RECEPTOR BIOCHEMISTRY AND METHODOLOGY, VOLUME 3

Series Editors: J. Craig Venter and Len C. Harrison

MOLECULAR AND CHEMICAL CHARACTERIZATION OF MEMBRANE RECEPTORS

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Membranes, Detergents, and Receptor Solubilization
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Volume 2
Receptor Purification Procedures
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J. Craig Venter, Claire M. Fraser, and Jon M. Lindstrom, *Editors*

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Preface

Cell hormone and neurotransmitter receptors are involved in the control and/or modulation of virtually every cellular and physiological process known. Changes in hormone receptors are implicated in major diseases including heart disease, hypertension, allergic respiratory diseases, metabolic disorders such as diabetes, and cancer. The recent molecular breakthroughs in the area of hormone and neurotransmitter receptor research have resulted from an interdisciplinary approach with the application of technologies derived from biochemistry, immunology, pharmacology, molecular biology, biophysics, and medicine.

The receptor field is undergoing a period of rapid advancement with a tremendous growth in the number of studies at the molecular level. This transition from receptor analysis using pharmacological techniques and radioligand binding studies toward the molecular resolution of the receptor molecules and their effector proteins requires working with a broad repertoire of new methodologies and techniques.

Receptor Biochemistry and Methodology is a new series devoted to the molecular advances involved in understanding the structure and biochemical basis of receptor action as well as to detailing the necessary methodologies.

The first three volumes are methodological reviews of approaches and technologies required by researchers involved in the isolation, purification, and biochemical characterization of cell surface proteins. Future volumes will deal in depth with single areas including Monoclonal Antibodies (Volume 4) and Radiation Inactivation (Volume 5), or will be devoted to the molecular basis of hormone and neurotransmitter action, with individual volumes on the structure, function, pharmacology, and biochemistry of individual receptors or effector proteins.

In Volume 3, **Molecular and Chemical Characterization of Membrane Receptors,** a broad spectrum of new and sophisticated approaches are detailed, from Chapter 1 on the use of monoclonal antibodies in receptor isolation and characterization to Chapters 10 and 11 on the application of recombinant DNA technology to receptor research. The intervening chapters contain the substantive methods available and essential to the receptor biochemist. These include Chapters 2, 3, and 4 on gel electrophoresis procedures, SDS-polyacrylamide gels, two-dimensional gels and peptide mapping—techniques that are applicable to purified proteins and/or affinity labelled receptors. Chapters 6 and 7 describe procedures for obtaining hydrodynamic parameters of receptor proteins, techniques that require only detergent solubilization of receptors and the binding of reversible ligands. Chapter 5 presents detailed methodology for dealing with an often ignored aspect of receptors, their carbohydrate moieties. All of these techniques require purified or partially purified receptor proteins; however, Chapter 8 introduces the technique of radiation inactivation which can be used to determine the functional molecular size of proteins while still in the native membrane. Chapter 9 is an excellent review of membrane receptor reconstitution, an approach that can be applied to the assay of solubilized membrane proteins, but more importantly, can ultimately provide the means of understanding the relationships between receptor and effector structure and function.

The contents of Volumes 1 to 3 reflect a strong prejudice, developed by us both in the course of our respective purification studies, that one key to success in receptor purification and structural analysis is to utilize many parallel approaches to insure, for example, that the isolated proteins are indeed those desired.

We feel fortunate in obtaining a unique collection of authors who are leaders in their respective fields and thank them for their help in making this series a reality. We also thank Paulette Cohen and the staff of Alan R. Liss, Inc. for their help and advice.

We believe that the reader will find that the articles are not only excellent reference sources but also extremely useful at the laboratory bench. We feel that the information contained in these volumes will be essential to researchers undertaking the molecular characterization of receptors or other integral membrane proteins. In addition, we hope that the reviews and discussions will interest researchers in biochemistry, immunology, pharmacology, physiology, biophysics, and cell and molecular biology as well as researchers and others who deal with the vast array of clinical disorders effected by or controlled through receptors.

J. Craig Venter
Len C. Harrison

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The Use of Monoclonal Antibodies in Receptor Characterization and Purification

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INTRODUCTION

In the few years since Kohler and Milstein first described "continuous lines of cultured cells secreting antibodies of pre-defined specificity" [Kohler and Milstein, 1975], hybridoma technology and monoclonal antibodies have had a tremendous impact on all areas of biomedical research.

A monoclonal antibody is the product of a single clone of B-lymphocytes. Each antibody produced by a single clone has the same amino acid sequence and, therefore, the same physical and chemical properties. It is possible to "immortalize" the production of predefined, specific monoclonal antibodies by fusing the relatively short-lived, normal antibody-producing splenic lymphocytes from immunized mice with cultured myeloma cells to generate clones of hybrid cells (hybridomas) that then secrete normal antibody continuously in culture [Kohler and Milstein, 1975, 1976].

The success of lymphocyte hybridization was due to the development of mutant myeloma cell lines adapted to grow permanently in culture in selective media [Littlefield, 1964]. Cotton and Milstein [1973] adopted the approach of using drug-resistant mutants of myeloma lines, one being a 5′-bromodeoxyuridine-resistant mouse line defective in thymidine kinase, the other an 8-azaguanine resistant rat line defective in hypoxanthine-guanine phosphoribosyl transferase (HGPRT). These lines were, respectively, unable to utilize thymidine and hypoxanthine from the culture medium when the de novo synthesis of these DNA precursors was blocked by aminopterin. Neither of the two cell lines, therefore, survived in selective HAT medium (hypoxanthine, aminopterin, thymidine) but complemented each other when fused, the hybrid cells being the only ones capable of continued growth. The fusion of these two myeloma lines resulted in a hybrid which expressed the product of each parent line (actually, new Ig molecules were produced as a result of mixing of heavy and light chains from the two parent cells, but there was no "scrambling" of V and C regions, indicating that the latter are not determined by cytoplasmic events). Finally, an 8-azaguanine-resistant line was derived from the mouse myeloma line MOPC-21 (P3) which secreted IgG_1 kappa and was designated P3/X63 Ag8. This line was then used by Kohler and Milstein [1975] in their revolutionary experiment to demonstrate that a hybrid formed with normal spleen cells from a BALB/c mouse immunized with sheep red blood cells (SRBC) secreted both IgG_1 myeloma protein and antibody to SRBC.

This apparently simple but elegant experiment has already revolutionized immunology and is having a major impact on many other areas of biology. The basic methodology has been used to generate monoclonal antibodies against a myriad of antigens including haptens, peptide hormones, enzymes, receptors and other proteins, polysaccharides, glycoproteins, lipopolysaccharides, differentiation antigens, histocompatability antigens, and other cell surface and cytoplasmic antigens.

ADVANTAGES OF HYBRIDOMA ANTIBODY PRODUCTION

As outlined in Table I, antibody production by hybridomas has many advantages over the conventional approach of harvesting sera from immunized animals.

1. Antibody production by hybridomas is continuous, theoretically unlimited in quantity, and constant in nature. Therefore, radioimmunoassays and related techniques may be standardized internationally. This is in contrast to the properties of antiserum obtained from immunized animals, which varies in specificity and affinity between animals and also with each harvest from the same animal.

2. Monoclonal antibodies are defined homogeneous reagents directed at only one antigenic determinant. This specificity ensures an unequivocal interpretation when such antibodies are used to purify receptors and map molecular function and structure. Polyclonal, heterogeneous antisera raised in animals contains antibodies to all components of the antigenic mixture and often to distinct antigenic sites on one particular antigen. The properties of polyclonal antisera greatly limit the interpretation and applicability of such preparations in many immunoassays.

3. The antigen used for immunization need not necessarily be pure, since hybridomas are selected for production of desired antibodies. In other words, specific antibodies can be obtained without specific immunization. This "dirty in/clean out" approach has been particularly useful in preparing monoclonal antibodies to some cell membrane receptors, whose low membrane concentration has made

TABLE I. Comparison of Properties of Monoclonal Antibodies and Conventional Antiserum Preparations

Property	Monoclonal antibodies	Antiserum
Composition of heavy and light immunoglobulin chains	Only one heavy chain, only one light chain	Heterogeneous mixture of antibodies of different heavy and light chain composition
Physical properties of antibodies (antigen specificity, affinity for antigen, etc)	Constant and homogeneous due to chemical identity of antibody molecules	Varies with each antiserum harvest due to heterogeneous nature of immunoglobulins in conventional antiserum
Recognizes and binds to:	A single determinant from immunogen	All antigenic determinants on all components of immunogen
Applicability for conventional immunological procedures	May not work	Applicable
Relevant immunoglobulin concentration	10–50 μg/ml (culture media) 1–10 mg/ml (ascites fluid)	0.1–1 mg/ml
Irrelevant proteins	Very small amount from fetal bovine serum (culture media) Some serum proteins and normal mouse immunoglobulins (ascites fluids)	All other serum proteins

complete purification extremely difficult [see chapters in Fellows and Eisenbarth, 1981; and Venter et al, 1983b].

4. Hybridoma technology may allow for the selection of antibodies to molecules that have little or no immunogenicity with respect to serum antibody production in immunized animals. Thus, the growth and expression of sensitized splenic B cells freed from the "immunoregulatory environment" in vivo could be amplified in a hybridoma, thereby increasing the chances for antibody generation.

5. Monoclonal antibodies can be selected and screened for on the basis of a particular desirable property, eg, low affinity, which could be advantageous in recovering receptors after adsorption to an immunoaffinity matrix.

6. C^{14}- or H^3-labeled monoclonal antibodies for use in immunoradiometric assays can be obtained by culture of clonal lines in medium containing labeled amino acids or sugars.

LIMITATIONS OF MONOCLONAL ANTIBODIES

Some of the properties of monoclonal antibodies that distinguish them from serum antibodies limit their applicability for certain experimental protocols. Fortunately, these limitations can usually be overcome provided they are understood.

1. Monoclonal antibodies generally can not precipitate their target antigens as they only cross-link antigens into dimers rather than form an antibody-antigen lattice. For this reason, monoclonal antibodies may not work in the Ouchterlony double-immunodiffusion assay and can not precipitate antigens from solution without addition of a second antibody.

2. Monoclonal antibodies do not readily fix complement, as complement requires at least two bound antibody molecules on neighboring determinants [Howard et al, 1979].

3. Conventional antisera contain a variety of antibody molecules that differ with regard to immunoglobulin class, specificity, and affinities for antigens and physical properties. A monoclonal antibody with a unique structure may differ from the majority of conventional immunoglobulins with regard to a particular property; ie, a monoclonal antibody may be more susceptible to denaturation due to temperature, pH or freezing and thawing than the bulk of serum immunoglobulins [Mason and Williams, 1980]. Furthermore, the rates of association and dissociation of monoclonal antibody-antigen complexes may be considerably slower or faster than preparations of conventional antisera.

4. Conventional antisera contain antibodies to a number of antigenic determinants on a given antigen, but monoclonal antibodies only recognize one determinant. Therefore, in some cases, conventional antisera may provide a more accurate identification of a target antigen in a radioimmunoassay, as any unknown antigen able to compete with all antibodies present in a serum preparation is most likely identical to the known antigen. In contrast, an antigenic determinant recognized by a monoclonal antibody may be present on unrelated antigens, and cross-reactivity elicited between a monoclonal antibody and several antigens may not necessarily be indicative of molecular identity between antigenic species. An appropriate combination of monoclonal antibodies to several determinants on one antigen is sufficient to overcome such a potential problem.

STRATEGIES FOR PREPARING MONOCLONAL ANTIBODIES TO CELL RECEPTORS

The use of monoclonal antibodies as probes of receptor structure and function and as specific reagents in the immunoaffinity purification of receptors offers unique insights heretofore not possible with more conventional biochemical and physiological approaches to receptor characterization. However, the generation of permanent lines of hybridomas secreting monoclonal antibodies with desired properties presents varying degrees of difficulty. Immunization of an animal with an antigen preparation results in the production of a heterogeneous population of immunoglobulin molecules against the antigen. Some of the antibodies produced may possess the desired characteristics, many will not. Immortalization of antibody secreting B-cell clones using hybridoma technology produces a random cross section of the overall population of immune cells. If the immune response to a particular component of an antigenic mixture is weak, this may be reflected in a small number of hybridomas producing antibody within the total population of hybrid cells. Successful production of a monoclonal antibody to a weak immunogen may involve a significant commitment in time and effort to perform several cell fusions and screen thousands of hybridomas for the presence of a desired antibody. Lastly, the properties of a monoclonal antibody identified to a specific antigen or determinant depend upon the experimental design and type of screening assay employed. In other words, if a low-affinity monoclonal antibody is sought, the immunization protocol and screening assays should be designed to maximize the chances of producing and detecting a clone of cells secreting a monoclonal antibody of low affinity. Similarly, if a monoclonal antibody directed to a determinant in the ligand-binding site of a receptor is desired, screening assays should be designed to detect this specificity.

In this chapter we will provide guidelines and suggestions for preparation of receptor-specific monoclonal antibodies with different properties. We will draw heavily on our own experience and present experimental protocols currently in use in our laboratories. This is not meant to imply that we consider our approaches more valid than those of others, but rather that these procedures have been found to work reproducibly in our hands. The following methodology is meant to serve as a basic introduction to hybridoma technology. Modification of all of these procedures have appeared, many of which may be more appropriate for the needs of a particular laboratory. The keys to success with hybridoma technology are simple: Become proficient with tissue culture techniques and use cell lines and fusion protocols that work reproducibly for you.

METHODOLOGY

Specific methodology (solutions, experimental protocols, etc.) will be found in Appendices I and II. The following sections will deal with general theory and approaches for hybridoma production.

Media Preparation

The most commonly used media for growth of myeloma cell lines and hybridomas are Dulbecco's Modified Eagle's Medium (DME) with 4.5 g glucose/liter or RPMI-1640 with glucose added to a final concentration of 4.5 g/liter.

Ready to use media (1× or 10×) is available commercially (GIBCO, Flow Laboratories) or may be prepared from dry powder using tissue culture grade water according to manufacturer's instructions. This requires equipment for filter sterilization.

We prefer to prepare our own media from dry powder, as it is more economical and the date of preparation is known. Media is stored at 4°C in the dark and is discarded if unused after 3 mo. Glutamine (0.3 mg/ml, final concentration) is added to media at biweekly intervals, as it is particularly susceptible to decay during storage of media and essential for cell growth.

Choice of Serum

Fetal bovine serum is presently the preferred supplement for support of hybridoma growth due to its relatively low immunoglobulin content and the observed efficiency of cell fusions in fetal bovine serum as compared to other sources of serum. We routinely use 20% fetal bovine serum for cell fusions and cloning and then reduce the serum concentration to 10% for growth of selected hybridomas.

Lots of fetal bovine serum from different commercial sources vary tremendously in their ability to support the growth of hybridomas. For this reason, careful testing of lots of serum is recommended. Purchasing a suitable lot of serum in quantity reduces the need for frequent serum screening (see Appendix I).

Tumor Cell Lines

Four major considerations in choosing an appropriate myeloma cell line for fusion should be taken into account. These are drug resistance, the species from which the line was derived, the question of whether or not the line secretes immunoglobulins, and the stability of the hybridomas produced.

As illustrated in Table II, the most commonly used myeloma cell lines are resistant to purine analogs such as 8-azaguanine or 6-thioguanine or to pyrimidine analogs such as 5-bromodeoxyuridine. Drug resistance allows for selection of growth of hybridomas following fusion in a selective medium (see below).

The majority of mouse myeloma cell lines used for production of hybridomas are derived from the BALB/c strain although rat and human lines are now also available. Generally, the wisest choice is to use a myeloma cell line from the same species as that of the immunized animal (which may not be a mouse, in specific cases). In this way, ascites tumors can easily be developed in syngeneic animals when specific hybridomas are selected.

In deciding which myeloma cell lines to use, a major consideration is the expression or lack of expression of myeloma immunoglobulins by the parent tumor cell. Hybridomas derived from immunized spleen cells and myeloma cells secreting heavy and light immunoglobulin chains express four immunoglobulin chains, usually designated G and K (heavy and light chains from parent myeloma) and H and L (heavy and light chains from spleen cell). The codominant expression of these four chains within the hybridoma can lead to the secretion of mixed species of immunoglobulin (such as KHHL, KGGL, KHGL, etc) in addition to the expression of the parental immunoglobulins (KGGK and LHHL) [Cotton and Milstein, 1973; Margulies et al, 1976]. For this reason, it is recommended that one start with a parental myeloma cell that expresses only light immunoglobulin chains (such as NS-1) or is a non-producer (653, SP-2/0). Using myeloma lines which do not produce any myeloma chains results in hybridomas expressing only the immunoglobulin chains of the parental spleen cells.

The stability of hybridomas is in part dependent on the choice of myeloma cell for a fusion partner. As is illustrated in Table II, some of the lines available are myeloma cell

TABLE II. Summary of Myeloma Cells

Cell line	Parental line	Drug resistance	Immunoglobulin chain	Reference
Mouse lines				
P3X63-Ag8 (P3)	MOPC 21	8-azaguanine	γ1, kappa	Kohler and Milstein [1975]
P3-NS-1/Ag14 (NS-1)	P3	8-azaguanine	---, kappa	Kohler et al [1976]
P3X63-Ag8.653 (653)	P3	8-azaguanine	---, ---	Kearney et al [1979]
SP2/0-Ag14 (SP2)	P3-spleen cell hybrid	8-azaguanine	---,---	Schulman et al [1978]
FO	SP2-SP2 hybrid	8-azaguanine	---,---	Fazekas de St. Groth and Scheidegger [1980]
S194/5.XXO.BU.5	S194	Bromodeoxyuridine	---,---	Trowbridge [1978]
Rat lines				
Y3-Ag1.2.3	Rat	8-azaguanine	---, kappa	Galfre et al [1977]