse. Even if a little ascites forms, the fluid can be transferred to another mouse (~0.5

ml/mouse), and large amounts of ascites should accumulate. Once the ascites is formed, the mouse-adapted cells can be frozen and used to reinoculate mice in the future.

Anticipated Results

Most culture supernatants will have saturating MAb titers of ≥1:10 when tested at 100 µl for 10^6 cells. If the spent culture supernatant is used for MAb purification by affinity chromatography (*UNIT 2.7 & APPENDIX 3*), 1 to 10 mg of purified MAb/liter can be anticipated. If a much lower titer or yield is achieved, recloning of the hybridoma line may be indicated. Hamster-mouse hybridomas are particularly notorious for instability.

Most hybridomas can be grown as ascites tumors. The saturating concentration of the MAb in such fluids should be detected at dilutions of 1:500. If MAb titers are significantly lower, the hybridoma may be a poor producer. If ascites do not form, see Troubleshooting above.

Most tumor cells that grow in suspension should be amenable to growth in roller flasks and densities of $>10^6$ cells/ml should be attained. Careful work should result in no contamination.

Time Considerations

For high-titer and large-scale production of MAb supernatants, a flask split 1:10 will be overgrown, with cell viability definitely decreasing by day 5 to 6, at which time the supernatants can be harvested. If several liters of supernatant are required, ~10 days are required to expand a 25-cm^2 flask (10 ml) to 2.4 liters.

For production of ascites fluid containing MAb, 4 to 6 weeks are necessary for growth of the cells for inoculation, ascites accumulation, tapping the fluid, centrifugation, and determination of the MAb titer.

Once the 175-cm^2 flasks are seeded for large-scale production of hybridomas and cell lines, <2 weeks are required to reach saturating cell densities in the roller bottles.

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