ABSTRACT

William Bruce Tannehill. POLYCLONAL AND MONOCLONAL ANTIBODIES THAT DEFINE AN IgA ASSOCIATED IDIOTYPE. (Under the direction of Dr. A. Mason Smith)
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Mineral Oil Plasmacytoma (MOPC) 467 is a monoclonal IgA producing plasmacytoma which was induced in BALB/c mice. Purified MOPC 467 IgA and IgA light chain were used to immunize New Zealand white rabbits and A/HeJ mice in order to produce anti-MOPC 467 idiotypic antisera.

Anti-MOPC 467 rabbit antisera were purified using affinity chromatography. The polyclonal antisera were tested for MOPC 467 idiotype specificity using a competitive enzyme linked immunosorbant assay (ELISA).

Hybridoma techniques were used to fuse spleen cells from immunized mice with FOX-NY myeloma cells. Hybridomas producing anti-MOPC 467 antibody were cloned for the production of monoclonal anti-MOPC 467. The monoclonal antisera were purified using affinity chromatography and clonotyped using enzyme linked immunosorbant assay (ELISA). Anti-MOPC 467 idiotype specificity of the monoclonal antibodies was tested using a competitive ELISA.

The anti-MOPC 467 polyclonal and monoclonal antibodies produced in this study defined, through their specificity, the location of the MOPC 467 idiotype as being on the IgA light chain and within the antibody binding site, or para-

tope. A discussion of the importance of anti-idiotypic antisera in medical research and possible future studies using anti-MOPC 467 idiotypic antisera is presented.

POLYCLONAL AND MONOCLONAL ANTIBODIES THAT DEFINE AN IgA ASSOCIATED IDIOTYPE

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INTRODUCTION

The integrity of the mammalian or vertebrate body is maintained by a multiple defense system including anatomic barriers, tissue secretions, phagocytic cells, and adaptive immune responses. These have enabled animals to survive in a world containing potential pathogens and other harmful agents. Specific immunocompetence among vertebrates involves the interaction of macrophages, lymphocytes, and immunoglobulin antibodies. The lymphocytes, a major component of the postnatal mammalian immune system, are primarily supplied by the bone marrow and thymus cortex. These lymphocytes are classified into T-cell and B-cell populations. T-cells develop in the thymus and are responsible for cell-mediated immunity, which is achieved by direct cell contact and release of non-antibody proteins. B-cells are lymphocytes which develop into plasma cells responsible for humoral immunity through the production of proteins called antibodies. These B cells originate in the bone marrow.

The interaction of cellular and humoral immunity produces several fundamental properties of the immune system. First, is the ability to discriminate "self" from "non-self". Cells of the immune system can recognize whether a molecule is foreign, and therefore requires a response, or is part of the host (self) and requires no antagonistic response. Next is the property of selective reactivity.

The immune system is able to recognize and distinguish one foreign substance, or antigen, from another. Therefore, immunity to one organism does not confer protection against unrelated organisms. The third property of the immune system is that of cytotoxic reactions following sensitization. Effector molecules are produced that promote neutralization or destruction of the antigen source once identified as antigenic. Fourth is the ability for inducible memory. The immune response is selectively altered on secondary contact, usually leading to a more vigorous response against an antigen previously encountered. Finally is the property of systemic dissemination, meaning a specific immune response at one site yields immunity to that antigen throughout the body (Hildemann, 1984). These five properties of the immune system allow it to be very diverse in its activities while at the same time be very specific.

In many instances, studies of the vertebrate immune system have been very difficult due to its complexities. The study of humoral immunity and the activities of antibodies has been made easier through the use of induced immunoglobulin-secreting plasmacytomas in mice. Plasmacytomas, the most widely studied immunoglobulin producing tumors in mice, appear to arise from fully differentiated cells that produce only a single molecular species of immunoglobulin. These tumors cells, with their increased proliferation abilities, are amplifications of normal

plasma cells. Thus, the plasmacytomas are extremely useful in studying the individual components of a vastly heterogeneous immunocyte system of cells (Potter, 1972). Mice, as well as other mammals including man, can develop plasmacytomas spontaneously, but these tumors are rare. A means for inducing plasmacytomas in mice was originally discovered by Merwin and Algire (1959). They were studying the long-term survival of allogeneic cells in millipore diffusion chambers. Highly inbred BALB/c mice received implants of diffusion chambers containing mouse mammary tumor tissue. It was noted that the mice carrying the chambers began developing hemmorrhagic ascites that were caused by either plasmacytomas or fibrosarcomas that developed on the peritoneal connective tissues. Subsequent experiments showed that plasmacytomas could be induced with plastic (Lucite) disks, which were components of the millipore diffusion chambers (Potter, 1973). In 1962, Potter discovered that mineral oils injected intraperitoneally could also induce plasmacytomas in BALB/c mice. One of the most effective plasmacytoma inducing mineral oils was found to be the hydrocarbon Pristane. Young BALB/c mice were injected intraperitoneally with three 0.5ml injections of Pristane, each about 2 months apart. Approximately 4 months after the first injection of oil, plasmacytomas began to appear in the peritoneal connective tissues. The tumors develop over a long period of time, generally about 180 days after the first injection of oil. However, a

plasmacytoma yield of about 60 - 70% is seen in BALB/c mice. A large number of these plasmacytomas, about 66%, are IgA producers. The reason for this is thought to possibly be due to an antigenic factor in mineral oil that attracts immunocytes from the stimulated gut and respiratory tracts that are already committed to IgA production (Potter, 1973). One such IgA producing plasmacytoma is Mineral Oil Plasmacytoma (MOPC) 467, induced in BALB/c mice in 1968 by Potter. The tumor which produces the monoclonal IgA, containing a kappa light chain, was found to be reactive against Salmonella spp. flagellin preparations (Smith and Potter, 1976).

The MOPC 467 IgA, as with all immunoglobulin proteins, exhibits an idiotypic marker which confers a degree of uniqueness to the molecule. Idiotypes are antigenic determinants on antibodies that distinguish one immunoglobulin variable region from all other variable regions. An idiotype is unique to immunoglobulin molecules produced by a given clone of B-cells. These antigenic determinants, or idiotopes, may reside within or outside of the actual antigen binding sites, but are coded by heavy or light chain variable region genes. Idiotypes were discovered simultaneously by Kunkel et al., (1963), and Oudin and Michel, (1963).

Jerne presented a network theory of immune regulation in 1974 in which the idiotype was an integral part. He described epitopes, which are antigenic determinants, and

paratopes, which are the actual antibody combining sites, as being two essential features of the immune system enabling it to accomplish its task of antigen recognition. Jerne described an idiotype as being a set of epitopes displayed by the variable regions of a set of antibody molecules. He used the term idiotope to describe each single idiotypic epitope. An idiotype then, in Jerne's theory, represents a certain set of idiotopes (Jerne, 1974).

Jerne's theory describes the immune system as a complex network of paratopes that recognize sets of idiotopes, and of idiotopes that are recognized by sets of paratopes. The interaction of these epitopes and paratopes lead to changes in B-cell and T-cell activities which ultimately result in immunoactivation followed by immunosuppression. An important concept introduced in the immune network theory is that of "internal imaging". When an antigen containing epitopes is introduced into the immune system, it is recognized by an antibody paratope (p_1), which binds the antigen. The p_1 antibody contains epitopes which are in turn recognized within the immune system by a second antibody paratope (p_2). The p_1 antibody also contains an idiotope (i_1) which is recognized by a third antibody paratope (p_3). Jerne has postulated that the p_2 antibodies are internal images of the original antigen and, therefore, stimulatory toward the p_1 set of antibodies. The anti-idiotypic p_3 antibodies are considered to be inhibitory

toward the p_1 set of antibodies, in the autologous immune system. The continued production and interaction of larger sets of antibodies within this network function to elicit the necessary immune response, and then to return the immune system to equilibrium. Since its introduction in 1974, Jerne's theory has gained support through published data such as that described by Urbain et al., (1984) and Bona, (1984).

Idiotypes and their importance in the immune system have been the subject of a great deal of study since their discovery in 1963. Idiotypic determinants of human (Kunkel et al., 1963), rabbit (Bordenave, 1971), and mouse antibodies (Wells et al., 1973), have been localized within the Fab fragment. Evidence for the relationships between idiotypic determinants and the antigen combining site of antibodies has also been provided for rabbit (Brient and Nisonoff, 1970) and mouse antibodies (Lieberman et al., 1975). Evidence that not all idiotypic determinants involve the combining site has also been presented for rabbit (Ghose and Karush, 1974) and mouse (Jorgense et al., 1977) antibodies.

A number of studies have been carried out to test whether idiotypic determinants of antibodies might be found on isolated heavy or light chains. Wang et al. (1970), obtained results suggesting that isolated heavy and light chains of some human myeloma proteins frequently express idiotypic specificities when the native myeloma protein is

lambda-type, but rarely when the native myeloma protein is kappa-type. Huser et al. (1975) have presented data suggesting that rabbit idiotypic determinants are rarely localized on isolated chains, but rather they require the interaction of heavy and light chains. In the mouse, studies have shown that the expression of idiotypic determinants can be either dependent on heavy and light chain interaction (Eichmann, 1978), isolated heavy chain (Eichmann, 1977), or isolated light chain (Pillemer and Weissman, 1981).

Anti-idiotypic antisera have been produced using autoimmunization, which is immunization with immunoglobulin antigen obtained from the individual being immunized, (Rodkey, 1974), isoimmunization, which is immunization with immunoglobulin antigen obtained within the same species and strain, (Eichmann, 1972), alloimmunization, which is immunization with immunoglobulin antigen obtained within the same species, but from a different strain, (Oudin and Michel, 1963), and heteroimmunization, which is immunization with immunoglobulin antigen obtained from a different species, (Kunkel et al., 1963). Detection of idiotypes by anti-idiotypic reagents can be done using standard immunochemical methods, such as double antibody precipitation (Hopper et al., 1970), hemagglutination (Kunkel, 1970), and enzyme linked immunosorbant assay (Stein and Soderstrom, 1984).

The study of antibody activity was further progressed

in 1975 by Kohler and Milstein when they successfully hybridized antibody producing mouse spleen cells with mouse myeloma cells. The result, following exposure to selective growth media, was a hybridoma cell line which produced antibody of the parent spleen cells while retaining the increased proliferation property of the myeloma cells.

The objective of the work presented in this paper was to identify the idiotype associated with the MOPC 467 IgA antibody through the use of hybridoma technology as well as polyvalent antisera production. Previous data from our laboratory had suggested that the MOPC 467 idiotype was located on the light chain (Smith, 1982). A/HeJ mice and New Zealand white rabbits were injected with intact MOPC 467 and purified MOPC 467 light chain. Spleen cells from immunized mice were fused with Fox-NY mouse myeloma cells for anti-idiotypic monoclonal antibody production. serum from immunized rabbits was collected and purified to obtain anti-idiotypic polyvalent antisera. The data collected from the study of the MOPC 467 idiotype provides relevant information regarding an idiotype associated with antibody molecules raised in response to a highly infectious organism. The data provides additional information pertaining to vaccine production and cancer research through the study of idiotypes and anti-idiotypes.

MATERIALS AND METHODS

Animals

Mice. Eight adult male A/HeJ mice were obtained from the Jackson Laboratory, Bar Harbor, Me. They were maintained in clean quarters, fed Wayne mouse chow and tap water ad libitum.

Rabbits. Three adult New Zealand white rabbits were obtained from the East Carolina University School of Medicine Breeding Facility.

Immunogen Preparation

IgA purification. The original MOPC467 plasmacytoma cell line was provided by Dr. Michael Potter of the National Institutes of Health, Bethesda, MD. The Myeloma cell line was carried in BALB/c mice produced by strict brother-sister matings in the Department of Microbiology & Immunology at East Carolina University School of Medicine.

Ascites fluid containing the IgA protein was collected from tumor bearing mice and an ammonium sulfate precipitation was performed using the method described by Smith, et al. (1977). The following reagents were used:

Ammonium Sulfate - 50% ammonium sulfate (Sigma Chemical

Co., St. Louis, MO)

TBS - 0.02M Hydroxymethyl Aminomethane (Tris)-HCl, 0.15M NaCl with 0.04% NaN_3, pH 8.0 (Fisher Scientific, Fair Lawn, NJ)

The clarified ascites fluid was precipitated by adding ammonium sulfate, under stirring, to a final concentration of 50%. The suspension was gently stirred for approximately 3 hrs, and was then centrifuged at 1600 x g. The resulting pellet was resuspended and washed 3x with 50% ammonium sulfate, and a final 47% ammonium sulfate precipitation was performed. The suspension was centrifuged, the pellet resuspended, and dialyzed against TBS.

The ammonium sulfate precipitated immunoglobulin fraction, containing the M467 IgA, was then passed over an affinity column of sepharose conjugated to *Salmonella spp.* flagellin monomer (MON). Conjugation of the ligand was carried out using the method described by Smith, et al. (1977), using the following reagents:

Gel - CH-Sepharose-4B (Pharmacia Fine Chemicals, Piscataway, NJ)

Ligand - *Salmonella spp.* flagellin monomer (MON)

Coupling reagent - N-ethyl-N´-(3-dimethylaminopropyl) carbodiimide hydrochloride (carbodiimide)

Start Buffer - TBS

Elution Buffer - 0.02M NH_2CH_2COOH, 1.0M NaCl pH 2.5 (Glycine Buffer)

MON, in a 10 - 20mg/ml solution, was mixed with an equal volume of swollen gel, and carbodiimide was added to a final 0.1M concentration. The components were mixed for 18h at room temperature then centrifuged and the supernatant removed. The gel was washed on a sintered glass funnel, resuspended and equillibrated in start buffer. A column with a bed volume of 10ml was stored at 4°C until used.

Ten ml of ammonium sulfate precipitated M467 was loaded into the affinity column and allowed to incubate for approximately 30 min at room temperature. The column was then washed with start buffer, and the optical density (O.D.) was monitored using a Beckman Model 26 Spectrophotometer, at 280nm. When the O.D. had reached baseline, the start buffer wash was discontinued. The remaining start buffer was allowed to settle into the gel until the top of the gel was dry. The column was then washed with elution buffer in order to elute the MON specific IgA that was bound to the column. Again, the O.D. of the elution was monitored until a baseline reading was obtained. The eluted Iga protein preparation was immediately brought to a physiologic pH of 8.0 using 19N NaOH. The affinity column was washed with start buffer until the eluent returned to a pH of 8.0.

The purified IgA, subsequently called M467, was concentrated in an Amicon gas concentrator using an Amicon

YM10 membrane (Diaflo Ultrafiltration Membranes, Danvers, MA). The preparation was concentrated to approximately 1/5 of the original eluent volume using nitrogen gas at a pressure of 20 psi. The protein was then dialyzed against TBS, using Spectrapor 1.0cm dialysis tubing with a MW cutoff of 12,000 - 14,000 (Fisher Scientific).

The purity of the IgA preparation was tested using an immunoelectrophoresis assay. Gel Bond 85mm x 100mm agarose gel support plates (FMC Corporation, Rockland, ME) were used. The hydrophobic/hydrophilic plates were cut in half lengthwise and placed on a leveling table with the hydrophilic side up. Agarose gel was made using 850mg of Seakem Highest Electroendosmotic (HEEO) Agarose (FMC Coroporation) added to 100ml of 0.05M Oxoid barbitone acetate buffer (Oxoid Ltd., England). The solution was placed in a boiling water bath until the agarose had dissolved completely. The 0.85% HEEO agarose was then poured onto the gel plates in a volume of 7.5ml per plate. The gels were allowed to solidify at room temperature and were then stored at 4°C in a humidity box. The immunoelectrophoretic assay was performed by using a template to produce a pattern of one trough and two wells in the gel. The gel was removed from the two wells on either side of the trough using suction. Capillary tubes formed from Pasteur pipettes were used to load an M467 sample into one of the wells and clarified M467 ascites fluid into the other well. The wells were loaded several times, allowing the

sample to diffuse into the gel between additions. Once loaded the gel containing the M467 samples was electrophoresed at 38v for 50 min at 4°C. The cathodal end of the gel was marked for reference. At the end of the 50 min electrophoresis, the gel was placed in a humidity box and the gel in the trough removed using suction. The trough was then filled with goat anti-whole mouse serum and allowed to incubate overnight at 4°C. The gel was then inspected for lines of precipitation.

Final purification of the M467 was performed using a preparative agarose gel to remove any remaining albumin. A 0.85% agarose gel was made using the HEEO agarose described above. The gel was heated in a boiling water bath until it dissolved, and was then poured into an 8.0cm x 25.0cm x 1.0cm gel plate and allowed to solidify. A 2.0cm x 4.0cm x 1.0cm trough was cut into the gel approximately 5.0cm from one end, and was loaded with M467. The gel was electrophoresed at 100v for 3 hrs at 4°C with the trough nearest the cathode. The cathodal section of the gel was then removed starting with the anodal edge of the trough. The gel, containing the M467, was minced and placed in a 30ml syringe. The gel was then forced through an 18 gauge needle into 50ml round bottom centrifuge tubes. The gel was centrifuged at 7000 x g for 15 min. The supernatant containing the M467 protein was removed, placed in an Amicon gas concentrator and concentrated using an Amicon YM10 membrane filter. The concentrate was tested for

purity using an immunoelectrophoresis assay as described above. Once M467 purity was confirmed, the preparation was dialyzed extensively against deionized water. The protein was transferred into a vacuum flask and shell frozen using liquid nitrogen. Once frozen the preparation was lyophylized. The freeze-dried M467 was stored until used as an immunogen.

Antigenic activity of the M467 was tested using double diffusion in agar gel (Ouchterlony). Gel plates were made using 850mg of Ion Agar No. 2 (Colab Laboritories, Inc.) added to 100ml of TBS and placed in a boiling water bath until the agar had dissolved completely. A base coat solution of 0.1% French Agarose in water (Fisher Scientific) was also placed in the boiling water bath. Glass microscope slides measuring 2.5cm x 7.5cm were coated with the base coat solution by adding one drop of the solution to a slide and placing a dry slide on top of it. The two slides were separated and placed on a leveling plate to dry. Dissolved ion agar solution was added to the coated plates in a volume of 7.5ml per plate. The 0.85% gel plates were allowed to solidify, placed in humidity boxes, and stored at 4°C until used.

M467 activity was tested by producing a pattern of test wells into the Ouchterlony gel and loading one well with M467 (1mg/ml in TBS). Opposing wells were loaded with rabbit anti-M467 and goat anti-mouse IgA. The gel was incubated in a humidity box at 37°C for 1-2 hrs, and then

inspected for lines of precipitation.

M467 Chain Separation. M467 chain separation was done using the reduction and alkylation method described by Rejewsky, et al. (1979) with modifications. Ten miligrams of M467 was dissolved in 1ml of 0.2M Tris-HCl pH 8.0. Reduction of the IgA was initiated by adding 10mM Dithiothreitol (DTT) (Bio-Rad, Richmond, CA) to the preparation, followed by incubation at 37°C for 2 hrs. The protein was then alkylated using an excess of Iodoacetamide (IoAc) at a 20mM concentration. The IoAc was dissolved and incubated in the preparation at 37°C for 30 min. The reduced and alkylated M467 was then dialyzed against TBS.

M467 chain separation was confirmed using a polyacrylamide gel electrophoresis (PAGE) System as described by Laemmli (1970). The following reagents were used:

Monomer Stock - 30% acrylamide, 2.7% bis acrylamide (Bio-Rad)

Resolving Gel Buffer - 1.5M Tris-HCl pH 8.8 (Sigma)

Stacking Gel Buffer - 0.5M Tris-HCl pH 6.8

SDS - 10% sodium dodecyl sulfate in water

Initiator - 10% ammonium persulfate in water (Bio-Rad)

Catalyst - N,N,N′,N′-Tetramethyl-ethylenediamine (TEMED)

Tank Buffer - 0.025M Tris pH 8.3, 0.192M glycine, 0.1% SDS

Fixative - 25% isopropyl alcohol, 10% acetic acid

Stain - 0.125% Coomassie Blue R-250 (Bio-Rad), 50% methanol, 10% acetic acid.

Destain - 25% methanol, 10% acetic acid

A solution containing 33.3ml acrylamide monomer, 25ml resolving gel buffer, and 40ml deionized water was prepared and degassed for 5 min. Following degassing, 1ml of 10% SDS was added to the acrylamide solution. Prior to preparing the acrylamide solution, six polyacrylamide gel plates were constructed using the Mighty Small Slab Gel Electrophoresis System (Hoefer Scientific, San Francisco, CA). Each plate contained one alumina plate, one glass plate, and two 1.5mm polypropylene spacers. Each plate was assembled separately and placed into the casting chamber. With the casting chamber assembled and upright on a leveling table, the previously degassed acrylamide monomer solution was prepared for polymerization. Prior to pouring the gels, 0.5ml initiator and 50ul of catalyst were added to the acrylamide solution. The 10% acrylamide solution was then poured into the stack of gel plates. The solution was allowed to come to the same previously determined level (10ml/plate) in all plates. Each gel was then overlayed with 1ml of water-saturated isobutanol. The gels were then allowed to polymerize at room temperature for 1 hr. While the resolving gel was polymerizing, the stacking gel was prepared by adding 6.7ml of acrylamide monomer and 12.5ml stacking gel buffer to 30ml deionized water. The solution

was degassed for approximately 5 min. After degassing, 0.5ml 10% SDS was added to the solution. Before adding the stacking gel to the polymerized resolving gel plates, the isobutanol was rinsed off the tops of the gels with deionized water. After rinsing off the resolving gel layers, 250ul initiator and 20ul catalyst were added to the degassed stacking gel solution. The 4% acrylamide stacking gel was then added to each gel sandwich separately using a Pasteur pipette. The stacking gel level in each plate was brought up to the notch in the alumina plate. Once all plates were filled, a 5 tooth comb was placed in each plate taking care not to trap air bubbles. The stacking gel was allowed to polymerize for approximately 1 hr at room temperature. Once the stacking gel had polymerized, the combs were removed. The gels were wrapped in Saran wrap and stored at 4°C.

Reduced and alkylated M467 was prepared for analysis on a 10% Laemmli gel as follows. Fifty ul of reduced and alkylated M467 were mixed with 5ul of 1x sample buffer, and prestained molecular weight markers (Pharmacia) were prepared in a 1:1 ratio with 1x sample buffer. A gel was placed in the electrophoresis unit, and the unit was filled with tank buffer. One lane of the gel was loaded with 55ul of the M467 sample while a neighboring lane was loaded with 5ul of the prestained marker sample. The samples were electrophoresed at 150v constant voltage, using power provided by an Electrophoresis Constant Power Supply (Pharma-

cia), until the lightest molecular weight marker had separated and moved to the bottom of the gel. The gel was removed from the unit, and placed in fixative for 15 min. The gel was passively stained in Coomassie blue overnight at room temperature. The gel was then destained in a gel destainer (GD-4, Pharmacia) at 24v for approximately 15 min. The destained gel was then removed for analysis.

M467 Light Chain Preparation (Laemmli PAGE). M467 light chain was collected using preparative PAGE gels. Laemmli gels were prepared as described above with the exception of the stacking gel addition. The volume of stacking gel poured per plate was approximately 1/2 that of the ana- lytical gels, and no combs were placed in the preparative gels. This allowed full use of the available gel surface for light chain separation.

Reduced and alkylated M467 was prepared by adding 250ul M467 to 10ul 1x sample buffer and 10ul bromphenyl blue with 10% glycerol. The electrophoresis unit was set up as described above and 270ul of the M467 preparation was loaded onto the gel. The sample was electrophoresed at 150v until the dye marker reached the bottom of the gel. The gel was removed from the gel plate, and a scalpel was used to cut a longitudinal section of the gel approximately 1cm in width. The remainder of the gel was placed in a small amount of tank buffer while the gel section was stained in coomassie blue to determine light chain location. Once destained, the gel section was placed on a glass plate along side of

the unstained remainder of the gel. A horizontal band was cut from the unstained gel which corresponded to the M467 light chain band in the stained gel section. This band was saved for light chain electroelution.

Electroelution of the purified light chain was carried out using a Unidirectional Electroeluter (Model UEA, IBI, New Haven, Connecticut). The unit was filled with approximately 450ml of electroelution buffer (Laemmli Tank Buffer). The V-channels of the unit were loaded with 125ul each of 7.5M ammonium acetate (NH_4OAc) salt cushion, using a Hamilton syringe. The polyacrylamide gel containing the M467 light chain was minced and placed into the horseshoe shaped slots on the eluter platform. The protein was eluted for 45 min at 125v constant power. The electroelution was carried out at room temperature. At the end of 45 min, the power supply was turned off and the salt cushion containing the eluted light chain was removed without draining the tank buffer or removing gel samples. A fresh 125ul salt cushion layer was placed in each V-channel, and the electroelution performed for an additional 45 min at 125v. Following electroelution, the salt cushion layers containing the protein from both runs were placed in dialysis tubing and dialyzed against TBS. Several PAGE gel bands containing M467 light chain were electroeluted and the protein dialyzed against TBS for purity analysis.

M467 Light Chain Preparation (Affinity Chromatography). An AH-Sepharose 4-B affinity column was prepared for light chain purification as described by Cambiaso, et al.(1974), using the following reagents:

Gel - AH-Sepharose 4-B (Pharmacia)

Ligand - Goat anti-mouse IgA, heavy chain specific, 5mg/ml (Cooper Biomedical, Malvern, PA)

Activation Buffer - 0.1M sodium carbonate pH 8.8 (Fisher)

Activator - 25% glutaraldehyde (Sigma)

Blocking Buffer - 0.2M ethanolamine pH 9.0

Start Buffer - 0.02M Tris, 0.15M NaCl pH 8.0 (Fisher)

Elution Buffer - 0.02M NH_2CH_2COOH (glycine), 1.0M NaCl pH2.5

The aminated gel was first washed with ten volumes of activation buffer on a sintered glass filter. To 3ml of packed gel was added, under stirring, 7ml activation buffer containing 2.5% activator. The gel was incubated at room temperature for 10 min followed by five washes with 20ml activation buffer. To the activated gel was added, under stirring, 7ml of ligand. The gel was incubated at room temperature for 15 min, followed by five washes of 20ml of activation buffer. A further washing with elution buffer was performed to remove non-conjugated proteins. The gel was incubated for 16 hr at 4°C with blocking buffer to block unreacted aldehyde groups. A 3ml column of anti-IgA conjugated AH-sepharose was then prepared and equilibrated

with start buffer.

Reduced and alkylated M467 was loaded into the column and allowed to incubate for 1 hr at room temperature. The column was then washed with start buffer and the effluent, containing M467 light chain, was collected. The optical density (O.D.) was monitored at 280nm. When the O.D. had reached baseline, the start buffer was discontinued. M467 heavy chain and unreduced M467 were then eluted from the column using elution buffer. The effluent, containing M467 light chain, was concentrated on an Amicon YM10 membrane for purity analysis.

M467 Light Chain Purity. M467 light chain purity and antigenic activity were tested using double diffusion in agar gel (Ouchterlony). A test pattern was cut into a gel and puirified light chain was loaded into a well as described above. Two opposing wells were loaded with rabbit anti-M467 and goat anti-mouse IgA, alpha chain specific. The gel was placed in a humidity box and incubated at 37°C for 1-2 hrs. The gel was then inspected for lines of precipitation.

The purified light chain, subsequently called M467 L-chain, was dialyzed extensively against deionized water. The protein was shell frozen, lyophilized,and stored until used as an immunogen.

Immunizations (Mice)

Two groups of four mice each were injected with either intact M467 or M467 L-chain, using the injection schedule described by Lieberman and Humphrey (1972) with modifications. Mice receiving intact M467 were given initial injections of 75ug M467 in Freund's Complete Adjuvant. The injections were given intraperitoneally (i.p.). Subsequent i.p. injections of 75ug M467 were given approximately 5 days later in Freund's Incomplete Adjuvant. Mice receiving M467 L-chain were given initial i.p. injections of 75ug L-chain using the Ribi Adjuvant System (ImmunoChem Research Inc., Hamilton, MN). Secondary i.p. injections of 75ug L-chain were also given in Ribi Adjuvant. Three additional injections of 75ug M467 or M467 L-chain, in sterile normal saline, were given subcutaneously (s.q.) at 4 - 5 day intervals. The s.q. injections were given at 2 axillary and 2 inguinal sites on each mouse. One week after the fifth injection, serum was collected from the mice using an intraorbital sinus bleed. The sera from the four mice in each group were pooled for testing.

Enzyme Linked Immunosorbant Assay. Antiserum activity against M467 was tested using an Enzyme Linked Immunosorbant Assay (ELISA) as described by Stein et al. (1984) with modifications. The following reagents were used:

Coating Buffer - 15mM Na_2CO_3, 35mM $NaHCO_3$, 0.05%, NaN_3, pH 9.6 (Fisher).

Blocking Buffer - 10% Bovine Serum Albumin (Sigma) in coating buffer.

PBS - 150mM NaCl, 7mM Na_2HPO_4, 3mM KCl, 1mM KH_2PO_4, 0.05% NaN_3, pH 7.4 (Fisher).

PBS-Tween - 0.05% Tween 20 (Sigma) in PBS

PBS-0.1% BSA - 0.1% BSA (Sigma) in PBS.

10% Diethanolamine Buffer - 10% bis 2-hydroxyethylamine, 4mM $MgCl_2 \cdot 6H_2O$, 0.05% NaN_3, pH 9.8.

M467 was coated onto a Linbro Microtitration Plate (Flow Laboratories, McLean, VA) at a concentration of 5ug/well in coating buffer. The plate was incubated overnight at 4°C. The plate was washed once with PBS-Tween, loaded with 200ul blocking buffer, and incubated at 37°C for 1 hr. The plate was then washed 3x with PBS-Tween. Three wells were loaded with 100ul anti-M467 neat serum, three with 100ul anti-M467 L-chain neat serum, and three with 100ul normal mouse serum (NMS) as a negative control. The plate was incubated for 2 hrs at 37°C, then washed as described. A secondary antibody of biotin conjugated goat anti-mouse IgA, IgG, IgM (Cooper Biomedical, Malvern, PA) was added to sample wells in a volume of 100ul/well. The high affinity antibody was diluted 1:1000 with PBS-0.1% BSA prior to addtion. Following incubation at 37°C for 1 hr, the plate was washed as described. A 1:250 dilution of

alkaline phosphatase labeled avidin (Zymed Laboratories, San Francisco, CA) in PBS-0.1% BSA was added to each well at a volume of 100ul/well. The plate was incubated at 37°C for 20 min, and washed 3x with PBS-Tween, at 10 min per wash. After the last wash, the plate was washed 3x consecutively and loaded with 200ul/well of 1mg/ml Sigma 104 Phosphate Substrate in 10% diethanolamine buffer. Color reactions in the wells were read at 405nm using a TiterTek Multiscan spectrophotometer (Flow Laboratories). Prior to reading, the color reactions were stopped by adding 25ul of 3M NaOH/well.

Once serum activity was confirmed, the two groups of mice received subcutaneous (s.q.) injections of 75ug M467 or M467 L-chain, respectively. Four days after the sixth injection, the spleens from two mice in each group were removed for fusion with myeloma cells.

Hybridoma Production

Spleen cells from immunized mice were fused with FOX-NY mouse myeloma cells using the protocol described by Hyclone Laboratories (1985). The following reagents were prepared prior to fusion:

Fusion Solution - 0.5ml DMSO and 5.0ml deionized water were filter sterilized and added to 5.0g polyethylene glycol (PEG M.W. = 4,000, Sigma). The mixture was placed in a boiling water bath until the PEG had

completely dissolved, and then autoclaved prior to use.

Complete Culture Media - 250ml of RPMI 1640, 10mM HEPES, 2mM L-glutamine (M.A. Bioproducts, Walkersville, MD) was added to a 500ml filter sterilizing flask (Corning Laboratory Sciences Co., Chicago, IL). Heat inactivated fetal calf serum (Collaborative Research, Lexington, MA) was added in a volume of 75ml. A 5ml volume of 100x Penicillin-Streptomycin-Antimycotic solution was added next. The medium was supplemented with 50ml of NCTC 109 culture medium (M.A. Bio-products), 5ml non-essential amino acids (Sigma), and 2ml each of 0.15mg/ml oxaloacetate, 0.05mg/ml pyruvate, 0.2U/ml of bovine insulin (Sigma). The complete media was brought up to 500ml volume with RPMI 1640 and filter sterilized. The sterile culture medium was stored at 4°C until needed and then warmed to 37°C before use.

AAT Media - 500ml complete culture medium plus 2.5ml 200x adenine, 5ml 100x aminopterine, 2.5ml 200x thymidine (Sigma).

ACT- 0.01M Ammonium chloride-Tris

serum Free Media - RPMI 1640

Macrophage Isolation. Isolation of peritoneal exudate cells (macrophages) was carried out using the following procedure. An adult male BALB/c mouse (Jackson Laboratory) was sacrificed by cervical dislocation. The mouse was

pinned to a dissecting board and disinfected with 70% ethanol (EtOH). Sterile forceps and scissors were used to cut a median sagittal incision in the abdominal skin. The abdominal muscle layer was lifted using forceps, and 5ml of a sterile 11.6% sucrose solution was injected using a syringe and a 21 gauge needle. While holding the abdominal muscle layer, the mouse was gently rocked from side to side for 30 sec. A small incision was made in the abdominal muscle layer using scissors, and a pasteur pipet was used to withdraw the sucrose. The clear sucrose solution was placed in a sterile 50ml conical tube and diluted 1:3 with serum free culture media. The solution was then centrifuged at 200xg for 10 min. The supernatant was decanted and the macrophages resuspended in 1ml of media. The cells were counted and diluted in AAT to a final concentration of 2.5×10^4 macrophages/ml. An addition of 1ml of the macrophage solution/well was made to 24-well fusion plates (Corning) 24 hrs prior to the addition of fusion products. The plates were incubated in a 5% CO_2 incubator at 37°C (Queue Systems, Parkersburg, WV).

Fusion. Immunized mice were sacrificed by cervical dislocation and saturated with 70% EtOH. The spleens were aseptically removed and placed into serum free media. The spleens were dissociated by forcing them through a stainless steel screen. The cell suspension was filtered through glass wool and centrifuged at 200xg for 10 min. The cells were resuspended in ACT and incubated for 5 min

at 37°C. They were then centrifuged at 200xg for 10 min. The spleen cells and FOX-NY myeloma cells were washed separately with serum free media. The cell preparation was resuspended in serum free media and counted. The myeloma cells and spleen cells were combined in a 50ml conical polypropylene tube at a ratio of 10 spleen cells to 1 myeloma cell, centrifuged at 200xg for 10 min, and the supernatant decanted. A drop by drop addition of 1ml of fusion solution was added over 60 sec with agitation. The cell mixture was incubated in a 37°C water bath for 90 sec with gentle agitation. The fusion mixture was then diluted with serum free media by adding 1ml dropwise over the first 30 sec, 3ml over the next 30 sec, and 16ml over the final 60 sec. The tube was filled with serum free media and allowed to stand for 5 min at room temperature. The fused cells were gently washed once in serum free media and resuspended in AAT culture media. Media was added to give a final cell concentration of 1 x 10^6 cells/ml. The fused cells were aliquoted into 24-well culture plates previously inoculated with the macrophage suspension. Fusion products were added in a volume of 1ml/well.

The cell cultures were incubated at 37°C for two weeks in the presence of 5% CO_2. The cells were fed AAT media twice a week and growth was monitored using a Nikon model TMS microscope. At the end of two weeks, culture supernatant was removed from those wells showing strong cell proliferation. The supernatants were tested for the

presence of anti-M467 antibody using an ELISA as previously described. M467 was coated onto a microtiter plate at a concentration of 10ng/well. Culture supernatants were used as primary antibodies at a volume of 100ul/well, and biotin conjugated goat anti-mouse IgA, IgG, IgM (Cooper) was used as a secondary antibody. Fresh AAT media was used as a negative control. Mouse anti-M467 and anti-M467 L-chain were used as positive controls. Color reactions were read at 405nm.

Anti-Idiotypic Monoclonal Antibody Production. Wells testing positive for anti-M467 and anti-M467 L-chain antibody were chosen for cloning purposes. The cells from one well in each group, showing the most proliferation, were counted and diluted in complete culture media to give a final concentration of 1 cell/100ul. The remaining cells producing anti-M467 activity were frozen using a Cryomed model 1010 cryopreservation unit (Cryomed, Mt. Clemens, MI). The diluted cells were added to 96 well sterile culture plates (Corning) at a concentration of 1 cell/ well. Five plates per fusion (5 for anti-M467 and 5 for anti-M467 L-chain) were used. The remaining cells were diluted 1:2 to give a final concentration of 1 cell/50ul. The cells were added to 5 additional plates per fusion at a concentration of 1/2 cell/well. The plates were incubated at 37°C for 24 hrs.

Isolation of Mouse Thymocytes. Mouse thymocytes were isolated for growth stimulation of the newly cloned

hybridoma cell lines using the following procedure. BALB/c mice (Jackson Laboratories) approximately 8 weeks old were selected for thymocyte isolation. A mouse was sacrificed by cervical dislocation, and disinfected with 70% ethanol (EtOH). Sterile forceps and scissors were used to open the chest cavity. The heart and thymus were exposed by removing the rib cage, and the thymus lobes were excised *in toto* and placed into complete culture media. The cells from the thymic lobes were dissociated by forcing them through a sterile stainless steel screen. The cells were centrifuged at 200 x g for 10 min, resuspended in 5ml of complete media, counted and diluted in a sterile 200 ml flask (Corning) to a final concentration of 5×10^6 cells/ml. The thymocytes were incubated at 37°C for 24 hrs before adding them to the 20 cloning plates previously inoculated with hybridoma cells. Prior to adding the thymocytes to the cloning plates, each plate was scanned to identify those wells containing only 1 hybridoma cell/well. The wells were marked and the thymocytes added at a concentration of 5×10^5 cells/well.

The hybridomas were incubated at 37°C for approximately 2 weeks and were fed complete culture media twice a week. Those previously marked wells showing cell confluency at the end of two weeks were expanded into 24 well culture plates (Corning) containing 2ml of complete media/well. The cells were cultured and fed until cell confluency was observed. The hybridoma cells were then

tested for anti-M467 antibody using an ELISA as described above. Wells showing anti-M467 activity were expanded into 75ml culture flasks (Corning) containing 10ml of complete media/flask, and were incubated at 37°C for two weeks. Five flasks from each fusion, showing optimal growth, were tested for monoclonality using an SBA Clonotyping System I (Southern Biotechnology Associates, Inc., Birmingham, AL). An ELISA plate was prepared as described with 10ng M467/well. Monoclonal culture supernatants were used as primary antibodies (100ul/well). Alkaline phosphatase labeled goat anti-mouse IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgM, kapa-chain, and lambda-chain antibodies were each diluted 1:250 with PBS-0.1% BSA, and were used as secondary antibodies. Complete culture media was used as a negative control. Color reactions were read at 405nm. One flask from each fusion showing monoclonal antibody production was chosen for final analysis.

Monoclonal Antibody Purification. Cell lines producing monoclonal antibodies against M467 and M467 L-chain were expanded into 200ml culture flasks (Corning) containing 100ml complete media, and were incubated at 37°C. Approximately every two weeks, the cells were "split" into fresh culture media to perpetuate the cell lines. A 1:10 split was routinely used (10ml cells into 90ml fresh media). The remaining cells were centrifuged at 200 x g for 10 min. The supernatants were collected for antibody purification, and the cells were cryopreserved.

Monoclonal hybridoma culture supernatants containing anti-M467 antibody or anti-M467 L-chain antibody were ammonium sulfate precipitated using 50% saturated ammonium sulfate as described above. The precipitated antibody was further purified using affinity column chromatography. A Protein A-sepharose affinity column was prepared as described by Pharmacia using the following reagents:

Gel - Protein A-sepharose CL-4B (Pharmacia)

Sample Buffer - 0.5M Na_2HPO_4, 0.5M NaH_2PO_4 pH 8.0

Start Buffer - 0.1M Na_2HPO_4, 0.1M NaH_2PO_4 pH 8.0

Elution Buffer - 0.02M NH_2CH_2COOH (glycine), 1.0M NaCl pH 2.5

Dry protein A-sepharose (1.5g) was swollen in start buffer for 30 min, then packed into a syringe to give a final bed volume of 5ml. Four volumes of precipitated monoclonal antibody was added to one volume of sample buffer. The sample was added to the column, and allowed to absorb for 30 min at room temperature. The column was washed with start buffer, and when the O.D. at 280nm reached baseline, the start buffer was discontinued. The column was then washed with elution buffer to elute the monoclonal antibody. The eluted antibody was brought to a physiologic pH of 8.0 using 19N NaOH, concentrated on an Amicon YM10 membrane and dialyzed into TBS.

Final purification of the monoclonal anti-M467 and

anti-M467 L-chain antibodies was performed by adsorption affinity chromatography. A CH-sepharose 4-B (Pharmacia) affinity column was prepared using the carbodiimide conjugation method described above. Mouse myeloma proteins TEPC-15 (IgA,k) and MOPC 315 (IgA,λ) were used as ligands for the adsorption column. Monoclonal antibody was loaded onto the column and incubated at room temperature for 1 hr. The column was then washed with start buffer (TBS), and the O.D. monitored at 280nm. The effluent containing anti-idiotypic monoclonal antibody was collected until the O.D. reached baseline. The anti-isotypic and anti-allotypic antibodies were then eluted from the column using elution buffer (glycine buffer). The column was reequilibrated with start buffer.

Monoclonal Antibody Purity (Double Diffusion in Agar Gel). Anti-M467 and Anti-M467 L-chain monoclonal antibodies, subsequently called BT/$3B_1$ and BTaL/$_1$ respectively, were tested for purity using double diffusion in agar gel (Ouchterlony) analysis as described above. Two patterns of test wells were produced in a gel and the center wells loaded with either BT/$3B_1$ or BTaL/$_1$. Five opposing wells were loaded with goat anti-mouse IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgM, respectively. The gel was incubated at 37°C in a humidity box for 1 - 2 hrs, and then inspected for lines of precipitation.

Monoclonal Antibody Purity (Immunoelectrophoresis). An additional test for antibody purity was performed using an

immunoelectrophoresis assay as described above. A pattern of one well and two troughs was produced in two separate gels. The wells were loaded with BT/$3B_1$ and BTaL/$_1$, and electrophoresis was carried out as described. The two troughs in each gel were filled with goat anti-mouse IgG_3 and goat anti-whole mouse serum, respectively. The gels were incubated overnight at 4°C, then inspected for lines of precipitation.

Monoclonal Antibody Specificity (ELISA). Idiotypic specificities of BT/$3B_1$ and BTaL/$_1$ were tested using a competitive ELISA. M467 was coated on a microtiter plate as described above using a concentration of 10ng/well. BT/$3B_1$ and BTaL/$_1$ were used as primary antibodies at a concentration of 100ng/well. Affinity purified M467 L-chain was used as a competitor at varying concentrations ranging from 1ng/well to 100ug/well. The competitor was added simultaneously with the primary antibodies. Biotin conjugated goat anti-mouse IgG (Cooper) was used as a secondary antibody at a 1:1000 dilution. TBS was used as a negative control. Color reactions were read at 405nm. The data was plotted to determine the concentration of competitor needed to achieve 50% inhibition of monoclonal antibody binding. Positive controls of uninhibited BT/3B1 and BTaL/1 were used. TBS was used as a negative control.

Immunizations (Rabbits)

Injection schedule. Three adult rabbits designated ECR-49,-50, and -51 were each given initial intramuscular injections (i.m.) of 1mg of immunogen in Freund's complete adjuvant. ECR 49 received intact M467 conjugated to key-hole limpet hemocyanin (KLH). ECR 50 received reduced and alkylated M467, and ECR 51 received purified M467 L-chain. Each rabbit was given a subsequent 1mg i.m. injection in Freund's incomplete adjuvant one week later. A third injection of 500ug of immunogen was given subcutaneously (s.q.) in saline one week later. Four days after the third injection, each rabbit was test bled from a marginal ear vein.

Each rabbit serum was tested for anti-M467 activity using Ouchterlony analysis as previously described. A pattern of test wells was produced in a gel, and M467 was loaded into the center well. Opposing wells were loaded with each of the rabbit antisera. The gel was incubated and inspected for lines of precipitation.

Bi-weekly s.q. injections of 100ug of immunogen were continued in each rabbit with periodic bleeding for serum collection. The immunizations were carried out for a total of four months. A final s.q. boost of 500ug of immunogen was given to each rabbit in saline. Four days after the injections, the rabbits were sacrificed, under anesthesia, using a femoral catheterization. The serum was collected for purification and analysis.

Polyvalent Antisera Purification and Analysis

Purification. The sera collected from ECR-49,-50, and -51 were purified by a 50% ammonium sulfate precipitation as described above. The precipitated immunoglobulin fractions were further purified using Protein A affinity chromatography.

A Protein-A column was prepared as described above and polyclonal antisera were loaded onto the gel. After incubation, the column was washed with phosphate buffer (start buffer) until the optical density (O.D.) had reached baseline. The anti-M467 IgG was then eluted using glycine buffer (elution buffer). The eluted fractions were neutralized and dialyzed into TBS for final purification.

Final purification of the rabbit antisera was performed by adsorption affinity chromatography. The CH-sepharose 4-B (Pharmacia) affinity column, with TEPC-15 (IgA,k) and MOPC 315 (IgA,λ) as ligands, was utilized following the same procedure used for final purification of monoclonal antibodies described above. Rabbit antisera was loaded onto the column and incubated at room temperature for 1 hr. The column was then washed with start buffer (TBS), and the O.D. monitored at 280nm. The effluent containing anti-idiotypic polyclonal antisera was collected until the O.D. reached baseline. The anti-isotypic and anti-allotypic antibodies were then eluted from the column using elution buffer (glycine buffer). The column was reequili-

brated into start buffer.

Purity of the polyclonal antibodies was tested using immunoelectrophoresis. Patterns were produced in three gels and purified antibodies from ECR-49,-50, and -51 were loaded into each of the respective wells. The gels were electrophoresed as described above and the three troughs were loaded with goat anti-whole rabbit serum. The gels were incubated and inspected for lines of precipitation.

Polyvalent antibody specificity. Polyvalent antibody titers were determined using the passive hemagglutination assay described by Stein and Soderstom (1984) with modifications. The following reagents were used:

Coupling Agent - 37.5mM $CrCl_3$ (Fisher)

Saline - 0.15M NaCl (Fisher)

SRBC - Sheep Red Blood Cells in Alsevers solution (Becton, Dickinson and Co., Cockeysvile, MD)

Diluent - Balanced Salt Solution (0.18M NaCl, 5mM KCl, 0.7mM Na_2HPO_4, 5.5mM dextrose, 0.02M Tris, 0.6mM $CaCl_2$, 5mM $MgCl_2$, pH 7.4)

FCS - Fetal Calf Serum (Collaborative Research)

The SRBC`s were washed 3x in saline and resuspended to 10% SRBC´s in saline. A 2ml sample of 10% SRBC`s was placed into a 15ml conical centrifuge tube along with 400ug of M467. The mixture was vortexed and 0.1ml of coupling agent was added. The mixture was vortexed and incubated at room temperature for 20 min. The coupled cells were washed once

with saline containing 5% FCS. The cells were then washed 2x with diluent containing 5% FCS. The cells were resuspended to 0.5% in diluent with 5% FCS for microtitration.

A 96-well Cooke microtitration plate (Dynatech Laboratories, Alexandria, VA) was loaded with 25ul/well of diluent. Purified antibody from ECR-49,-50, and -51 were loaded into the first well of three separate rows in a volume of 25ul/well. Serial twofold dilutions were carried out on each sample with final dilutions ranging from 1:2 - 1:4096. M467-coupled SRBC's were added to each dilution well in a volume of 25ul/well. Three wells containing diluent and SRBC's, but no antisera were used as negative controls. The plate was incubated at 37°C for 45 min, then allowed to incubate 10 min at room temperature before being inspected for agglutination.

Polyvalent Anti-idiotypic Specificity. Idiotypic specificity of purified antibody from ECR-49,-50, and -51 was tested using a competitive ELISA as described above. M467 was coated onto a microtiter plate at a concentration of 10ng/well. ECR-49,-50, and -51 were used as primary antibodies at a concentration of 10ug/well (1:1000 dilution). Eighteen different mouse myeloma proteins, in clarified ascites form, were used as competitors at a 1:10 dilution. M467 L-chain was also used as a competitor at a concentration of 100ug/well. The competitors were added to the wells simultaneously with the primary antibodies.

Biotin conjugated goat anti-rabbit IgG (Cooper) was used as a secondary antibody at a 1:1000 dilution. TBS was used as a negative control. Color reactions were read at 405nm.

RESULTS

Purification of M467 Protein

Clarified Ascites Containing M467. An immunoelectrophoresis assay of the clarified ascites fluid was used to test for the presence of M467 (Fig. 1). The IgA protein band was present along with several other serum protein bands, including IgG and albumin, when electro- phoresed against rabbit anti-whole mouse serum.

Affinity Chromatography Purification. M467 eluted from a CH-sepharose affinity column conjugated to *Salmonella spp.* flagellin was tested for purity using an immunoelectrophoresis assay. The IgA band was observed along with one other anodal serum protein band, thought to be albumin (Fig. 1).

Agarose Gel Purified Protein. Final purification of M467 was carried out on agarose gel. An immunoelectrophoresis assay was used to test final purity. The IgA band, minus any other serum proteins, was observed (Fig. 2).

Purified L-chain. M467 L-chain collected by polyacrylamide gel electroelution (Fig. 3) and AH-sepharose affinity chromatography was tested for purity using double diffusion in agarose gel (Ouchterlony). As seen in Figure 4, reduced and alkylated M467 exhibited lines of precipitation

between the sample well and the wells containing rabbit anti-M467 and alpha chain specific goat anti-mouse IgA. Purified L-chain exhibited lines of precipitation only between the L-chain sample well and the well containing rabbit anti-M467. No lines of pre- cipitation were observed between the L-chain sample well and the well containing alpha chain specific goat anti- mouse IgA. These results indicate the L-chain purity needed for immunizations and final inhibition analysis.

Anti-Idiotypic Polyvalent Antisera

Purification of Polyvalent Antisera. Following ammonium sulfate precipitation, Protein-A affinity column purification, and immunoglobulin adsorption, the three polyvalent rabbit antisera were tested for purity. ECR-49, prepared against KLH-conjugated intact M467, ECR-50, prepared against reduced and alkylated M467, and ECR-51, prepared against purified M467 L-chain, were tested for purity using immunoelectrophoresis. Single IgG precipi- tation lines were observed for all three polyvalent antisera, when electrophoresed against goat anti-whole rabbit serum (Figs. 11, 12, 13).

Specificity of Polyvalent Antisera. M467 idiotypic specificity of ECR-49, -50, and -51 was confirmed using a competitive ELISA. Eighteen mouse myeloma proteins of different immunoglobulin subclass and light chain types

were used as competitors along with purified M467 L-chain (Table 3). A t-Test performed on the mean optical densities of the test wells compared to the negative controls indicated a significant difference at the 5% significance level for all wells except those where M467 L-chain was the competitor. The fact that none of the eighteen myelomas successfully competed with either of the three polyvalent antisera, suggested M467 specificity of the antisera. These results coupled with the fact that M467 L-chain caused 100% inhibition, indicated that ECR-49, -50, and -51 polyvalent antisera were specific for the M467 idiotype, located on the M467 light chain.

Anti-Idiotypic Monoclonal Antibodies

Hybridoma Production. Two weeks after fusion, anti-M467 and anti-M467 L-chain culture plates were inspected for fused cells. Approximately 80% of the wells for both fusions exhibited proliferating hybrid cells, in the presence of selective AAT media. Wells from both fusions showing cell proliferation were tested for the production of anti-M467 antibody using an ELISA described in Materials and Methods. Over 90% of the wells tested were producing anti-M467 activity.

Anti-M467 Monoclonal Antibody. Cloned hybridoma cells from both anti-M467 and anti-M467 L-chain cell lines, showing anti-M467 activity, were tested for monoclonality

using a clonotyping ELISA. Five clones from each cell line were chosen for testing. The immunoglobulin subclasses produced by the cells are shown in Tables 1 and 2. The presence of the predominant IgG_3 kappa-chain subclass, produced by the majority of the clones tested, is similar to the results reported by Nahm, et al., 1982. Other immunoglobulin subclasses observed from the cell lines tested included IgG_{2A}, IgG_{2B}, and IgM in the anti-M467 cells, and IgG_1 and IgG_{2A} in the anti-M467 L-chain cells. The additional subclasses seen in the two fusion cell lines were present in combination with other subclasses indicating their lack of true monoclonality. They were cryopreserved for (possible) future cloning experiments. One IgG_3 producing, true monoclonal cell line, from each fusion was chosen for final analysis. The clones were designated $BT/3B_1$ for the anti-M467 cell line and BTaL/1 for the anti-M467 L-chain cell line.

Purification of Monoclonal Antibodies. Purification of $BT/3B_1$ and BTaL/1 was verified using immunoelectrophoresis and Ouchterlony analysis. Immunoelectrophoresis resulted in a single line of precipitation against goat anti-whole mouse and goat anti-mouse IgG_3 for both monoclonal antibodies (Figs. 5 and 6). Ouchterlony analysis resulted in a single line of precipitation against goat anti-mouse IgG_3 for both monoclonal antibodies. No precipitation was seen against the other anti-mouse immunoglobulin subclasses (Figs. 7 and 8). These

results coupled with the immunoelectrophoresis results indicated monoclonal antibody purity.

Specificity of Monoclonal Antibodies. A competitive ELISA was performed on both BT/$3B_1$ and BTaL/1 antibodies to confirm idiotypic specificity. M467 L-chain was used as the competitor for both ELISA's and the inhibition curves are shown in Figures 9 and 10. As shown in Figure 9, the concentration of M467 L-chain needed for 50% inhibition of 100ng BT/$3B_1$ was 700ng/0.1ml. The concentration of L-chain needed for 50% inhibition of 100ng BTaL/1 was 500ng/0.1ml as shown in Figure 10. These results suggest that BT/$3B_1$ and BTaL/1 monoclonal antibodies are specific for the M467 idiotype, located on the M467 light chain.

M467 Idiotope Location. An experiment was performed, using a competitive ELISA, to determine whether or not the M467 idiotope was located within the immunoglobulin (IgA) binding site. An ELISA plate was prepared identical to the one used in monoclonal antibody specificity testing, described in Materials and Methods. BT/$3B_1$ and BTaL/1 were added to wells at a concentration of 100ng/well. The competitor used, instead of M467 L-chain, was an M467-*Salmonella spp.* flagellin polymer (POL) complex. M467, which is specific for POL (Smith and Potter, 1976), was incubated with excess POL for 30 min at 37° C, prior to addition to ELISA test wells. The competitor complex was added to the test wells at a concentration of 10ug M467-POL/well. Negative control wells of 100ng BT/$3B_1$ or

BTaL/1 per well plus 10ug unbound M467/well were also prepared. Biotin conjugated goat anti-mouse IgG was used as secondary antibody as previously described. $BT/3B_1$ experimental wells showed a mean optical density value of 0.97 at 405nm compared to a negative control value of 0.42. A t-Test performed on the data indicated a significant difference between the experimental wells and the control wells at the ($p<.05$) significance level. BTaL/1 experimental wells showed a mean optical density value of 0.98 at 405nm compared to a negative control value of 0.41. A t-Test again indicated a significant difference between the experimental wells and the negative control wells at the ($p<.05$) significance level. The fact that the competitor, M467 with its binding site blocked by POL, did not successfully compete with either $BT/3B_1$ or BTaL/1 for the M467 coated test wells, suggested that the M467 idiotope was located inside the binding site.

Figure 1. Immunoelectrophoresis of clarified M467 ascites fluid.

Well (A) contains ammonium sulfate precipitated M467, and well (B) contains affinity purified M467 with albumin in anodal region of gel. Trough 1 contains rabbit anti-whole mouse serum. Trough 2 contains rabbit anti-M467. Electrophoresis was carried out as described in "Materials and Methods".


A
1
B
2

Figure 2. **Immunoelectrophoresis of agarose purified M467.** The sample well contains agarose purified M467 and the trough contains rabbit anti-whole mouse serum. Electrophoresis was carried out as described in "Materials and Methods".

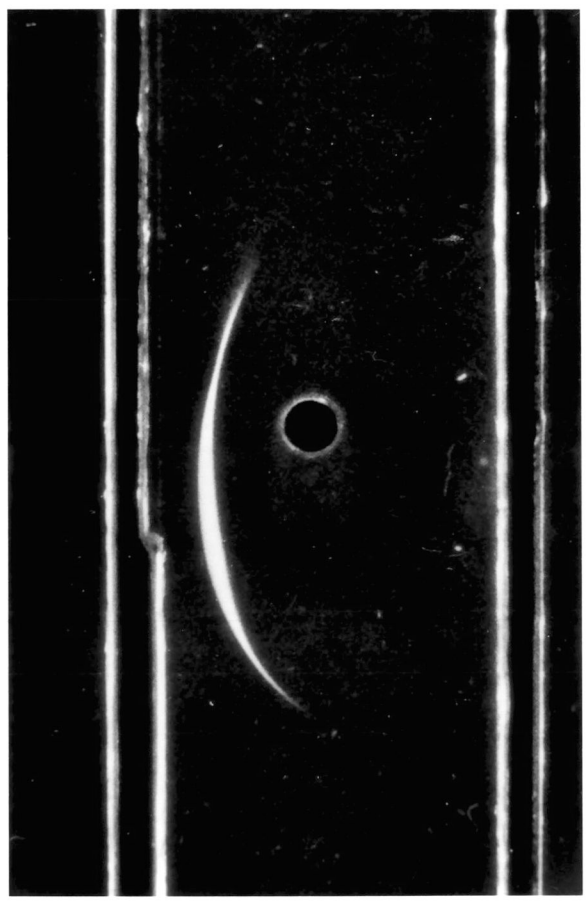

Figure 3. **Reduced and alkylated M467 on a 10% Laemmli polyacrylamide gel.**

Lane 1 contains prestained molecular weight (MW) markers. Lane 2 contains the M467 L-chain band (25,000 MW), which was cut from the gel and the L-chain electroeluted. Electrophoresis was carried out as described in "Materials and Methods".

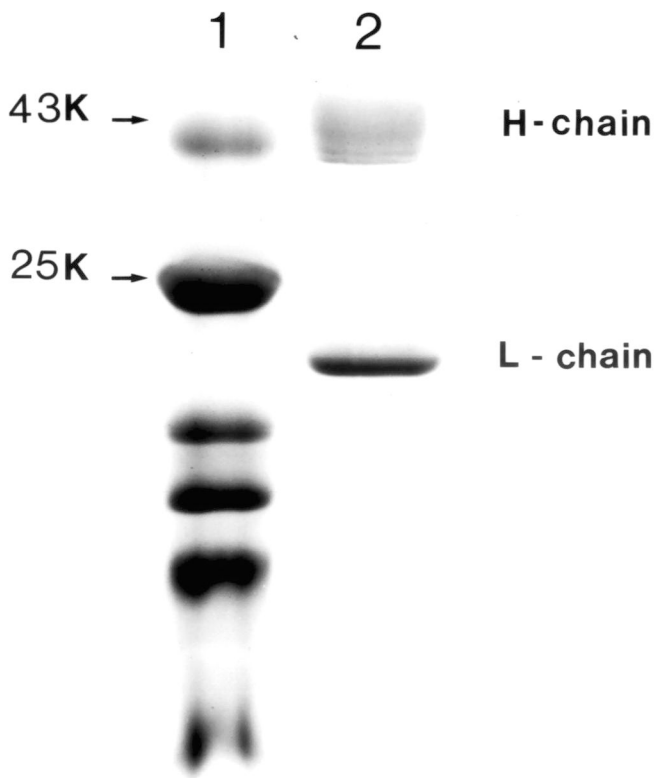
1
2
43K
25K
H-chain
L - chain

Figure 4. **Double diffusion in agarose gel of reduced and alkylated M467 and purified M467 L-chain.**

Pattern A contains reduced and alkylated M467 (A-1) against goat anti-mouse IgA, alpha chain specific (A-2) and rabbit anti-M467 (A-3). Pattern B conatains purified M467 L-chain (B-1) against goat anti-mouse IgA, alpha chain specific (B-2) and rabbit anti-M467 (B-3). The absence of precipitation between wells B-1, purified L-chain, and B-2, heavy chain specific antisera, indicate M467 L-chain purity. Diffusion was carried out as described in "Materials and Methods".


3
2
1
A
3
2
1
B

Figure 5. **Immunoelectrophoresis of purified BT/$3B_1$.** The sample well contains purified BT/$3B_1$. Trough 1 contains goat anti-mouse IgG_3, and trough 2 contains goat anti-whole mouse serum. Electrophoresis was carried out as described in "Materials and Methods".

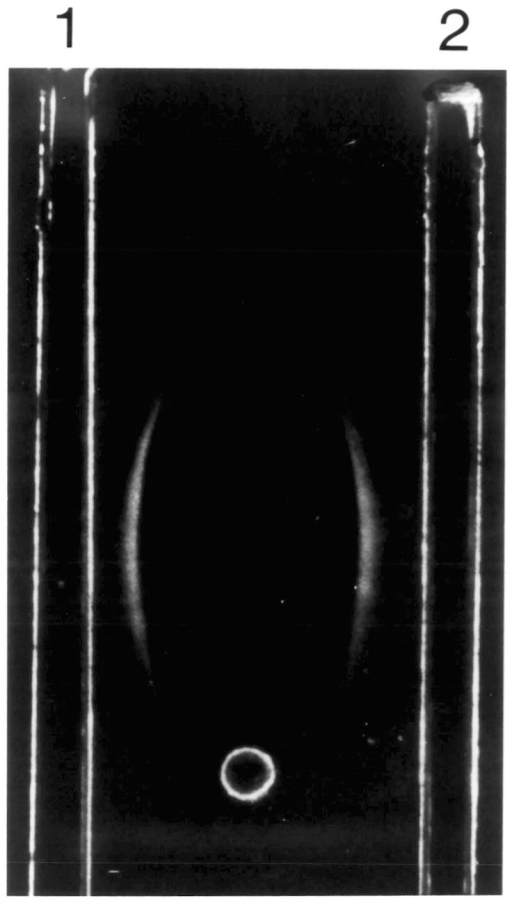
1
2

Figure 6. Immunoelectrophoresis of purified BTaL/1.

The sample well contains purified BTaL/1. Trough 1 contains goat anti-mouse IgG_3, and trough 2 contains goat anti-whole mouse serum. Electrophoresis was carried out as described in "Materials and Methods".

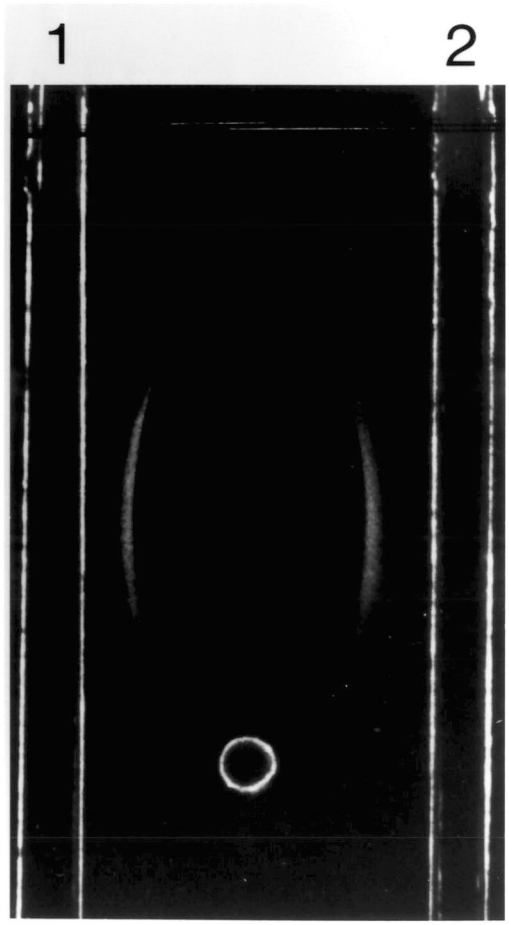
1
2

Figure 7. **Clonotyping by double diffusion in agarose gel of purified BT/3B$_1$.**

The center well contains purified BT/3B$_1$. The surrounding wells contain goat anti-mouse IgG$_1$ (1), IgG$_{2a}$ (2), IgG$_{2b}$ (3), IgG$_3$ (4), and IgM (5), respectively. Diffusion was carried out as described in "Materials and Methods".

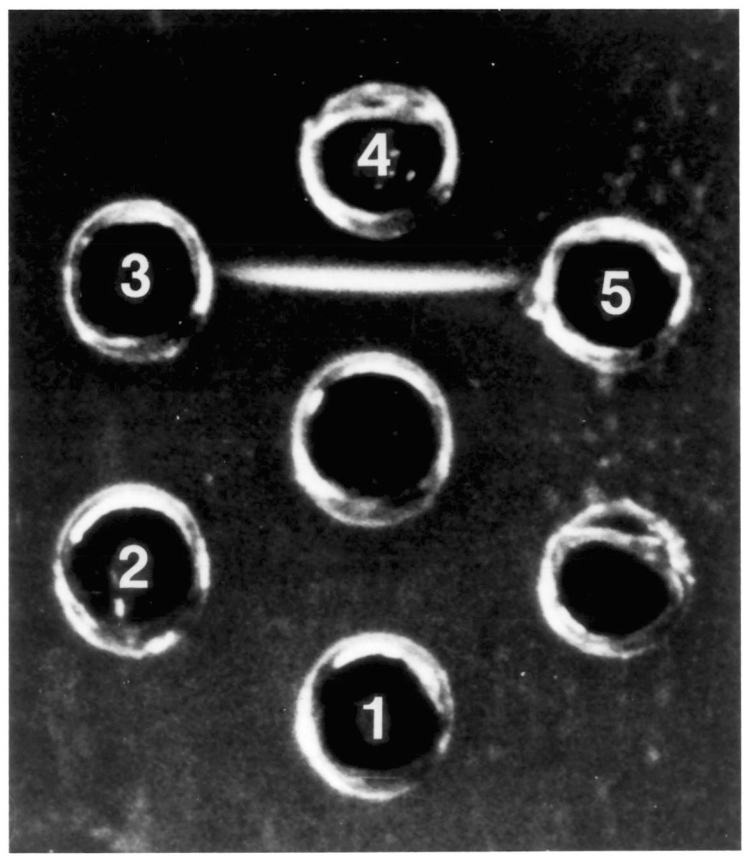
4
3
5
2
1

Figure 8. **Clonotyping by double diffusion in agarose gel of purified BTaL/1.**

The center well contains purified BTaL/1. The surrounding wells contain goat anti-mouse IgG_1 (1), IgG_{2a} (2), IgG_{2b} (3), IgG_3 (4), and IgM (5), respectively. Diffusion was carried out as described in "Materials and Methods".

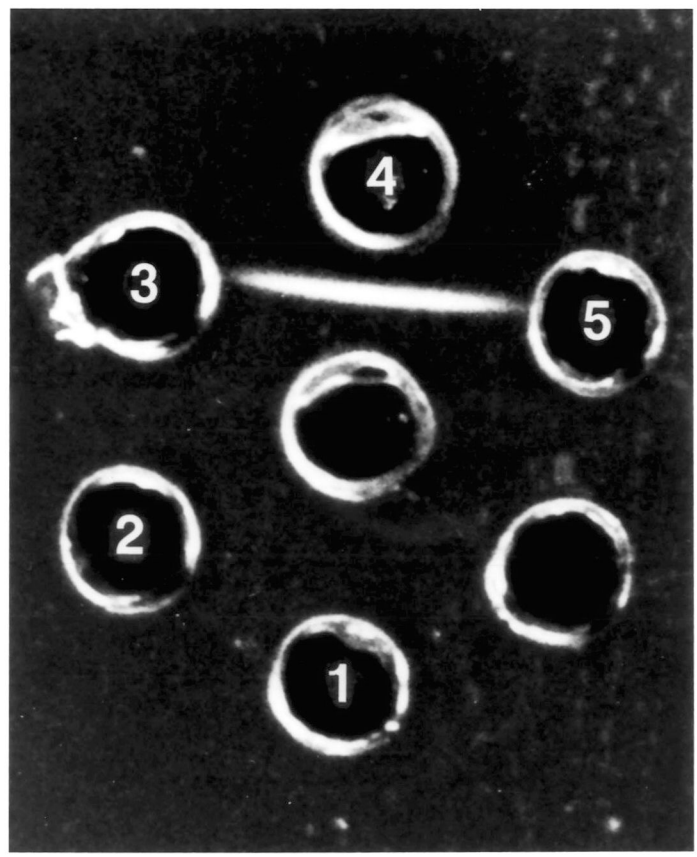
4
3
5
2
1

Figure 9. Competitive ELISA of BT/3B$_1$.

M467 was coated onto a microtiter plate at a concentration of 10ng/well. BT/3B$_1$ was added to test wells at a concentration of 100ng/well. Varying concentrations of purified M467 L-chain (competitor) were added to test wells ranging from 1ng - 100,000ng. Biotin conjugated goat anti-mouse IgG was diluted 1:1000 and added to the test wells. Negative controls of 100ul/well TBS and 100ul/well conpetitor at varying concentrations were used. Optical density at 405nm was used to determine the amount of M467 L-chain needed for 50% inhibition of BT/3B$_1$.

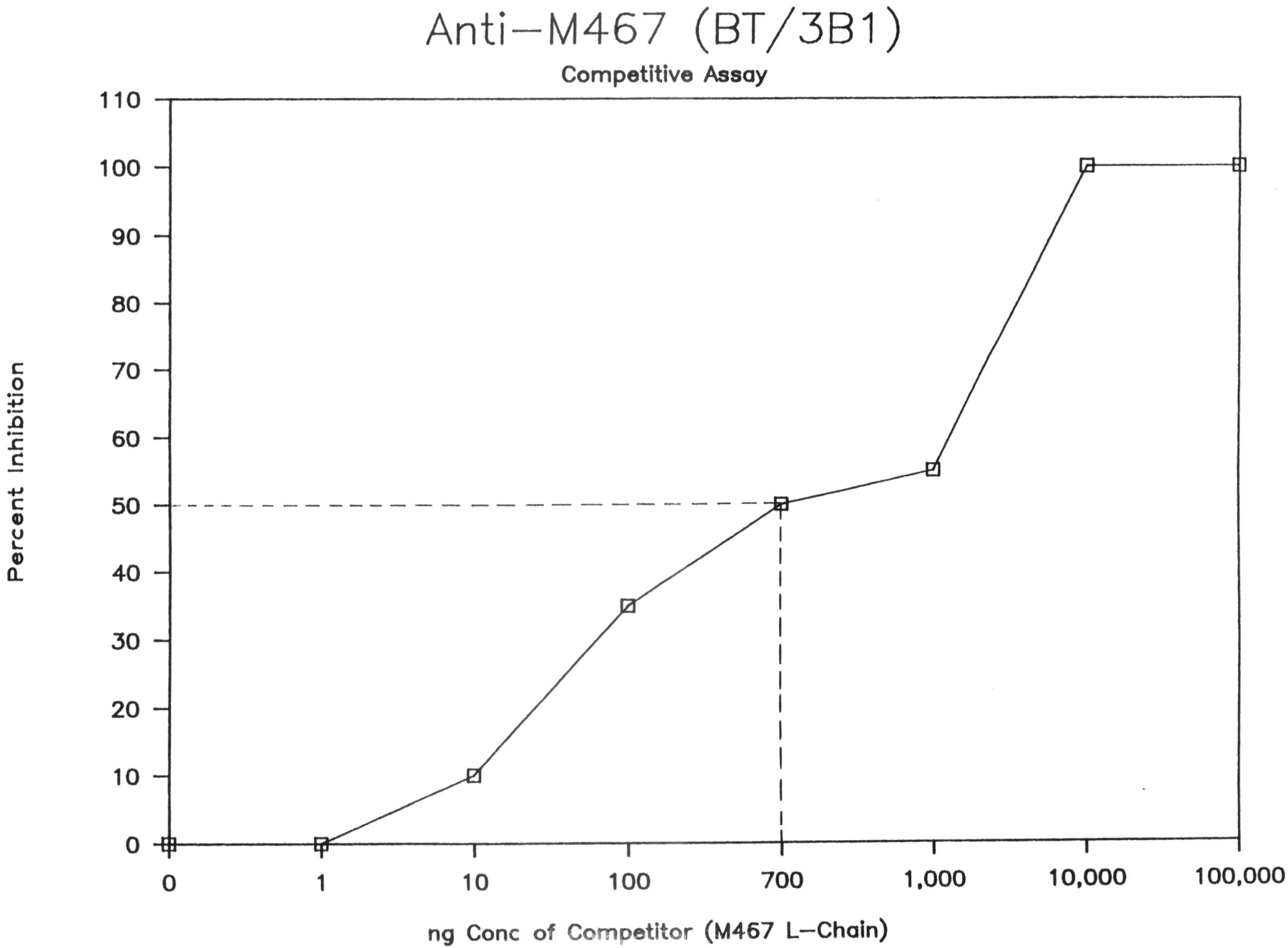
Anti–M467 (BT/3B1)
Competitive Assay
Percent Inhibition
110
100
90
80
70
60
50
40
30
20
10
0
0
1
10
100
700
1,000
10,000
100,000
ng Conc of Competitor (M467 L–Chain)

Figure 10. Competitive ELISA of BTaL/1.

M467 was coated onto a microtiter plate at a concentration of 10ng/well. BTaL/1 was added to test wells at a concentration of 100ng/well. Varying concentrations of purified M467 L-chain (competitor) were added to test wells ranging from 1ng - 100,000ng. Biotin conjugated goat anti-mouse IgG was diluted 1:1000 and added to the test wells. Negative controls of 100ul/well TBS and 100ul/well conpetitor at varying concentrations were used. Optical density at 405nm was used to determine the amount of M467 L-chain needed for 50% inhibition of BTaL/1.

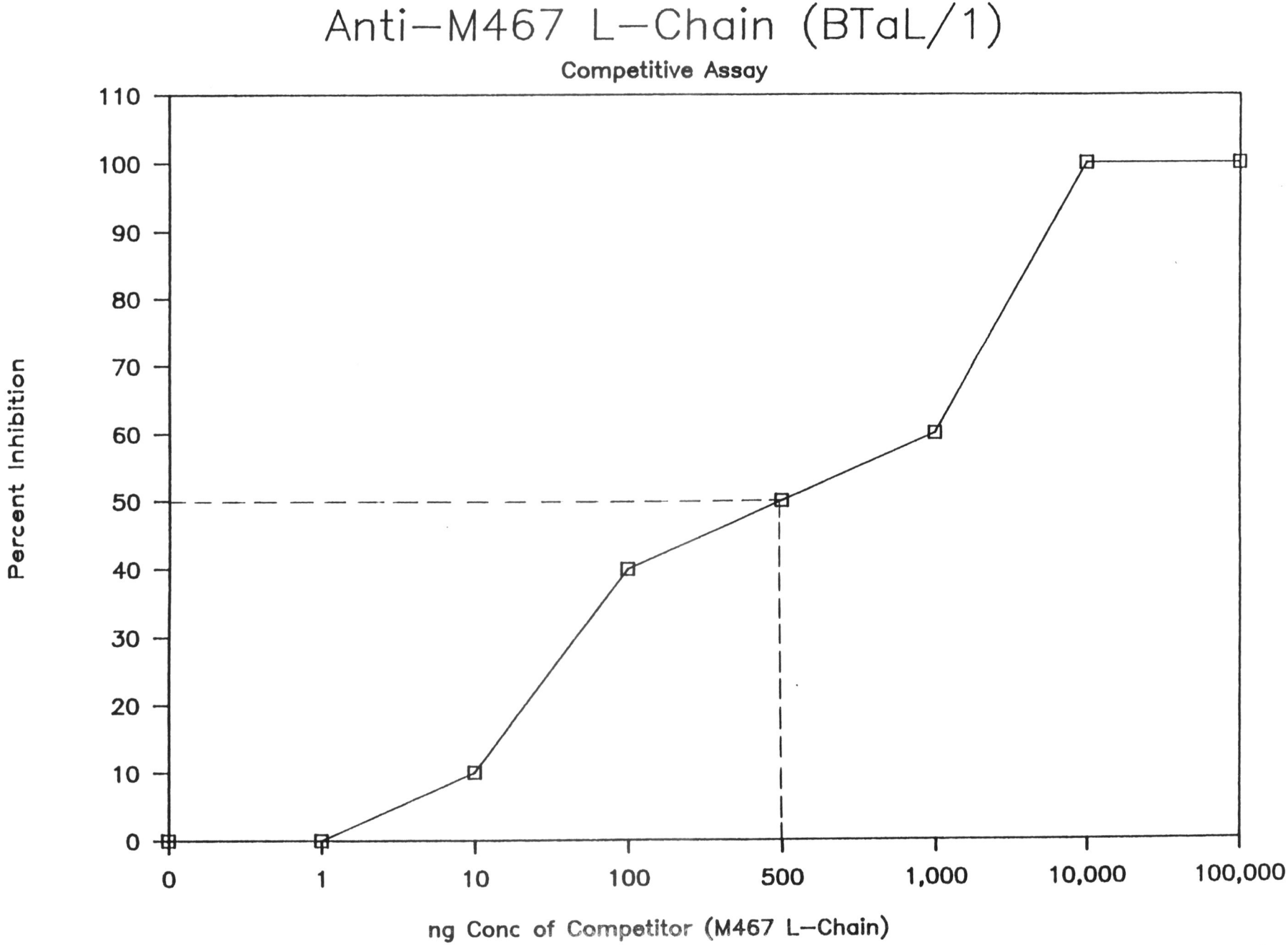
Anti–M467 L–Chain (BTaL/1)
Competitive Assay
Percent Inhibition
110
100
90
80
70
60
50
40
30
20
10
0
0
1
10
100
500
1,000
10,000
100,000
ng Conc of Competitor (M467 L–Chain)

Figure 11. Immunoelectrophoresis of purified ECR 49.

The sample well contains purified ECR 49. The trough contains goat anti-whole rabbit serum. Electrophoresis was carried out as described in "Materials and Methods". ECR 49 was immunized with KLH-conjugated intact M467.

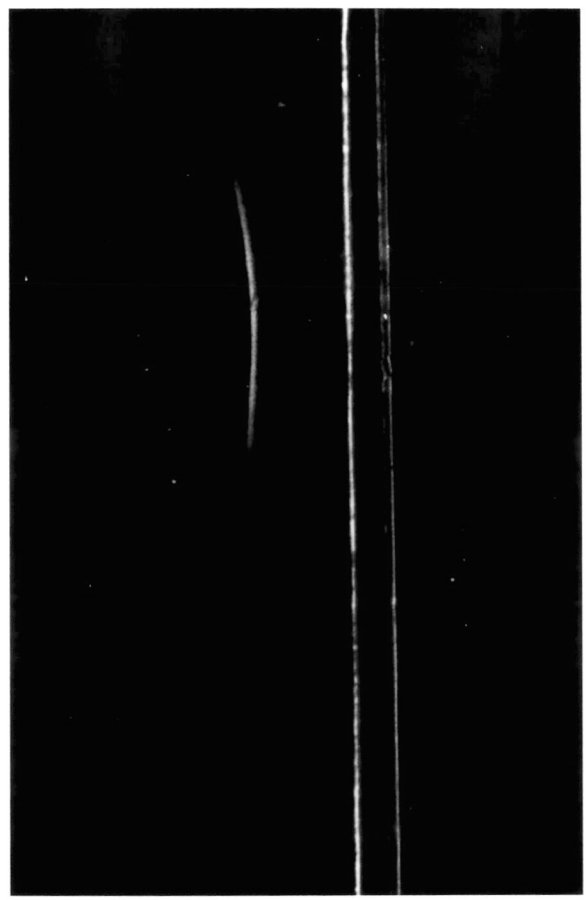

Figure 12. Immunoelectrophoresis of purified ECR 50.

The sample well contains purified ECR 50. The trough contains goat anti-whole rabbit serum. Electrophoresis was carried out as described in "Materials and Methods". ECR 50 was immunized with reduced and alkylated M467.

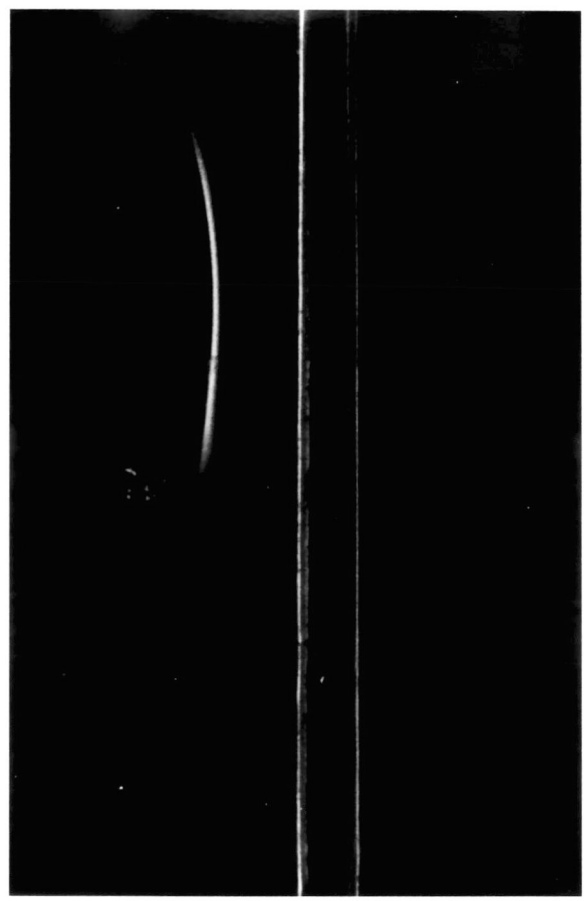

Figure 13. Immunoelectrophoresis of purified ECR 51.

The sample well contains purified ECR 51. The trough contains goat anti-whole rabbit serum. Electrophoresis was carried out as described in "Materials and Methods". ECR 51 was immunized with purified M467 L-chain.

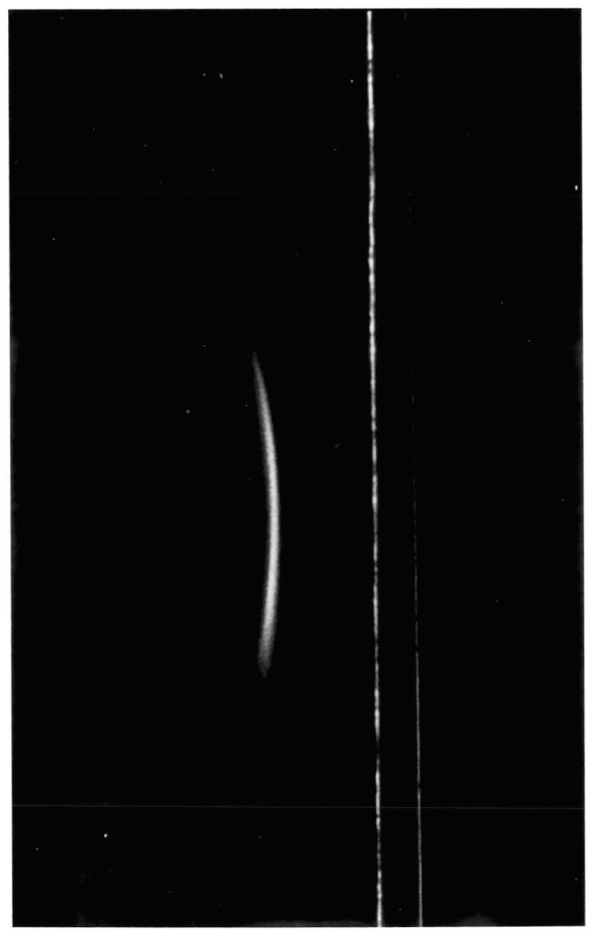

Table I
Anti-M467 Clonotype Assay

Cell Culture	Alkaline Phosphatase Labeled Antisera (Optical Density at 405nm)						
	anti-IgG1	anti-IgG2A	anti-IgG2B	anti-IgG3	anti-IgM	anti-kappa	anti-lambda
2A1	0.444	0.285	0.367	0.663	0.399	0.561	0.293
2A4	0.332	0.241	0.323	0.531	0.317	0.403	0.318
2B4	0.476	0.422	0.515	0.644	0.373	0.768	0.983
*3B1	0.233	0.219	0.261	0.578	0.249	0.465	0.218
4A1	0.418	0.957	0.411	1.135	0.503	0.701	0.751
Negative	0.250	0.248	0.251	0.253	0.250	0.249	0.251

Clonotype ELISA of anti-M467 clones. M467 was coated onto a microtiter plate at a concentration of 10ng/well. Culture supernatants containing anti-M467 antibody were added in a volume of 100ul/well. Alkaline phosphatase labeled antisera was diluted 1:250 and added in a volume of 100ul/well. Negative controls of 100ul/well culture media and 100ul/well labeled antisera were used. Cell clone *3B1, IgG3 kappa-chain, was chosen for final analysis and designated BT/3B1.

Table II
Anti-M467 L-Chain Clonotype Assay

Cell Culture	Alkaline Phosphatase Labeled Antisera (Optical Density at 405nm)						
	anti-IgG1	anti-IgG2A	anti-IgG2B	anti-IgG3	anti-IgM	anti-kappa	anti-lambda
9	0.570	0.500	0.350	0.880	0.300	0.425	0.575
10	0.519	0.454	0.399	0.481	0.424	0.628	0.496
11	0.452	0.560	0.400	0.480	0.350	0.511	0.286
12	0.705	0.638	0.405	0.710	0.403	0.558	0.489
*31	0.310	0.308	0.311	0.910	0.306	0.605	0.312
Negative	0.305	0.299	0.300	0.308	0.303	0.300	0.305

Clonotype ELISA of anti-M467 L-Chain clones. M467 was coated onto a microtiter plate at a concentration of 10ng/well. Culture supernatants containing anti-M467 antibody were added in a volume of 100ul/well. Alkaline phosphatase labeled antisera was diluted 1:250 and added in a volume of 100ul/well. Negative controls of 100ul/well culture media and 100ul/well labeled antisera were used. Cell clone *31, IgG3 kappa-chain, was chosen for final analysis and designated BTaL/1.

Table III
Polyvalent Antisera Competitive ELISA

			ECR 49			ECR 50			ECR 51		
Myeloma	Subclass	Light(a) Chain	Optical(b) Density	Standard Error	Percent Inhibition	Optical Density	Standard Error	Percent Inhibition	Optical Density	Standard Error	Percent Inhibition
MOPC 467 L-Chain	IgA	k	0.175	0.078	100.000	0.178	0.080	100.000	0.169	0.075	100.000
Pry 10	IgG1	k	0.434	0.194	0.392	0.437	0.195	1.145	0.424	0.189	0.000
MOPC 511	IgA	k	0.424	0.189	4.314	0.429	0.191	4.198	0.417	0.186	1.200
MCPC 870	IgA	k	0.425	0.190	3.922	0.430	0.192	3.817	0.412	0.184	3.200
Sana 368	IgA/G2b	k	0.436	0.195	0.000	0.448	0.200	0.000	0.429	0.191	0.000
XRPC 44	IgA	k	0.426	0.190	3.529	0.429	0.191	4.198	0.412	0.184	3.200
TEPC 817	IgA	k	0.427	0.185	3.137	0.431	0.192	3.435	0.424	0.189	0.000
TEPC 525	IgA	-	0.437	0.195	0.000	0.437	0.195	1.145	0.466	0.208	0.000
TEPC 821	IgA	-	0.428	0.190	2.745	0.438	0.195	0.763	0.427	0.190	0.000
MOPC 460	IgA	k	0.457	0.204	0.000	0.461	0.206	0.000	0.461	0.206	0.000
RPC 5	IgG2a	k	0.427	0.190	3.137	0.431	0.192	3.435	0.424	0.189	0.000
TEPC 173	IgA	k	0.454	0.198	0.000	0.500	0.223	0.000	0.464	0.205	0.000
CBPC 112	IgM	k	0.431	0.192	1.569	0.443	0.198	0.000	0.428	0.191	0.000
TEPC 105	IgA	k	0.434	0.194	0.392	0.437	0.195	1.145	0.423	0.188	0.000
TEPC 119	IgA	-	0.432	0.193	1.176	0.433	0.193	2.672	0.416	0.186	1.600
J606	IgG3	k	0.435	0.194	0.000	0.446	0.199	0.000	0.431	0.193	0.000
UPC 10	IgG2a	k	0.429	0.191	2.353	0.434	0.193	2.290	0.420	0.188	0.000
MOPC 104E	IgM	lambda	0.433	0.193	0.784	0.437	0.195	1.145	0.430	0.192	0.000
Positive	-	-	0.435	0.200	0.000	0.440	0.199	0.000	0.420	0.187	0.000
Negative	-	-	0.180	0.081	100.000	0.178	0.079	100.000	0.170	0.075	100.000

Competitive ELISA of polyvalent antisera. M467 was coated onto a microtiter plate at a concentration of 10ng/well. ECR-49, -50, and -51 were diluted 1:1000 (100ug/ml) and added to test wells in a volume of 100ul/well. Competitive myeloma proteins, which were in the form of clarified ascites fluid, were diluted 1:10 and added to the test wells in a volume of 100ul/well. Purified M467 L-chain (1mg/ml) was added to other test wells in a volume of 100ul/well. Biotin conjugated goat anti-rabbit IgG was diluted 1:1000 and added in a volume of 100ul/well. Positive controls of 100ul/well rabbit antisera, 100ul/well TBS, and 100ul/well biotin conjugated antibody were used. Negative controls of 100ul/well TBS, 100ul/well competitive myeloma, and 100ul/well biotin conjugated antibody were used. Optical densities reported for negative controls are mean values of 18 myeloma test wells for each of the three antisera.
(a) - Light chain type (k = kappa, lambda)
(b) - Mean optical density at 405nm of 5 test wells

DISCUSSION

The development of anti-idiotypic antisera has been studied for over a decade. Xenogeneic, allogeneic and syngeneic anti-idiotypic antisera have been researched with varying results. Lieberman and Humphrey (1972), using BALB/c myelomas to immunize different strains of mice, noted that when allotypic determinants were the same for the myeloma protein immunogen and the strain immunized, no antibody was produced against the idiotype. However, when two to three allotypic determinants were similar to those present on the BALB/c myeloma proteins used for immunization, antibody specificities were directed only toward the idiotype. Sakato and Eisen (1975) reported the production of syngeneic anti-idiotypes in BALB/c mice against four out of five BALB/c myelomas used as immunogens. Davie (1980) has reported that while the production of syngeneic anti-idiotypic antiserum is certainly possible, its success is limited by the fact that relatively small quantities of antibodies are achieved with isologous immunizations. The most common approach to the production of anti-idiotypic antiserum has been to generate xenotypic or allotypic antisera to a homogeneous immunogen, and then to absorb the antisera with normal immunoglobulins to remove anti-isotypes and anti-allotypes leaving only anti-idiotypes. This method has been very successful and is the method which was used in our study for both the polyvalent

and monoclonal antisera.

We have presented data to show the production of both polyvalent and monoclonal antibodies directed against the idiotypic marker, located on the light chain of a mouse myeloma IgA protein. The specificities for the M467 idiotope of both antisera have been shown using inhibition studies. These inhibition studies have also shown that the expression of the M467 idiotype is independent of heavy and light chain association. The fact that the M467 IgA light chain can be completely dissociated from the heavy chain and still successfully compete for an anti-idiotype, which was generated against intact M467, suggests that idiotypic expression of M467 is localized on the light chain. Failure of M467, with its binding site blocked by flagellin polymer, to compete for anti-M467 idiotype suggests that the idiotope is not only located on the light chain, but is also located within the M467 paratope. These findings suggest that the M467 idiotype may be highly conserved in the germ line of the mouse, because it is apparently a group of amino acid peptides on the IgA light chain which is not associated with the three dimensional structure of the binding site.

The knowledge gained from the study of idiotypes through the use of anti-idiotypic reagents, such as the ones presented here, has become increasingly valuable to medical science. Two of the functions of idiotypes and anti-idiotypes which have received a great deal of recent

attention are their importance as immunoregulators in the immune system and their use as surrogate antigens for vaccines.

Anti-idiotypic antibodies and their roles in immunoregulation have been studied in several animal systems, and have been found to be involved in both helper and suppressor activities. McNamara and Kohler (1984) induced the production in mice, of T-helper cells which recognized two mouse IgA myeloma proteins, by injecting free light chains of anti-idiotypic hybridoma antibody. Milburn and Lynch (1983) studied the in vitro effect of anti-idiotypic antibodies and anti-idiotypic T-cells on the expression of MOPC 315, a mouse myeloma IgA protein. Evidence was presented showing that M315 anti-idiotypic antibodies eliminated cell surface membrane M315, but did not influence M315 secretion. In contrast, idiotype-specific T-cells caused inhibition of M315 secretion without influencing surface M315 expression. These observation supported a view that regulatory effects of anti-idiotypes are focused on specific molecules to elicit specific effects. Saito et al. (1986) induced chronic suppression of idiotypically defined anti-(4-hydroxy-3-nitrophenyl)-acetyl (NP) antibody using neonatal administration of isologous monoclonal anti-idiotypic antibodies. The suppression inducing anti-idiotopes were specific for the anti-NP variable region. Campa et al. (1986) showed anti-idiotypic regulation of B- and T-cells.

by injecting mycobacteria into mice via different pathways. One set of mice was injected subcutaneously (s.q.) with mycobacteria and another set was injected intravenously (i.v.) with an equal dose of mycobacteria. Mice receiving s.q. mycobacteria injections produced a specific delayed type hypersensitivity response whereas the mice receiving i.v. injections of mycobacteria did not. It was determined that anti-idiotypic B-cells specific for existing idiotype positive B-cells were produced 4 days after the i.v. injections. These anti-idiotypic B-cells activated antigen specific suppressor T-cells affecting the induction of the delayed type hypersensitivity response. These results indicated the presence of an immunosuppressive circuit by which mycobacterium may interfere with the host's immune response during early stages of infection.

Idiotope-bearing polypeptides, in addition to anti-idiotype reagents have been shown to have immunoregulatory effects. Sakato et al. (1986) induced suppressor T-cells in mice that blocked a delayed type hypersensitivity response to the M315 (IgA) idiotype. The suppression was induced by injecting the M315 lambda light chain into mice. Injection of M315 heavy chain failed to block the delayed type hypersensitivity response. These results agreed with the earlier work of Sakato et al. (1982). Bogen et al. (1985), and Hannestad et al. (1986) reported the production of T-helper cells specific for the M315

L-chain idiotope. Results in both cases showed the high specificity of the T-helper cells induced. Although tested with other light chains differing only in a small number of amino acids from M315, neither paper reported cross-reactivity of the T-helper cells. Cerny et al. (1986) immunized mice with Streptococcus pneumoniae and charted the T-cell activity toward the dominant idiotype present in the immune response. It was determined that idiotype specific T-cells were present throughout the immune response, however their effector functions varied. Anti-idiotypic T-cells were found to act as both suppressor and helper cells depending on the stage of the immune response. These results provide evidence for the importance of idiotypes and anti-idiotypes in immunoregulation.

Another important and increasingly promising function of anti-idiotypes is their use as surrogate antigens for vaccines. Anti-idiotypic antibodies, specific for the idiotype on antibodies which react with a particular antigen, have been found to act as "internal images" of the original antigen (Nisonoff and Lamoyi, 1981). Sege and Peterson (1978) found that anti-idiotypic antibodies prepared against anti-bovine-insulin antibodies mimicked the action of insulin, in that they were able to interact with insulin receptors on tissues, as well as stimulate the physiological action of insulin itself.

Roitt et al. (1985) proposed three ways by which an

anti-idiotype might act as an internal image of the original antigen. First, there is the possibility of a true internal image of the antigen through identical amino acid sequences in the hypervariable region of the anti-idiotype which are physically analogous to the epitope of the antigen. Second, there is the possibility of internal imagery through identical amino acid contact residues between the anti-idiotype and the antigen, while at the same time being physically different from each other. Finally, there is the possiblilty of "obligatory" non-binding site, or non-paratopic idiotypes. Here, a structure eliciting an anti-idiotypic response may be a consistent or obligatory feature of antibodies within a given specificity even though it is non-paratopic, i.e. does not make contact with the antigen. An anti-idiotype directed to this obligatory structure will be combining with the same set of antibodies as would the antigen itself, and therefore would behave as a surrogate antigen. To date, a number of studies have been done to test the effectiveness of anti-idiotypes as vaccines against infection in several animal systems. Stein and Soderstrom (1984) reported protection in mice against E. coli infection by neonatal injection of a monoclonal mouse anti-idiotype. Mice were shown to have protection when challenged with E. coli at 5 weeks of age. McNamara et al. (1984) likewise showed protection in mice against a lethal Streptococcus pneumoniae infection when previously

vaccinated with a monoclonal anti-idiotope coupled to a carrier protein.

More recently, anti-idiotypic reagents have been shown to provide protection against parasitic infections when used as vaccines. Schistosomiasis is a parasitic infection of man which is widespread in tropical countries, and which so far has resisted attempts at control. Grzych et al. (1985) used a rat monoclonal antibody active against Schistosoma mansoni to produce monoclonal anti-idiotypic antibodies in rats. These anti-idiotypic antibodies were used as vaccines for a third set of rats. Results indicated 50 - 80% protection in immunized rats to a Schistosoma challenge.

Protection against viral infections, using anti-idiotypic antibodies as surrogate antigens, has also been reported by Kennedy et al., (1986). Anti-idiotypic antiserum against hepatitis B active rabbit antibodies were produced in rabbits and used to immunize both rabbits and chimpanzees. The immunizations resulted in protection of both rabbits and chimpanzees against hepatitis B infection. These results suggest that genetic restrictions associated with the induction of an interspecies immune response may not be a limitation of anti-idiotype based vaccines. They also demonstrate a viable alternative approach to vaccination against viral agents such as hepatitis B which can cause human disease. Gaulton et al. (1986) used mouse anti-reovirus type 3 antisera to induce

an anti-idiotypic syngeneic response in mice. A BALB/c monoclonal IgM anti-idiotope was used to vaccinate unimmunized mice. Although a lengthy immunization protocol was necessary, the authors reported viral neutralizing antibodies in the mice at levels comparable to those obtained after inoculation with the virus.

Another important possibility for the use of idiotypes and anti-idiotypes is cancer research and treatment. Myumi et al. (1982) used a monoclonal anti-idiotypic antibody to study the clonal origin of human B cell leukemia, and to make a postulation about malignant transformation of B cell clones. Stevenson et al. (1984) used monoclonal anti-idiotypic antibodies directed against neoplastic lymphocytes to enhance the removal of cancer cells. They found that by linking the Fab' regions of the monoclonal anti-idiotypes to normal immunoglobulin prior to injection, the ability of the complex to invoke complement and natural killer cell killing of target lymphocytes was far superior to that of parent antibody. More recently, Raychaudhuri et al. (1986) used monoclonal antibodies active against tumor-associated antigens of a mouse lymphoma subline to make hybridoma anti-idiotypic antibodies. The monoclonal anti-idiotypes, when used to immunize mice, elicited a delayed-type hypersensitivity reaction against tumor challenge. As with viral and bacterial internal image anti-idiotypes described above, these results represent the successful passive transfer of protection against tumor challenge

using anti-idiotypes. Dunn et al. (1987) used rat monoclonal anti-idiotypic antibodies directed against tumor reactive rat monoclonal antibodies for protective immunization. Rats receiving the anti-idiotype showed reduced tumor production following intravenous challenge with tumor cells. The anti-M467 idiotypic antisera produced in our study has the potential for use in cancer research both as an enhancer to cancer cell removal through the linkage of the anti-idiotype to normal immunoglobulin prior to injection, as well as passive transfer of protection against tumor challenge through internal imagery.

Idiotypes have also recently been shown to be effective in providing resistance to tumor challenge. Kaminski et al. (1987) used an idiotype derived from a mouse B cell lymphoma to study the induction of idiotype specific tumor immunity. Mice were immunized with the idiotype protein coupled to keyhole limpet hemacyanin. The resulting anti-idiotypic antibodies produced by the host induced a state of resistance to tumor growth. It was noted, however, that while tumor immunity could be induced in animals with pre-existent serum idiotype protein, such as that found in tumor bearing mice, the immunity was inhibited in a dose-dependent manner.

The monoclonal and polyvalent anti-idiotypic antibodies produced in our study, along with those produced in other studies have a very promising future in medical research.

Their applications in the study of immunoregulation, surrogate antigen vaccine, and cancer therapy are milestones in immunology.

One of the most obvious and important future studies using the anti-M467-idiotypic antibodies we have generated is to test their effectiveness as surrogate antigens for vaccine purposes. First, it is important to remember that the M467 IgA protein used in our study has been shown to react with many species of Salmonella. Using this knowledge, one could use the anti-idiotypic monoclonal and polyvalent antibodies, which may represent internal images of the Salmonella epitopes recognized by M467, and immunize both syngeneic and xenogeneic animals in an attempt to induce protection against Samonella infection without previous exposure to the original antigen. Our anti-idiotypic antisera could also be used to study the immunoregulatory effects that the M467 idiotype might have on the immune system of mice, as well as identify the possible location of the idiotope on other immunoglobulin proteins found in the mouse immune system. These studies will aid in our further understanding of the functions of idiotypes and anti-idiotypes in the imune system, and hopefully provide additional support for their use in human vaccine and cancer treatments.

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