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APPLICATION OF MONOCLONAL ANTIBODIES IN BASIC RESEARCH, DIAGNOSIS, AND THERAPY[1]

CREED W. ABELL* and RICHARD M. DENNEY

Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, Texas 77550

ABSTRACT.—Monoclonal antibodies are the natural products of hybrid cells called hybridomas, which are obtained from fusion of cultured myeloma cells with spleen cells from an immunized animal. Monoclonal antibodies can exhibit high specificity and affinity for distinct determinants (epitopes) in low-molecular-weight compounds and macromolecules. The availability of an inexhaustible and easily reproducible source of such antibodies, which can be harvested from cell culture supernatants of permanent hybrid cell lines and animal ascites fluids, promises to advance considerably our knowledge of biology at the cellular, subcellular, and molecular levels. In the future, hybridoma technology should allow preparation of many well-characterized diagnostic tools and development of novel therapeutic reagents. This presentation provides an overview of the rapidly growing areas in which monoclonal antibodies are being implemented. A few specific examples illustrate their value in advancing our knowledge of biomedical systems.

Fewer than ten years ago, Köhler and Milstein (1) reported the successful fusion of mouse spleen cells with cells of a murine myeloma line to yield hybrids (called hybridomas) that can be grown indefinitely and that synthesize and secrete antibodies of predetermined specificity. This discovery has permanently changed the scientist's approach to the production of antibody preparations from immunizing laboratory animals for harvesting of antisera, to selection and growth of antibody-secreting, cloned cells. The revolutionary nature of Köhler's and Milstein's discovery was recently recognized when, along with the immunologist Jerne, they were awarded the Nobel Prize for Medicine in 1984.

Monoclonal antibiodies from these hybridomas can recognize determinants (epitopes) in the vast majority of foreign substances and can be produced in unlimited quantities. In March 1984, the American Type Culture Collection (ATTC) published an index listing 218 hybridomas, identified by the antigenic determinant with which the secreted antibody is reactive.[2] These cell lines are available to investigators for study. Although this collection represents only a small fraction of the hybridomas that have been produced worldwide, the listing illustrates the diverse nature of the epitopes that can be recognized by these immunoglobulins, and supports the contention that unlimited quantities of reagents reacting with virtually any biological or chemical substance of interest can be obtained. Table 1 shows an abbreviated summary of this index with references (2-53).

More recently, a Committee on Data for Science and Technology of the International Council of Scientific Unions and the International Union of Immunological Societies, in cooperation with the World Health Organization and the American Type Culture Collection, are sponsoring development of a computerized data bank[3] on cloned cell lines and their immunoreactive products. The purpose of this data-sharing plan is to provide scientists with information on hybridoma technology and its prod-

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[2]ATCC Index (ATCC, 12301 Parklawn Drive, Rockville, MD 20852).

[3]Hybridoma Data Bank, 12301 Parklawn Drive, Rockville, MD 20852-1776.

TABLE 1. Summary of ATCC Hybridoma Index (March 1984)

CELL TYPES

B cells (2-4)
Common leukocyte antigen (5-7)
Erythrocytes (8)
Granulocytes (9-11)
Leukocytes (12)
Lymphocytes, activated (10, 13)
MAC-1 (5, 7, 14)
Myeloid cells (15)
Monocytes (9-11)
Null cells (9-11)
T cell subset, helper (9, 10, 16)
T cell subset, suppressor (9, 10, 17)
T cell, peripheral (10)
Thymocytes (18)

VIRAL ANTIGENS

Dengue complex (19)
Hepatitis B (20)
Influenza (21)
SV 40 (22, 23)

SURFACE PROTEINS, GLYCOPROTEINS, AND GLYCOLIPIDS

B cell surface glycoprotein (24)
Blood group H antigens (25)
HLA (26-28)
Lewis (29)
Glycophorin A (29)
Glycolipid, asialo GM_2 (30)

RECEPTORS

Acetyl choline (31)
E Rosette receptor (18)
Transferrin receptor (32, 33)

OTHER PROTEINS

Actin[a]
Brain clathrin (34,35)
CEA (36)
Conconavalin A (37)
Fibronectin (38)
Forssman antigen (5, 7, 39)
Insulin (40)
Neural Antigen (41)

ENZYMES

Acetyl cholinesterase (42)
DNA polymerase (43, 44)

IMMUNOGLOBULINS AND TRANSPLANTATION ANTIGENS

δ, γ and μ heavy chains (45)
κ Light chains (45)
IgA_1 and A_2 (46)
IgE (47)
IgG (48)
IgM (49)
I-A (50)
I-E (51)

NUCLEIC ACIDS

ss and ds DNA (52)

HAPTENS

2,4,6-Trinitrophenyl (53)

[a]Hybridomas that recognize these antigens are on deposit in connection with a NIAID contract.

ucts. Thus, it is likely that a large body of new and important information will be immediately available to scientists with an interest in this field.

Before discussing the specific applications of monoclonal antibodies, we will review briefly the molecular nature of antibodies and how monoclonal antibodies are produced. An antibody molecule is composed of two light chains and two heavy chains that are held together by interchain disulfide bonds. Each chain contains a constant and a variable region that confer binding specificity on the antibody. The mammalian genes that code for the constant and variable regions are derived from dispersed segments of DNA that come together in a developing antibody-producing cell. Because these genes can be formed by selecting one of several hundred subgenic elements that are included in the final form of the gene, mammals have the capacity for enormous immunoglobulin diversity through introduction of variability in the antigen binding region [see Leder (54) for a review of this topic]. Thus, when an animal is immunized with an antigen, each spleen cell may produce a unique, homogeneous antibody that recognizes a distinct epitope on the foreign substance introduced. When Köhler and Milstein (1) discovered

that cultured mouse myeloma cells, which have an indefinite life span, could be fused to spleen cells from immunized animals, they had found a method for obtaining unlimited quantities of homogeneous antibodies. The hybrids resulting from such fusions are cloned and subcloned to ensure that all cells in a culture are identical, i.e., that they contain the genetic information required for the synthesis of a unique antibody species and thus produce homogeneous antibodies.

The advantages of hybridoma technology are profound. Because many hybrids arising from each spleen are screened for their potential to produce antibodies to the compound of interest and only a few are selected, it is possible to immunize with impure antigens and yet obtain specific antibodies. The immortality of the cell lines assures that an unlimited supply of a homogeneous, well-characterized reagent is available for use in many laboratories for a variety of applications, including identification and resolution of cell types (55); immunocytochemical localization of antigens in subcellular fractions (56) and tissues (57, 58); comparison of antigens in different species (59); examination of the structure and function of surface membrane receptors (60); rapid purification of proteins by immunoaffinity chromatography (61); quantitation by immunoassay of hormones (40), vitamins (62), and small molecular weight components (63); identification and mapping of functionally significant sites on major domains in macromolecules (64-66); identification of expressed epitopes in *in vitro* translation systems (67); and diagnosis and immunotherapy of pathological disorders (68-72). A selection of articles demonstrating these and other applications of hybridoma technology can be found in Kennett *et al.* (73) and McMichael and Fabre (74).

A general procedure used by many laboratories for producing monoclonal antibodies is shown in Figure 1. BALB/c mice are immunized with the antigen of interest and then repeatedly boosted. Although most foreign substances elicit an antibody response in mice, the degree of success in immunization depends on the number of specific antibody-forming cells in the spleen at the time of fusion, which depends, in turn, on the innate immunogenicity of the antigen used. Thus, antigens that induce high titers of antisera usually will generate large numbers of antibody-producing hybrids. Many different schedules and routes of immunization and adjuvants have been

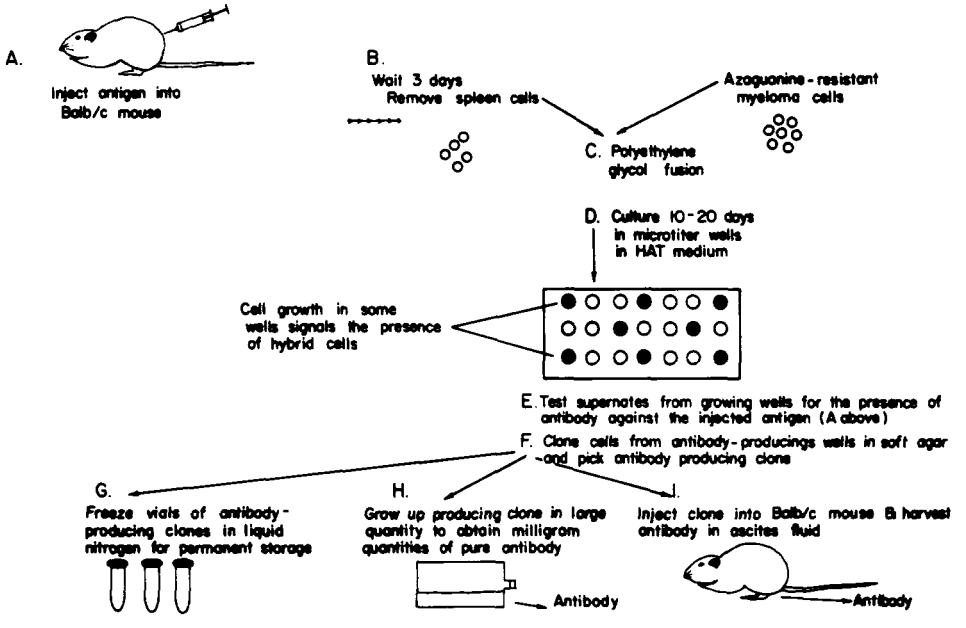

FIGURE 1. Isolation of Hybrid Cell Lines Which Secrete Antibodies Against Defined Antigens.

used, but in general, at least three injections of antigen, the first one usually in complete Freund's adjuvant, are used in the immunization procedure. After the initial immunization, the animals are then rested for four to six weeks before boosting. It is often useful to bleed the animal during this period to determine if circulating antibodies have indeed been elicited to the antigen of interest and that the level of circulating antibody has dropped appreciably prior to the final boost. The mice are then given the final immunization, and fusion of spleen cells with myeloma cells is carried out three to four days later when the greatest number of spleen cells are producing antibodies to the immunogen.

The spleen is then removed and a single-cell suspension of spleen cells is mixed with cultured myeloma cells in the presence of polyethylene glycol. Several mouse myeloma cell lines have been shown to be suitable as partners in spleen fusions. The myeloma line P3/X63 AG8 was used in early experiments (1), but others have been employed in a variety of studies (75-78). Myeloma P3/X63 AG8 cells produce their own heavy and light chains; hybrids formed with these lines produced immunoglobulins containing mixtures of immunoglobulin-light and -heavy chains from the parental myeloma cells and spleen cells. Consequently, hybridomas derived from this myeloma line, which are selected for their ability to produce antibodies to the antigen of interest, also produce inactive immunoglobulin molecules, which dilute the specific immunoglobulin reagent under production. As a solution to this problem, variant mouse myeloma cell lines have been developed [e.g., SP2/0 AG14 (77) and P3/X63 AG8.653 (78)] that do not produce their own heavy and light chains.

Most of the myeloma lines used are deficient in the enzyme hypoxanthine phosphoribosyl transferase (HPRT), an enzyme coded by a gene on the X chromosome which permits cells to utilize exogenous purines for nucleic acid synthesis. These myelomas do not survive in medium containing hypoxanthine, aminopterin, and thymidine (HAT). The $HPRT^-$ myeloma cells die because they cannot utilize the exogenous hypoxanthine to synthesize purines, and the addition of aminopterin prevents *de novo* synthesis of purines and pyrimidines. The unfused spleen cells are not killed by HAT medium, but they do not proliferate under the culture conditions used. In contrast, hybrids between spleen and myeloma cells contain spleen cell HPRT and thus can utilize exogenous hypoxanthine and thymidine for cell growth.

A critical step in the procedure is the screening of hybridoma cultures for hybrids secreting useful antibodies. Because a fusion may generate hundreds of hybrids growing at different rates, it is crucial to have relatively fast and accurate methods for identifying those hybrids that produce antibodies to the antigen of interest. This is particularly true when impure antigenic preparations have been used to immunize the spleen-donor mouse, for then the screening process substitutes for purification of the immunogen. Many assays are used for screening, including enzyme-linked immunosorbent assays (ELISA) (79), binding assays (80), indirect immunoprecipitation of functional activity (81-82), immunofluorescent staining of a cell preparation known to contain the antigen of interest (83), immunoblotting of the material of interest (84), as well as other assays employing variations on these methods. Each assay has advantages and disadvantages. The ELISA assay is very rapid but may require use of pure antigen and may detect antibodies that do not work well in immunoprecipitation assays (85). Alternatively, functional assays are usually sensitive and accurate but can be very time consuming, since they require independent measurements (e.g., indirect immunoprecipitation of a catalytically active enzyme requires enzymatic assay of the supernatants and immunoprecipitates). Because different screening methods can reveal different antibodies, it is generally advisable to use a screening method appropriate for the expected application of the antibody.

After screening, positive hybridomas are subcloned by plating in multi-welled culture dishes at concentrations that will give rise, on the average, to less than one hybrid clone per well. The cones are then maintained in culture for two to three weeks, and after they have become grossly visible, the cell culture supernatants are assayed again for antibody that recognizes the antigen of interest. Clones producing antibody are then transferred to large culture dishes, grown up, and retested; confirmed positive cultures are frozen and maintained in liquid nitrogen for future use. To produce a large amount of antibody, cultured cells are injected into BALB/c mice and grown in the peritoneum to produce ascites fluid, which usually contains concentrations of antibody 100-1000 times higher than that in cell culture medium. Monoclonal antibodies from ascites fluids can be purified by ammonium sulfate fractionation (86); by resolution on antigen affinity or Protein A columns (86), which bind most classes of immunoglobulin; by ion exchange chromatography (86); or by hydroxylapatite chromatography (87).

We now turn to some specific examples of the applications of monoclonal antibodies to biomedical research, drawing from past and present work in our laboratories. Our studies have focused on monoamine oxidase (MAO), the major intracellular enzyme responsible for the metabolic degradation of biogenic amines in mammals (88). Among its natural substrates are the monoamine neurotransmitters, dopamine, norepinephrine, and serotonin, whose levels are thought to be controlled in part by MAO. This enzyme, which is located in the outer mitochondrial membrane and exists in two forms (A and B), catalyzes the oxidation of amines to aldehydes, which are rapidly converted to their corresponding acids or alcohols by dehydrogenases or reductases, respectively. This enzyme has generated clinical interest because patients with a wide variety of neurological and psychiatric disorders have been found to express altered levels of the enzyme compared to age- and sex-matched healthy subjects (89-96). Despite the interest in MAO at both the basic and clinical levels, the protein structure of the enzyme has proved elusive, and its neuronal location and physiological function are obscure. We are currently using monoclonal antibodies to MAO A and B in a variety of ways to elucidate these important questions. Before describing these applications, however, a brief summary of the characteristics of the enzyme will be useful.

MAO has a molecular weight of 100,000-115,000 and consists of two subunits of identical or nearly identical molecular weight (97, 98). One subunit has an essential molecule of covalently bound flavin adenine nucleotide (FAD) (98), which is presumably coupled to the polypeptide by an as yet uncharacterized enzyme. MAO may have carbohydrate residues of unknown function added to its polypeptides, and the composition of its lipid microenvironment is either essential or contributes significantly to its catalytic activity (99-101). The measured level of MAO activity in most tissues consists of a mixture of both forms of the enzyme (A and B). MAO A is inhibited by the irreversible active site inhibitor, clorgyline, and preferentially oxidizes serotonin (102), while MAO B is inhibited by the related compounds pargyline and deprenly, and preferentially oxidizes phenylethylamine and benzylamine (103). These inhibitors enter the active site of the enzyme and bind covalently to the cysteinyl-flavin residue (104-105). Studies with ^{3}H-pargyline-labeled enzyme have shown that the FAD-containing subunits of human MAO A from placenta and MAO B from platelets have molecular weights of 63,000 and 60,000 respectively (106). Evidence from studies of peptides generated by the digestion of ^{3}H-pargyline-labeled MAO A and B with proteases suggests that MAO A and B differ in amino acid sequence (107). Nevertheless, a pentapeptide fragment around the FAD-bound residue is identical in both forms of the enzyme (108, 109). MAO A and B were not, until recently, physically separated from a tissue containing both forms (see below), and the molecular basis of the differences observed between the two enzymes has not been defined.

We have approached these problems using hybridoma technology. In our studies (110-112), we partially purified platelet MAO-B by ammonium sulfate precipitation and DEAE cellulose chromatography, and then we labeled the enzyme with ^{3}H-pargyline (this irreversible inhibitor binds only to MAO in these preparations) to provide a convenient method for following the enzyme during subsequent purification steps. MAO was further purified by isoelectric focusing or chromatofocusing (113), and a preparation in which MAO represented 20-30% of the total protein was used to immunize BALB/c mice. Spleen cells from an immunized mouse were then fused to mouse myeloma P3/X63 AG8 cells, using polyethylene glycol.

Products of this hybridization were first screened for their ability to recognize any antigen in the chromatofocused preparation, and then for their ability indirectly to immunoprecipitate ^{3}H-pargyline-labeled MAO B. The fusion produced approximately 300 clones. Thirty-four of the clones secreted antibody that bound to microtiter wells coated with the chromatofocused MAO and could be detected by a peroxidase-linked immunosorbent assay (ELISA). One clone produced an antibody, MAO B-1C2, which recognized both pargyline-inactivated and catalytically active MAO. To verify the specificity of the protein(s) recognized, MAO B-1C2 was linked to a cyanogen bromide-activated Sepharose 4B column, and crude mitochondrial preparations obtained from human liver (which contains both MAO A and MAO B) were passed through the immunoaffinity column. The majority of the protein (97%) did not bind to the column, but a single polypeptide was eluted when the column was treated with 4 M potassium thiocyanate. The polypeptide eluted had a molecular weight of 58,000 on SDS polyacrylamide gels as visualized by silver staining. Furthermore, when MAO was prelabeled with ^{3}H-pargyline, all of the radioactivity eluted with the 58,000 MW component.

Unfortunately, the MAO eluted from the immunoaffinity column with potassium thiocyanate was catalytically inactive, although indirect immunoprecipitation by MAO B-1C2, rabbit antimouse IgG, and heat inactivated *Staphylococcus aureus* had little or no effect on the catalytic activity of the enzyme. Control experiments showed that treatment of the enzyme with potassium thiocyanate under similar conditions destroyed enzyme activity, probably by unfolding the molecule, or possibly by dissociating essential lipids from it. Enzyme activity could not be recovered after removal of the potassium thiocyanate by dialysis. Consequently, to determine whether MAO B-1C2 recognized both MAO A and B, activities of both forms of the enzyme were compared in extracts applied to the immunoaffinity column and in the material that passed directly through the column (unadsorbed fraction). Phenylethylamine was used as a preferred substrate for MAO B and 5-hydroxytryptamine as preferred substrate for MAO A. The specificities of these substrates for MAO A and B were verified by treatment with the type-specific inhibitors, deprenyl and clorgyline. As expected, when phenylethylamine was used as a substrate, most of the activity (95%) was sensitive to deprenyl but insensitive to clorgyline. In contrast, when 5-hydroxytryptamine was used as a substrate, 98% of the material was clorgyline sensitive (but not deprenyl sensitive). The results of these experiments clearly showed that MAO B, but not MAO A, was retained on the MAO B-1C2 immunoaffinity column. Binding of MAO B was also shown by preincubating the liver mitochondrial preparations with unlabeled clorgyline (to block the A sites) and then labeling the remainder of the enzyme with ^{3}H-pargyline. The column was loaded with approximately 100,000 cpm, and only 3% failed to adsorb to the column.

Our identification of a monoclonal antibody that discriminates between MAO A and MAO B is consistent with recent evidence of structural differences in the active site subunits of the two forms of the enzyme (107, 114). Our results also agree with

McCauley's and Racker's (115) finding that bovine liver and brain MAO B have at least one antigenic site not found on bovine brain MAO A. Nevertheless, the structure of the antigenic determinant recognized by MAO B-1C2 is presently unknown, and consequently, the molecular basis for the antigenic difference(s) between MAO A and MAO B cannot be established at this time. Differences in one or both of the polypeptide subunits should be revealed through structural and sequencing studies of the two enzymes, and it is likely that the sequence of MAO B will become available in the foreseeable future because milligram quantities of nearly pure human MAO B have recently been prepared using MAO B-1C2.

The availability of large amounts of our well-characterized monoclonal antibody makes it possible to measure MAO protein concentration in extracts of platelets from a large population of patients with neurological and psychiatric disorders for comparison with normal controls. We have developed a radioimunoassay that is a highly sensitive method for determining concentrations of MAO protein independent of its catalytic activity. By determining the amount of MAO protein and the catalytic activity of the enzyme in the sample, we can determine the molecular activity of MAO B in crude extracts of human platelets. In clinical studies of patients with schizophrenic, affective, and other psychiatric disorders, our methodology will permit us to determine whether any differences detected are due primarily to altered molecular activity or to differences in enzyme concentration. Genetic changes or the presence of drugs could alter the rates of synthesis or degradation of MAO in the platelet, resulting in a change in platelet MAO protein concentration (and necessarily, specific activity). Alternatively, the amount of protein could be unaffected but catalytic activity reduced, for example, as a result of alterations in the sequence of the enzyme or the presence of an endogenous or drug-induced inhibitor that binds tightly to the active site of the enzyme. Such studies are of paramount importance to our understanding of MAO malfunctions, if they exist in psychiatric disorders, or the effects of neuroleptics on MAO in schizophrenics and MAO-inhibitors in depressed patients.

Recently, we have been successful in using MAO B-1C2 for immunoperoxidase cytochemical staining of cells in the human brain fixed 5 h postmortem (116). These results represent the first localization study of MAO B in the human brain by immunocytochemical methods. Our studies show that many neurons and some glial cells were stained in specific regions of the brain. In those areas so far studied, staining of MAO B was widely distributed among cells in regions that contain catecholamines and serotonin, and in other regions that do not appear to stain for these monoamines. MAO B staining was observed in some but not all neurons in these regions. Quantitative analysis using computer-assisted graphics reveals that numeric determinations of MAO concentration can be derived from photomicrographs of stained regions. Thus, it should be possible in the future to perform quantitative comparisons between appropriately matched sections of autopsy brain from controls and individuals with neurological and psychiatric disorders. Finally, recent studies using several newly developed monoclonal antibodies to MAO A (85) should provide the first histochemical data available about the relative location of MAO A and MAO B in the same or different neurons in the brain. This information should provide us with important clues about the physiological functions of these two forms of MAO.

In conclusion, these few examples serve to illustrate the power of applying hybridoma technology to basic and clinical problems. This field is expanding rapidly and holds the promise of resolving fundamental issues in biology and of using highly specific immunological reagents for the accurate diagnosis and effective treatment of human disease.

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