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MONOCLONAL HYBRIDOMA ANTIBODIES:

TECHNIQUES *and* APPLICATIONS

John G. R. Hurrell

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Monoclonal Hybridoma Antibodies: Techniques and Applications

Editor

John G. R. Hurrell, Ph.D.
Head, Immunochemistry R & D Group
Commonwealth Serum Laboratories
Parkville, Victoria
Australia

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PREFACE

The landmark paper of Köhler and Milstein (*Nature (London)*, 256, 495, 1975) describing the production and characterization of the first somatic cell hybrids capable of indefinite production of antibody of predetermined specificity, heralded a new era in biology and initiated a virtual "avalanche" of scientific literature. The potential of the somatic cell hybridization (hybridoma) technique was quickly realized by investigators in many areas of biology and applied to a wide variety of antigens. The technique has been refined and many alternative protocols developed, but all with the same basic core element, the fusion of primed splenocytes with biosynthetically defective myeloma cells.

The first section of this volume was aimed to provide a comprehensive review of the many varied and often empirically derived techniques and procedures currently in use to produce monoclonal hybridoma cell lines and to characterize the antibodies secreted. This goal has been achieved with the chapter contributed by Zola and Brooks who, as each step in the process of hybridoma production and antibody characterization is reviewed, have provided an experimental procedure found to be satisfactory in their laboratory. Investigators about to begin a hybridoma program are thus presented with background information for the rapid assessment of alternative procedures. The advantages, faults, and known pitfalls of each method are discussed and, if confused by the numerous procedures in the literature, the investigator may choose the experimental procedure of the authors to serve as a starting point.

The second section of this volume was designed to provide a review of areas in which monoclonal hybridoma antibodies have been of particular advantage. This is a rapidly advancing field which could not be thoroughly reviewed in a single volume. The particular examples reviewed were selected from the broad areas of soluble antigens (somatotrophic hormones, cardiac myosin, alpha fetoprotein, and carcinoembryonic antigen), viral antigens (influenza virus and herpes simplex virus), parasite antigens, tumor associated human tissue antigens, and nontumor tissue antigens (macrophage membrane, lymphocyte membrane, and histocompatibility antigens). These specific applications of monoclonal hybridoma antibodies are intended as general reviews as well as illustrating and expanding on many of the points brought out by Zola and Brooks in the first chapter.

It is apparent that the surface has only just been broken regarding the information that monoclonal hybridoma antibodies will provide. The future will see the widespread development and use of monoclonal hybridoma antibodies for routine immunotherapy and diagnosis, and for furthering our understanding of the molecular biology of the cell and of disease processes. There is no doubt that this is an exciting time to be a biological scientist!

JOHN G. R. HURRELL
Parkville, Australia
December 1981

THE EDITOR

John G. R. Hurrell, Ph.D., is Head of Immunochemistry Research and Development at the Commonwealth Serum Laboratories in Parkville, Australia. He is also an Associate in the Department of Pathology and Immunology, Monash University, Melbourne.

Dr. Hurrell graduated from the University of Melbourne in 1971 with a B.Sc. in Chemistry and Biochemistry. In 1974, after 3 years as a Research and Development scientist at the Commonwealth Serum Laboratories, he returned to the University of Melbourne to study under Professor Sidney J. Leach in the Department of Biochemistry. Dr. Hurrell received a Ph.D. in 1977. After a postdoctoral year at the Commonwealth Serum Laboratories, Dr. Hurrell was awarded a Fulbright Fellowship in the Cellular and Molecular Research Laboratory, Massachusetts General Hospital, Boston. Following a short period as a Visiting Scientist at Centocor Inc., Philadelphia, Dr. Hurrell returned to Australia in July 1980 to take up his present position.

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Chapter 1

TECHNIQUES FOR THE PRODUCTION AND CHARACTERIZATION OF MONOCLONAL HYBRIDOMA ANTIBODIES

H. Zola and D. Brooks

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I. INTRODUCTION

When two cells are brought into close contact and their membranes caused to fuse together, the resulting fusion product contains both the nuclei. A cell with two or more dissimilar nuclei is called a heterokaryon and in due course the nuclei can fuse together producing a single nucleus with genetic information from both of the original cells. This fusion product is called a hybrid. The fusion of somatic cells has been carried out for many years with a variety of different aims. The hybrids usually lose some chromosomes but retain some of the properties of each of the parental cells. In 1975 Köhler and Milstein[1] fused antibody-producing mouse spleen cells with mouse myeloma cells. The hybrids they obtained secreted antibodies of the specificity dictated by the parent spleen cell, but in the quantity characteristic of a myeloma. This experiment has led to greatly increased use of somatic cell hybridization. Because many laboratories are now producing hybrids, there has been a rapid development and proliferation of techniques.

Several articles are available giving either detailed technical descriptions or overviews to enable the reader to make an informed choice of technique.[2-8]

In this chapter, we describe in detail the techniques used in our laboratory, while discussing some of the variations which can be used successfully. An attempt will be made to differentiate between procedures which are based on sound evidence and practices which appear to be beneficial but may turn out to have only ritual significance.

Successful fusion and selective growth of antibody-secreting hybrids depends on a complex interplay of experimental variables. The complexity of the interaction between variables can lead to contradictory results between laboratories. For example, it is conceivable that the beneficial effect of feeder cells (see Section VI) is much greater if the fetal calf serum and media are suboptimal. Thus, a laboratory using a good batch of serum may find feeder cells do not help very much, while another laboratory, using a batch of serum which is less able to support growth of hybridomas, will find feeder cells indispensable. Since there is no entirely satisfactory test to predict the suitability of the fetal calf serum, it is not easy to prove that the need for feeder cells is related to calf serum batch. Experiments to study all these variables are tedious and relatively uninteresting, at a time when most investigators are anxious to produce some useful antibodies, irrespective of the efficiency of the process. Thus, it is not surprising that successful procedures become entrenched, and that dogmatic statements about technical variables are accepted unchallenged. As the initial excitement wears off it is to be expected that much more work will be done on technical aspects and that the procedures will lose much of their empiricism and mysticism.

The newcomer to hybridization is well advised to learn the technique in a laboratory which is already practicing fusion. It has been a frequent observation that newcomers to the techniques are relatively unsuccessful initially and obtain many hybrids after some practice, although an experienced observer cannot see any difference between the technique used on the first day and in subsequent, successful experiments. The best approach therefore is to learn from an experienced laboratory and practice until hybrids are obtained. During this development stage all possible variables should be noted and controlled as far as possible, so that once a successful procedure has been established, it can be maintained.

The different stages in the production of monoclonal hybridoma antibody, starting from immunization and finishing with a characterized monoclonal antibody, are illustrated schematically in Figure 1. Each stage is dealt with in detail in subsequent sections of this chapter.

II. PREREQUISITES TO STARTING A HYBRIDOMA PROJECT

In this section, an attempt is made to list what is needed in order to successfully execute a hybridoma production exercise, in terms both of materials and expertise.

A. Major Equipment

Hybridoma work does not require any sophisticated equipment, but three major items of equipment are needed and they must be of good quality.

1. Cabinet for Sterile Work

The investment of time and effort into hybridoma production is such that the frequency of lapses in sterility must be kept low. An efficient cabinet in which sterile air is blown across the work area towards the operator is adequate, provided it is maintained well and disinfected after use. These cabinets incorporate ultraviolet lamps to sterilize the cabinet between use, but these lamps have a rapid fall-off in effectiveness and liberal use of alcohol to swab the surfaces is recommended.

If the project involves the use of pathogenic material, a laminar flow cabinet with a vertical curtain of sterile air separating the work from the operator is needed.[9] These cabinets are variable in performance, and are only effective if used properly.

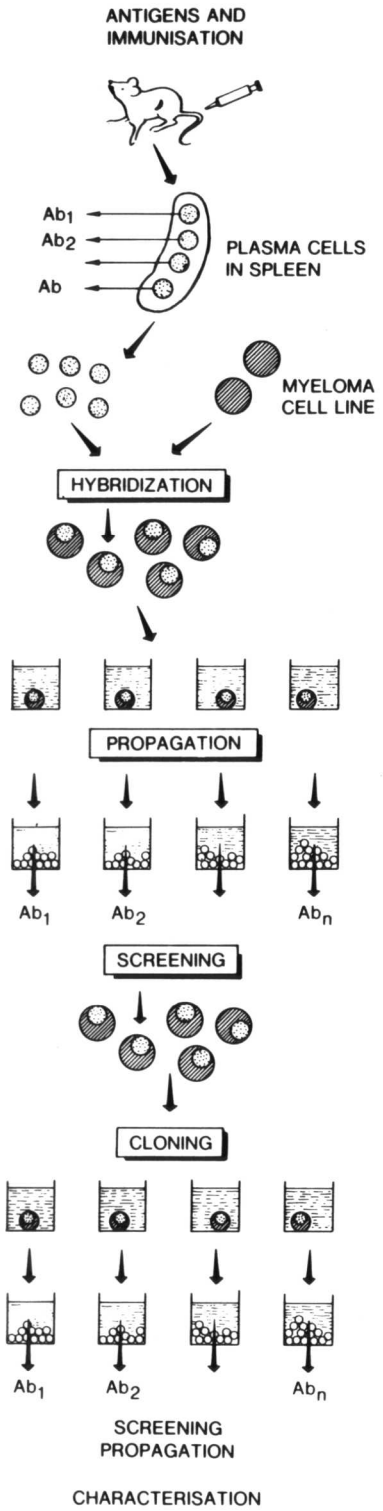

FIGURE 1. Schematic representation of the stages of a hybridization, from immunization to characterization of monoclonal antibody.

2. Gassed Incubator

Hybridoma cultures, in their early phase, are kept in unsealed culture vessels which depend on free and rapid exchange of CO_2 for pH control. Thus an incubator is needed with:

1. Temperature control
2. Arrangement for maintaining humidity
3. Arrangement for maintaining a stable CO_2 concentration

Many instruments are available commercially. The temperature control should be precise and large fluctuations (for instance, when the doors have been opened) are undesirable. Of particular importance is that the CO_2 concentration, which drops when the doors are opened, should be readjusted rapidly. This requires a method for rapid gassing. For the same reason, it is unsatisfactory to use an incubator which is shared for several different uses and is opened frequently.

3. Liquid Nitrogen Storage Facility

Once produced, hybridomas must be maintained and a low-temperature store for cryopreservation is essential (see Section IX). A simple but large cryogenic tank and a source of liquid nitrogen are needed. A programmed freezing instrument (see Section IX) is useful but not essential.

B. Minor Equipment

1. Animal holding facilities.
2. Sterile surgical equipment for mouse dissection.
3. Water baths, 37 and 56° C.
4. Centrifuges (bench top, preferably refrigerated).
5. Tissue culture ware (see Sections VI, VII, IX).
6. Inverted microscope (desirable but not essential).
7. Laboratory microscope, hemocytometers.

Further equipment needs will depend on the assay system to be used (see Sections VIII, XI) but typically a fluorescence microscope and a γ counter might be used.

C. Expertise

It is not necessary to have extensive cell culture experience or to be an immunologist to undertake hybridoma work, although it helps. On the basis that the hybridoma technique has many applications outside immunology, this chapter has been written in a style that does not assume an extensive familiarity with immunology. Hybridomas are rather fastidious cells and the chances of producing them and maintaining them are certainly higher if the worker has previous cell culture experience. This chapter is not intended to serve as a tissue culture primer and the reader who lacks experience is referred to available books.[10–13]

The most important prerequisite in terms of expertise relates to the antigen type to be used and the assay for antibody against the antigen. Hybridoma technology is secondary and can be learned, but it is essential to have experience working with the material which is the subject of the project, be it a virus or a peptide, a lymphocyte

differentiation antigen or a pathogenic parasite. Specific examples of the production of hybridoma antibodies to each of these types of antigens are described in later chapters.

III. MATERIALS AND MEDIA

A. Materials/Chemicals to be Ordered

1. Cell Culture Medium: RPMI 1640, or Alternative (Dulbeccos; Iscoves)

2. Fetal Calf Serum: Selected Batch

Not all serum batches are suitable, and the critical stage is most probably the initial postfusion stage, when hybrids are present at low concentration in company with large numbers of dead or damaged cells. The only certain way of screening to select a batch is to do a fusion and plate out in the different serum batches (test 4 to 6 at a time), ensuring that the serum is the only variable. The plate that gives the most hybrids indicates the best serum batch.

This approach is not always used, for a variety of reasons. For the worker who is just starting fusion, it does not make sense to use a procedure with a variable chance of success to check his serum; failed hybridizations are as likely to result from other technical factors. The newcomer to fusion should try to get enough of a tested batch from an established fusion laboratory to get his technique working before he screens batches.

Even in the established fusion laboratory, serum selection by fusion essentially poses problems because it is difficult to ensure that the serum is the only variable (for instance, the different cell batches will inevitably be left to stand for different periods before plating out). Nevertheless this is the most relevant screen for selecting a batch of serum, and any simpler screen may, at least theoretically, not pick the right batch. The screening procedure that is used most often is simply to test the ability of serum batches to support cloning at limiting dilution (see Section IX) of either the parent myeloma or an established hybrid.

Some laboratories have used mixtures of fetal calf serum and horse serum for fusion, and established myeloma lines can be maintained in cheaper sera than fetal calf, including horse and calf sera (see Section VII).

3. HAT Selective Medium Components

1. Hypoxanthine
2. Aminopterin
3. Thymidine

4. Polyethylene Glycol (PEG)

This material varies according to manufacturer, batch, and molecular weight. For many fusions one 500 g bottle will suffice, so a batch number recommended by a colleague should be obtained. PEG is toxic to cells, and the toxicity probably depends on molecular weight. The molecular weight quoted on the label represents an average value, and the range of molecular weight will depend on the batch, even when the average molecular weight is the same. Successful fusions have been reported with batches ranging in nominal molecular weight from 500 to 6000. We have done all of our fusions with a single batch of PEG 4000 mol wt from British Drug Houses (BDH), Poole, U.K.

5. Dimethyl Sulfoxide

B. Working Solutions

1. Medium

RPMI 1640 is supplemented with: FCS 10%, Glutamine 2 mM, Penicillin 100 IU/mℓ, and Streptomycin 100 μg/mℓ.

Glutamine and penicillin/streptomycin mixture can be obtained as frozen stock solution, and should be kept frozen. Glutamine has a half-life of about 2 weeks in liquid media; thus, even though media can be obtained with glutamine already added, the level should be supplemented if the medium is stored for 2 weeks or more. Other media (e.g., Dulbecco's MEM, Iscoves) may be used. While these media are essentially equivalent and most cells can be grown in any of these media, changing from one to another can set the cell growth back or even cause cell death. Thus when receiving a new line from another laboratory, it is advisable to get it established in its original medium and change over to your own medium gradually. Other additives are used by some workers, for instance, pyruvate and 2-mercaptoethanol.

2. HAT Medium

Medium as above with added hypoxanthine (136 μg/mℓ), aminopterin (0.19 μg/mℓ), and thymidine (3.88 μg/mℓ). We make up 100x HAT stock with hypoxanthine (13.6 mg/mℓ), aminopterin (0.019 mg/mℓ), and thymidine (0.388 mg/mℓ), which we store aliquotted, frozen, and in the dark (aminopterin is light-sensitive). For use, add 1 mℓ stock to 100 mℓ medium.

3. HT Medium

This is prepared similar to the HAT medium but without the aminopterin. Again, we use a 100x stock HT, and dilute for use.

4. Phosphate-Buffered Saline (PBS)

The Ca^{++}/Mg^{++}-free Dulbecco's PBS is used because it generally does not cause cell clumping. It can be bought, or made up according to the following formula:

- Sodium chloride 8.0 g
- Potassium chloride 0.2 g
- Disodium hydrogen orthophosphate (anhydrous) 1.15 g
- Potassium dihydrogen orthophosphate (anhydrous) 0.2 g

Dissolve in sequence in 0.8 ℓ distilled water. Adjust pH to 7.3, make volume to 1 ℓ with distilled water. Sterilize by autoclaving at 15 lb/in.^{2} for 15 min.

5. Gey's Hemolytic Medium

This is not generally available commercially (another medium known as Gey's is a growth medium and cannot be used for hemolysis). Stock solutions made up as follows:

Gey's solution A	
Ammonium chloride	35.0 g
Potassium chloride	1.85 g
Dipotassium hydrogen	

orthophosphate (KH_2 PO4)	0.119 g
Glucose	5.0 g
Phenol red	0.05 g
Gelatine (Difco®)	25.0 g
Distilled water	1000 mℓ

Gelatine is the variable component of this mixture, and not all gelatines will be effective. We have used Difco® gelatine successfully.

The mixture is dispensed into 20 mℓ aliquots in screwcap glass vials and autoclaved (15 lb/in.^{2}, 15 min). It can then be stored indefinitely at room temperature.

Gey's solution B

Magnesium chloride	($MgCl_2$ $6H_2O$)	4.20 g
Magnesium sulfate	($MgSO_4.7H_2O$)	1.40 g
Calcium chloride	($CaCl_2$)	3.40 g
Water		1000 mℓ

Dissolve salts with stirring, dispense in 10 mℓ aliquots in screwcap glass vials, and then autoclave (10 lb/in.^{2}, 10 min).

Gey's solution C

Sodium bicarbonate available commercially from media manufacturers, or make up:	
Sodium bicarbonate	5.6 g
Water	100 mℓ
Sterilize by membrane filtration.	

Working solution: Gey's hemolytic medium is made up from the stock solutions no more than 30 min before use.

Mix: 14.5 mℓ distilled water
4 mℓ Gey's solution A
1 mℓ Gey's solution B
add Gey's solution C (bicarbonate) to give pH 7.2 to 7.4 as judged by the indicator color.

Note: Bicarbonate solutions lose CO_2 and the amount to be added depends on the age of the solution. The volume required should be 0.1 to 0.5 mℓ.

6. PEG/DMSO Fusing Agent Contains 42% PEG W/V and 15% DMSO (V/V)

PEG will not dissolve unless heated, and the fusing mixture is made up as follows: weigh out 10 g PEG into a screwcap glass bottle; autoclave (15 lb/in.^{2}, 15 min) to sterilize and liquefy; while the PEG is still hot, add 14 mℓ of 15% DMSO in PBS; then store at +4° C.