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(54) Title: HUMAN MONOCLONAL ANTIBODIES BINDING DETERMINANTS OF GRAM NEGATIVE BACTERIA

(57) Abstract

The present invention provides novel methods for producing human monoclonal antibodies capable of binding an epitope on gram negative bacterial core lipopolysaccharide comprising the steps of (1) screening monoclonal antibodies secreted from transformed human cell lines by employing a competitive assay against non-human monoclonal antibodies capable of binding such an epitope and (2) selecting human cell lines which secrete monoclonal antibodies competitive with the non-human monoclonal antibodies. The present invention further contemplates monoclonal antibodies produced by those methods and the hybridoma cell lines producing such monoclonal antibodies. Preferably the human cell lines are derived from human B lymphocytes from a person hosting an infection of gram negative bacteria and are transformed with Epstein-Barr virus. Most preferably the Epstein-Barr virus is that released by B95-8 marmoset lymphoma cells having A.T.C.C. Accession No CRL 1612 and the non-human hybridoma cell line is selected from the group consisting of the cell lines designated A.T.C.C. Accession Nos HB 9081, HB 9082, HB 9083 and HB 8909. The monoclonal antibodies produced by the cell lines of the present invention are useful in the detection of bacterial infections, therapy and prophylaxis of bacterial endotoxemia and infection caused by gram negative bacteria.

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HUMAN MONOCLONAL ANTIBODIES BINDING DETERMINANTS OF GRAM NEGATIVE BACTERIA

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates generally to infectious diseases and, more particularly, to the prevention, diagnosis and treatment of infections caused by gram negative bacteria.

Bacterial sepsis and related septic shock are frequently lethal conditions caused by infections which can result from certain types of surgery, abdominal trauma and immune suppression related to cancer, transplantation therapy or other disease states. It is estimated that over 700,000 patients become susceptible to septic shock-causing bacterial infections each year in the United States alone. Of these, 160,000 actually develop septic shock, resulting in 50,000 deaths annually.

Gram negative bacterial infections comprise the most serious infectious disease problem seen in modern hospitals. Two decades ago, most sepsis contracted in hospitals was attributable to more acute gram positive bacterial pathogens such as Staphylococcus and Streptococcus. By contrast, the recent incidence of infection due to gram negative bacteria, such as Escherichia coli and Pseudomonas aeruginosa, has increased.

Gram negative bacteria now account for some 200,000 cases of hospital-acquired infections yearly in the United States, with an overall mortality rate in the range of 20% to 60%. The majority of these hospital-acquired infections are due to such gram negative bacilli as E. coli, Klebsiella pneumoniae, P. aeruginosa, and Acinetobacter. Bacteremia is more likely to occur with

local infections caused by Bacteroides species, Serratia, Acinetobacter, and Providencia.

Gram negative sepsis is a disease syndrome resulting from the systemic invasion of gram negative rods and subsequent endotoxemia. The severity of the disease ranges from a transient, self-limiting episode of bacteremia to a fulminant, life-threatening illness often complicated by organ failure and shock. The disease is often the result of invasion from a localized infection site, or may result from trauma, wounds, ulcerations or gastrointestinal obstructions. The symptoms of gram negative sepsis include fever, chills, pulmonary failure and septic shock (severe hypotension).

Gram negative infections are particularly common among patients receiving anti-cancer chemotherapy and immunosuppressive treatment. Infections in such immuno-compromised hosts characteristically exhibit resistance to many antibiotics, or develop resistance over the long course of the infection, making conventional treatment difficult. The ever increasing use of cytotoxic and immunosuppressive therapy and the natural selection for drug resistant bacteria by the extensive use of antibiotics have contributed to gram negative bacteria evolving into pathogens of major clinical significance.

Fortunately, more than a decade ago, investigators in the United States and Germany demonstrated that gram negative endotoxins of many different bacterial genera have a "common core structure." In other words, while many infectious gram negative organisms contain individual capsule and surface polysaccharides, there is a core lipopolysaccharide (LPS) structure that is widely shared among the diverse gram negative bacterial genera and their endotoxins.

This core structure contains material identified as "lipid A" that is felt to be responsible for all of the biologic properties of "endotoxin."

including pyrogenicity, activation of the complement and clotting systems, hypotension and death in experimental animals. This core or LPS structure is therefore significant for at least two reasons; its association with endotoxicity and its conservation in gram negative bacterial genera.

Because antibiotic treatment remains largely suboptimal against gram negative sepsis, particularly that associated with P. aeruginosa bacterial infection, (antibiotics are only effective in treating the bacteria and not in reducing the effects of microbial endotoxins) attention has increasingly focused on immunologic methods to prevent and control such infections. Immunotherapy involves the administration of immunoglobulins (antibodies or active fragments thereof) to bolster the host's native defenses against the toxic effects of the bacteria, for example, by enhancing opsonization and phagocytosis of the infecting bacterial cells, or by neutralization of the biological effects of LPS. Antibodies, or active fragments thereof, that bind with the core structure or lipid A, i.e., LPS, could have a broad reactivity with a number of gram negative endotoxins.

Antibodies directed against epitopes or antigenic determinants on the O-specific side chains of smooth gram negative bacteria have limited utility for use in immunotherapy. This is because they are effective against only those strains of bacteria having complementary or cross-reactive antigenic determinants. Such strain-specific antibodies are of only limited utility. While the core oligosaccharide and lipid A of all strains are thought to share antigenic determinants, the few previous attempts to produce and utilize monoclonal antibodies reactive with these regions in Pseudomonas have been largely unsuccessful. Recent immunological studies describe significant structural differences between the core oligosaccharide of P. aeruginosa

and those of other gram negative genera (Rowe and Meadow, Eur. J. Biochem. (1983)132:329-37).

Immunoglobulins that bind most of the clinically significant gram negative pathogens are essential to the success of immunotherapy. P. aeruginosa organisms, which account for 5% to 15% of bloodstream infections, have at least 16 different serotypes (O-antigenic types). Klebsiella organisms have more than 80 capsular types, and E. coli organisms, which are far more common, have more than 130 serotypes.

Patients with bacteremia often do not have a confirmed specific diagnosis as to the type of bacterial infection until bacteriologic results are available, which may take several days. Therapy often must be started based on an empirical diagnosis in order to prevent a patient's condition from rapidly deteriorating during the critical first 24 to 48 hours of illness.

There therefore exists a longstanding need for the production of monoclonal antibodies (MoAb), or active fragments thereof, reactive with an epitope or antigenic determinant present on all important pathogenic strains of gram negative bacteria, thus permitting effective diagnosis, prophylaxis, control of bacterial infection and neutralization of associated endotoxemia attributable to gram negative bacterial genera. It would also be beneficial to have available MoAbs which are cross-reactive with gram positive bacteria useful in the diagnosis, treatment and prevention of bacterial infections generally.

EP 0 217 527, published August 4, 1987, which is incorporated by reference herein, describes hybridoma cell lines that produce monoclonal antibodies that bind epitopes found on lipopolysaccharide most commonly associated with the endotoxin core of gram negative bacteria and exhibit broad cross-reactivity with gram negative bacteria. At least one of the MoABs disclosed

binds an epitope found on gram positive bacteria. Some of these hybridoma cell lines disclosed by that application are: XMMEN-OE5, given A.T.T.C. Accession No. HB 9081, XMMEN-LY1, given A.T.T.C. Accession No. HB 9082, and XMMEN-J5D, given A.T.T.C. Accession No. HB 9083 were deposited with the American Type Culture Collection, Rockville, MD (A.T.C.C.) on April 24, 1986. XMMPS-605 was deposited with the A.T.C.C. on September 26, 1985 and given A.T.C.C. Accession No. HB 8909.

Human monoclonal antibodies, or active fragments thereof, reactive with an epitope or antigenic determinant present on the lipopolysaccharide of gram negative bacteria would be preferable to non-human derived monoclonal antibodies as they are less likely to cause antigenic reaction in a human patient and will thus be more useful in long-term therapy.

Description of the Relevant Literature

Bacterial infections have received widespread treatment in the scientific and patent literature. Much of this treatment has focused on sepsis due to gram negative bacterial endotoxin. The following is a list of relevant articles and published applications and a brief description of each:

EP O 101 039 A2, published February 22, 1984, discloses a monoclonal antibody to Pseudomonas aeruginosa and methods for its use in diagnosis and therapy;

WO 84/04458, published November 22, 1984, discloses MoAbs reactive with endotoxin core;

WO 85/01659, published April 25, 1985, discloses MoAbs against endotoxin of gram negative bacteria;

EP O 163 493, published April 12, 1985, discloses human MoAbs against gram negative bacteria and specific for serotypic determinants of lipopolysaccharide useful for treating or preventing P. aeruginosa infection;

Feingold, et al., Arch. Int. Med. (1965) 116:326-28, describe the use of polyclonal antisera

derived from human patients recovering from gram negative infection to effectively treat gram negative sepsis in a human patient;

Abe, et al., Jpn. J. Exp. Med. (1975) 45:355-59, describe the use of polyclonal antisera produced in response to immunization of mice with P. aeruginosa endotoxin;

Apicella, et al., Infect. Immun. (1981) 34:751-56, report the analysis of lipopolysaccharide from Neisseria gonorrhorae and N. meningitidis using monoclonal antibodies;

Zeigler, et al., N. Eng. J. Med. (1982) 307:1225-30, report the results of a double-blind trial wherein gram negative bacteremic human patients were treated with human antiserum prepared by vaccinating healthy donors with heat-killed E. coli J5 mutant;

Hancock, et al., Infect. Immun. (1982) 37:166-71, describe MoAbs specific for Pseudomonas aeruginosa outer membrane antigens;

Hiernaux, et al., Eur. J. Immunology (1982) 12:797-803 describe MoAbs specific for E. coli 0113 lipopolysaccharide (LPS);

Mackie, et al., J. Immunol. (1982) 129:829-32, describe MoAbs which bind gram negative bacteria of different genera;

Young, L.S., Clin. Res. (1982) 30:518A, describe MoAbs prepared using S. minnesota RS-95 LPS as the immunogen;

Pollack, et al., J. Clin, Invest. (1983) 72:1874-81, report enhanced survival of P. aeruginosa septicemia associated with high levels of circulating antibody to E. coli endotoxin core;

Sawada, et al., J. Infect. Dis. (1984) 150:570-76, report protection in mice against infection with P. aeruginosa by passive transfer of MoAbs to lipopolysaccharides and outer membrane proteins;

Three relevant abstracts were included in Abstracts of the 24th Interscience Conference on Antimicrobial Agents and Chemotherapy (1984) 106: Black and Cannon, "Monoclonal antibody to common pathogenic neisseria antigen (H8Ag) protects against meningococcemia (ME) in mice"; Williams, et al., "Panreactive monoclonal antibody (MCA) to Porin Protein F of Pseudomonas aeruginosa (PA): Passive immunotherapy in mice"; and Kim, et al., "Studies of the protective mechanism of monoclonal antibodies against E. coli";

Mutharia, et al., Infect. Immun. (Sept. 1984) 45:631-36, describe MoAbs cross-reactive with gram negative bacteria of different genera believed to bind lipid A and not reactive with gram positive bacteria;

Nelles and Niswander, Infect. Immun. (Dec. 1984) 46:677-81, describe two mouse monoclonal antibodies reactive with lipopolysaccharide derived from the J5 mutant of E. coli 0111:B4 which binds lipopolysaccharide from both smooth and rough phenotype, gram negative bacteria;

Young, et al., Clin. Res. (1984) 32:522A, describe a MoAb directed against the "core" of glycolipid of enterobacterial endotoxin;

Young, L.S., Principles and Practice of Infectious Disease (1985) John Wiley and Sons, N.Y., NY, pp. 452-75, provides an overview of gram negative sepsis;

Sadoff, et al., Antibiot. Chemother. (1985) 36:134-46, describe the characterization of mouse monoclonal antibodies directed against P. aeruginosa lipopolysaccharides;

Teng, et al., Proc. Nat. Acad. Sci. USA (March 1985) 82:1790-94, report the protection of mice against gram negative bacteremia and endotoxemia with human monoclonal IgM antibodies. The MoAbs showed no significant protection from gram positive bacterial infection;

Gigliotti and Shenep, J. Infect. Dis. (June 1985) 151:1005-11, describe MoAbs that bind LPS from E.

coli rough mutant J5 but do not bind intact smooth strains of E. coli 0111:B4 or K1:07;

Peters, et al., Infect. Immun. (Nov. 1985) 50:459-66, describe MoAbs to enterobacterial common antigen and E. coli lipopolysaccharide outer core and demonstrate a shared antigenic determinant believed to be at least in part 4-linked α-N-acetylglucosamine;

Dunn, et al., Surgery (August 1985) 98:283-90, report the production of a strain specific binding MoAb using E. coli smooth strain 0111:B4 as the immunogen and the production of a gram negative bacteria cross-reactive MoAb using E. coli rough mutant J5 as the immunogen. These MoAbs were not reactive with gram positive bacteria;

Dunn, et al., Arch. Surg. (Jan 1986) 121:58-62, report on the immunotherapy of gram negative sepsis employing a single murine monoclonal IgG antibody, demonstrating reactivity with a variety of gram negative organisms, promotion of phagocytosis and providing protection during experimental gram negative sepsis. Also, the MoAb showed no reactivity with gram positive bacteria tested;

Miner, et al., Infect. Immune. (April 1986) 52:56-62, describe the characterization of murine MoAbs to E. coli J5.

Brade and Galanos, J. Med. Microbiol (1983) 16:203-10, describe a new lipopolysaccharide antigen identified in Acinetobacter calcoaceticus.

Nurminen et al., Infect. Immun. (1984) 44:609-13, described immunologically related ketodeoxyoctonate-containing structures Acinetobacter calcoaceticus anitratus.

Thornley et al., J. Gen. Microbiology (1985) 131:7-15, described monoclonal antibodies cross-reactive with a strain of Acinetobacter.

Brade and Rietschel, Eur. J. Biochem. (1985)

153:249-54, report the identification of 2-keto-3-deoxy-17-dicarbozyheptonic acid as a constituent of lipopolysaccharide of Acinetobacter calcoaceticus NCTC 10305.

Of general interest on the subject of endotoxin shock is Handbook of Endotoxin, R.A. Proctor, Ed., Elsevier (1986).

Sawada et al., J. Infect. Dis. (1985)152: 965-70, describe serotype-specific human monoclonal antibodies which are reactive with Pseudomonas aeruginosa. Human monoclonals were derived both from fusion between human lymphocytes and a murine myeloma and from viral transformation of human lymphocytes.

Siadak and Lostrom, Human Hybridomas and Monoclonal Antibodies (1985) Plenum Press, New York, NY, pp. 167-85, describe serotype-specific human monoclonal antibodies which are reactive with Pseudomonas aeruginosa. Human monoclonals were derived from viral transformation of human lymphocytes.

Stoll et al., Infect. Immun. (1986)53: 656-62, describe monoclonal antibody XMMPS-OP1 (PIC9.1.1), its specific reaction with P. aeruginosa Fisher immunotype 1 LPS, and a Western blot derived from it.

Although a wide variety of core LPS-specific monoclonal antibodies have been described to date, to our knowledge, only antibodies XMMPS-605 (described here) and 11.14 (described by Sidberry et al., J. Immunol. Methods (1985)76:299-305) have been described as specifically reactive with Pseudomonas core LPS. Antibody 11.14, unlike XMMPS-605, detects a type-specific determinant (serotypic determinant) which is not broadly cross-reactive with Pseudomonas LPS preparations. The core-reactive nature of antibody 11.14 was determined by Western blot analysis. This analysis also determined that higher molecular weight forms of LPS which contain oligosaccharide side chain and core were recognized by antibody 11.14.

SUMMARY OF THE INVENTION

The present invention provides novel methods for producing human monoclonal antibodies capable of binding an epitope on gram negative bacterial core lipopolysaccharide comprising the steps of (1) screening monoclonal antibodies secreted from transformed human cell lines by employing a competitive assay against non-human monoclonal antibodies capable of binding such an epitope and (2) selecting human cell lines which secrete monoclonal antibodies competitive with the non-human monoclonal antibodies. The present invention further contemplates monoclonal antibodies produced by those methods and the hybridoma cell lines producing such monoclonal antibodies. Preferably the human cell lines are derived from human B lymphocytes from a person hosting an infection of gram negative bacteria and are transformed with Epstein-Barr Virus. Most preferably the Epstein-Barr virus is that released by B95-8 marmoset lymphoma cells having A.T.C.C. Accession No. CRL 1612 and the non-human hybridoma cell line is selected from the group consisting of the cell lines designated A.T.C.C. Accession Nos. HB 9081, HB 9082, HB 9083 and HB 8909.

Further, this invention contemplates monoclonal antibodies produced by the above method wherein the antibody is attached to a detectable label. In addition, diagnostic methods are provided for detecting the presence of bacterial cells in a solution suspected of harboring the cells, comprising the steps of:

attaching the cells in the solution to a solid support;

contacting the solid support with labeled human monoclonal antibodies of the invention; and

monitoring the solid support for the presence of labelled antibody thereon. The solution may be derived from the body fluid or tissue of a human patient and the label may be one capable of emitting radiation or a component of an enzymatic reaction.

Further, the present invention provides a method for detecting a localized infection in a human patient due to bacteria comprising the steps of:

administering to the patient labelled monoclonal antibodies of the present invention;

permitting the labeled monoclonal antibodies to accumulate in the localized infection; and

monitoring the patient so as to determine the site of the localized infection. The monitoring step may further comprise whole body imaging. The label may be one which exhibits nuclear magnetic resonance.

The invention further contemplates kits for use in a method for detecting, treating or preventing gram negative infection in a human patient comprising a labelled monoclonal antibody of the present invention.

Further contemplated are methods of prophylactically treating human patients at risk for bacterial infection, comprising administering to the patient a prophylactically effective amount of a monoclonal antibody of the present invention, whereby the risk for infection is reduced, and methods of therapeutically treating patients infected with bacterial cells comprising administering to the patient a therapeutically effective amount of monoclonal antibody of the present invention whereby said infection is alleviated.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 shows sodium dodecylsulfate-polyacrylamide gel electophoresis (SDS-PAGE) analysis of whole cell extracts of P. aeruginosa binding with XMMPS-605 monoclonal antibodies by Western Immunoblot;

Fig. 2 shows SDS-PAGE analysis of P. aeruginosa lipopolysaccharide binding with XMMPS-605 monoclonal antibody by Western Immunoblot;

Fig. 3 shows a Western blot analysis of the Pseudomonas Fisher 1 LPS- and PAC 605 LPS-associated

epitopes recognized by antibodies XMMPS-605 and XMMPS-OP1;

Fig. 4 shows the companion silver-stained gel for the Western blot shown in Fig. 5; and

Fig. 5 shows a Western blot analysis of the Pseudomonas Fisher 1 LPS-, Fisher 2 LPS-, and PAC 605 LPS-associated epitopes recognized by antibodies XMMPS-605 and XMMPS-OP1.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention involves certain hybrid cells, viral transformants and their functional equivalents, capable of producing monoclonal antibodies which bind one or more epitopes present on lipopolysaccharide commonly associated with gram negative bacteria, usually Pseudomonas species and Pseudomonas aeruginosa in particular. Of particular interest to those skilled in the art will be human monoclonal antibodies produced by viral transformants.

The invention further provides methods for employing such compounds in the detection, treatment and prevention of bacterial infections.

Hybridoma formation and monoclonal antibody production may be effected by many different techniques which are well-known in the art. (See e.g., Goding, J.W., Monoclonal Antibodies: Principles and Practice, Second Ed., Academic Press (1986), which is incorporated by reference.) Basically, the process involves first obtaining immune cells, such as those from the spleen of a mammal, which have been previously stimulated with an antigen either in vivo or in vitro. These cells are then fused to cells, such as myeloma cells or transformed cells, which are capable of replicating indefinitely in cell culture, thereby producing an immortal, immunoglobulin-secreting cell line. The resulting fused cells, or hybridomas, are cultured and the resulting colonies screened for the production of the desired monoclonal

antibodies. Colonies producing such antibodies are cloned, and grown either in vivo or in vitro so as to produce large quantities of antibody (for description of the theoretical basis and practical methodology of fusing such cells, see Köhler and Milstein, Nature (1975) 256:495, the disclosures of which are hereby incorporated by reference). While such methods are described in further detail hereinafter, it will be appreciated by those skilled in the art that modifications and additions to the techniques may be made without departing from the scope of the present invention.

Mammalian lymphocytes are immunized by in vivo immunization of the animal or in vitro contact with whole cells or cell extracts of gram negative bacteria or free lipopolysaccharide. Such immunization is repeated as necessary at intervals of up to a few weeks so as to obtain a sufficient titer of antibodies. The cells or cell extracts are carried in appropriate solutions or adjuvants. Following the last antigen boost, the animals are sacrificed and spleen cells removed.

Fusion with mammalian myeloma cells or other fusion partners capable of replicating indefinitely in cell culture is effected by standard and well-known techniques (e.g., Milstein and Köhler, Eur. J. Immunol. (1976) 6:511, the disclosures of which are hereby incorporated by reference), for example by using polyethylene glycol (PEG) or other fusing agent. This immortal cell line, which is preferably murine, but may also be derived from cells of other mammalian species, including but not limited to rats and humans, is selected to be deficient in enzymes necessary for the utilization of certain nutrients, to be capable of rapid growth and to have good fusion capability. Many such cell lines are known to those skilled in the art, and others are regularly described. Enzyme deficiencies may include, for example, thymidine kinase (TK) or hypoxanthine-guanine phosphoribosyl transferase (HGPRT). These deficiencies

allow selection for fused cells according to their ability to grow on, for example, hypoxanthine-aminopterin-thymidine medium (HAT). Preferably, the immortal fusion partners utilized are derived from a line which does not secrete immunoglobulin.

Individual fused cells are grown in individual tissue culture wells. Feeder cells, such as irradiated thymocytes or other cells, may be used to increase the viability of the cells. Hybridoma culture supernatants from the individual wells are assayed for antibody binding to purified lipopolysaccharide or whole gram negative bacteria, or by other suitable detection methods known in the art, such as enzyme-linked immunoassay (EIA) and immunodot assay. For the former, culture supernatants are placed in reaction wells which have been coated with lipopolysaccharide. After incubation, the reaction wells are washed, and remaining antibody bound to the antigen is detected through a labelled antibody reactive with the anti-LPS antibody. Appropriate labels include radioisotopes, luminescent substrates such as fluorescing agents and components of enzymatic labels.

The Immunodot method is also utilized to screen for clones expressing anti-LPS antibodies (Towbin, et al., Immunol. Method (1984) 72:313, the disclosures of which are hereby incorporated by reference). Purified LPS is applied to cellulose nitrate membrane as "dots" and allowed to dry. After blocking in a gelatin solution, the membranes are sequentially immersed in culture supernatant, an anti-mouse Ig-peroxidase conjugate solution and a 4-chloro-1-naphthal solution, with phosphate buffered saline (PBS) washes in between. Clones expressing reactive immunoglobulin appear as colored dots. Other screening systems known to those in the art may be utilized.

Due to the frequent immune response elicited in humans by administration of non-human immunoglobulins

and the inability of those skilled in the art to deliberately immunize humans to obtain immune cell fusion partners, a variety of techniques have been developed for producing human MoAbs. According to the present invention, the preferred method of producing human monoclonal antibodies is to isolate B lymphocytes from patients following natural exposure to gram negative pathogens such as cystic fibrosis patients. These B lymphocytes are transformed with a transforming agent such as Epstein- Barr virus (EBV) to produce an immortal cell line stabily secreting human MoAbs. An example of a cell line producing EBV is the EBV transformed marmoset lymphoma cell line B95-8, as described by Casali et al., Science (1986) 234:476-79 (A.T.C.C. Accession No. CRL 1612). Alternative EBV transformation methodologies, such as that disclosed in U.S. Patent No. 4,464,465 may be employed as well. These transformed human antibody secreting cell lines are screened for the production of gram negative LPS binding activity similar in activity to that observed in non-human (preferably murine) MoAbs known to be capable of binding an epitope on gram negative bacterial lipopolysaccharides by employing a competitive assay.

Procedures for competitive assays are well known in the art. An example of such a competitive assay is to screen EBV transformed B lymphocytes isolated from cystic fibrosis patients for XMMPS-605-like MoAb activity. Essentially, transformant supernatants are applied to LPS adsorbed to a solid support, with and without preincubation with XMMPS-605, and binding is determined by standard ELISA techniques well known in the art and described below. B cells from samples showing positive reactions with LPS which has not been preincubated with XMMPS-605 and showing reduced reactions with LPS preincubated with XMMPS-605 are expanded and cloned using limiting dilution techniques.

The non-human MoAbs act as the competing MoAbs. The competing MoAbs may be any MoAbs known to be capable of binding an epitope on gram negative bacterial lipopolysaccharide. For example, the murine hybridoma cell lines described in EP Publication No. 0217527, supra, may be used to produce competing monoclonal antibodies. Human MoAbs, for example, having XMMPS-0E5-like activity may be selected for by employing XMMPS-0E5 hybridoma-produced murine MoAbs as the competing MoAbs.

The level of the competing antibodies needs to be of sufficient level to properly interfere with the human monoclonal antibodies to be screened. Typically the affinity binding constant (K) will be about 10^8, preferably about 10^9, and most preferably about 10^{10}.

According to the present invention, in order to increase the stability of specific antibody secreting transformant cells they may be fused with a suitable enzyme-deficient myeloma, lymphoblast cell line or heteromyeloma, such as, respectively, NS-1 (A.T.C.C. No. T1B 18), WIL2-NS (A.T.C.C. No. CRL 8155), or SI-IM-D33 (A.T.C.C. No. CRL 1668). A less preferred method of producing human monoclonal antibodies is to directly fuse B lymphocytes as previously described with a variety of fusion partners. In addition to those listed above are the 6-thioguamine-resistant human lymphoblastoid B-cell line UC 729-6 as described by Glassy et al., Proc. Natl. Acad. Sci. U.S.A. (1983) 80:6327-31; human myeloma RPMI 8226 (A.T.T.C. No. CCL 155); and a variety of heteromyelomas as disclosed by Kaplan et al., U.S. Patent No. 4,574,116.

Alternatives to cell line transformation and hybridoma production include chimeric antibodies as described by Sun et al., Hybridoma (1986) 5:517-20 and synthetic immunoglobulins and peptide-linked fragments having binding-activity.

Large quantities of monoclonal antibodies from secreting hybridomas or transformed cell lines are produced by injecting the clones into the peritoneal cavity of mice and harvesting the ascites fluid therefrom. The mice, preferably primed with pristane or other tumor-promoter and immunosuppressed chemically or by irradiation, may be of various strains, such as New Zealand Black or Balb/c strains. The ascites fluid is harvested from the mice and the monoclonal antibody purified therefrom, for example, by CM Sepharose column or other chromatographic means. High titers of antibodies may be so recovered. Alternatively, the hybridomas or transformed cell lines may be cultured in vitro in a variety of ways, utilizing either perfusion cultures or suspension cultures, both in batch or continuous culture processes, and monoclonal antibodies recovered from the culture medium or supernatant.

The monoclonal antibodies so produced have a number of diagnostic and therapeutic uses. They are used as in vitro diagnostic agents to test for the presence of gram negative bacteria or bacteria generally in human patients by subjecting body fluids and tissues or other human-derived substances or fluids to standard immunoassay protocols. Additionally, extracts of inanimate objects of which contamination by bacteria would be detrimental, such as medical devices, foodstuffs or water, may also be tested. Such assays may be of a radioimmunoassay, EIA or chemiluminescent format. In one such assay, body fluid is contacted to antibodies of the present invention and a labelled second antibody used to detect the presence of bacteria to which the antibodies are bound. Alternatively, a competitive immunoassay or a "sandwich" type assay can be employed. Such histochemical methods are well-known in the art; protocols are found, for example, in Methods in Immunodiagnosis, 2nd edition, Rose and Bigazzi, eds., John Wiley and Sons, 1980, which is incorporated by

reference, and in Campbell et al., Methods of Immunology, W.A. Benjamin, Inc., 1964.

Further, monoclonal antibodies of the present invention are used for the in vivo detection of localized areas of bacterial infection, for example, abscesses or cysts in the soft tissue and osteomyelitis in bone. For example, labelled antibody is administered to a patient suspected of having a bacterial infection. The antibody selectively binds to the bacteria present in the patient, thereby concentrating the label in the area of infection. Labels appropriate for such use include radioisotopes, such as ^{123}Iodine, ^{125}Iodine, ^{131}Iodine, ^{99}Technetium and ^{111}Indium, which can then be detected using standard radiographic techniques. Alternatively, the monoclonal antibodies can be labelled with paramagnetic contrast agents, and detected by nuclear magnetic resonance methods. The labelled antibodies thus produce a detectable image of the bacterial abscess. Similarly, these monoclonal antibodies may be employed in a method for detecting and quantifying microbial endotoxins in body fluids, secretions, and extracts as well as in drugs, diagnostic agents or liquid intermediates produced in the manufacture of diagnostic and therapeutic agents.

The term "detectable" in the phrase "attached to a detectable label" will vary in accordance with the selected label as well as the particular test used to monitor that label. For example, different radioisotopes have different specific activities. One of skill in the art will readily recognize that the quantity of radio-label required for detection in a specific assay will vary in accordance with the specific activity of the particular label. Experimental strategies for selecting a detectable amount of labeled immunogen is routine.

Monoclonal anti-LPS antibodies are used prophylactically in patients at risk for gram negative

bacterial infection. Administration of effective amounts of these monoclonal antibodies serves to enhance the body's potential ability to defend against the particular organism, thereby lessening the risk of subsequent infection.

The monoclonal antibodies of the present invention may be used therapeutically to treat potentially lethal bacterial infections and septic shock. The antibodies are administered either intravenously or intramuscularly in a physiologically acceptable solution, either alone or in combination with antibiotics. Although to do so may affect the binding characteristics of the present monoclonal antibodies, they may be lyophilyzed for storage and shipment and reconstituted prior to administration.

Hosts determined to have a bacterial infection may preferably be treated with human monoclonal antibodies reactive with an antigenic determinant common to all strains of the bacterium. The monoclonal antibodies are administered venously, intramuscularly, intraperitoneally, or the like, in a physiologically acceptable carrier solution, such as phosphate buffered saline. The dosage is determined by the body weight of the host, it preferably being in the range of about 0.1 mg/kg to about 40 mg/kg body weight, and usually about 1 mg/kg to about 10 mg/kg of host body weight. Alternatively, the dosage is established by evaluating the extent of the remaining infection, as by quantitatively standardized EIA radioimaging or other methods. Treatment is repeated at intervals as necessary, to effect enhancement of the hosts' ability to recover from the infection.

Because the human monoclonal antibodies have special affinity for lipopolysaccharide, they provide selective treatment for life-threatening symptoms of endotoxemia, such as septic shock associated with gram negative infections, which are otherwise often unresponsive to antibiotic treatment. Among the effects of

treatment with several of these monoclonal antibodies are the facilitation of opsonization and phagocytosis of some bacteria, presumably by binding to the bacterial cell wall. The monoclonal antibodies thus aid in combatting the toxic effects of the bacterial infections.

For all such diagnostic, prophylactic and therapeutic uses, the monoclonal antibodies and other necessary reagents and appropriate devices and accessories may be provided in kit form so as to be readily available and easily used.

The following examples are offered by way of illustration and not limitation.

EXAMPLE I - HYBRIDOMA XMMPS-605

A. Production of XMMPS-605 Hybridomas

New Zealand Black mice (Jackson Laboratories, Bar Harbor, ME) were immunized with 1 x 10^8 formalin-killed cells of Pseudomonas aeruginosa strain PAC 605 (A.T.C.C. No. 53273) in Complete Freund's Adjuvant (Difco Laboratories, Detroit, MI). The LPS of PAC 605, a bacteriophage-resistant mutant of strain PAC 1R, lacks outer O-specific side chains and consists only of the core LPS (lipid A and core oligosaccharide), as shown by sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Meado et al., J. of General Microbiology (1984) 130:631-644). After primary immunization, the mice were boosted with an intraperitoneal injection of 1 x 10^8 formalin-killed P. aeruginosa cells lacking O-specific side chains at one month intervals.

Four days following the last antigen boost, spleen cells from an immunized mouse were aseptically removed. Following procedures as outlined elsewhere (St. Groth, J. Immuno. Meth. (1908) 35:1), 5 x 10^7 spleen cells were fused with an equal number of SP2/O-AG14, a nonsecreting mouse myeloma cell line of Balb/c origin, using polyethylene glycol 4000 (American Type Culture Collection, Rockville, MD). Hybrid cells were placed into 96-well culture plates (Costar, Cambridge, MA, #3596) on medium which had been pre-incubated with a feeder layer of normal New Zealand Black thymocytes (1 x 10^5 cells per well, one day before fusion). Cells were cultured at 37°C, in a 10% CO_2 atmosphere, in the following medium for the first two weeks: Dulbecco's Modified Eagle's Medium, with glutamine, and glucose at 4.5 g/l (Gibco, Santa Clara, CA, #320-1965), Fetal Bovine Serum (10%) (Microbiological Associates, Walkerville, MD), sodium pyruvate (1 mM) (Gibco, Santa Clara, CA, #320-1360), penicillin (50 µ/ml) -- Streptomycin (50 µ/ml) (Gibco, Santa Clara, CA, #600-5070), and

hypoxanthine-aminopterin-thymidine (HAT) which was prepared by using 1.0% v/v (100X) hypoxanthine-thymidine supplement (Microbiological Associates, Walkersville, MD, #17-782A) combined with 0.04 mM aminopterin (Sigma Chemical Co., St. Louis, MO). Medium for regular maintenance (past two weeks fusion date) was identical to the above, except that no aminopterin was included in the medium (HT medium).

Between two to four weeks post-fusion, cultures of hybrid cells were tested for antibody binding to P. aeruginosa purified LPS by EIA and immunodot assay as described in Examples II and VII of EP Publication No. 0217527, supra. Cultures that were positive on repeated testing were then cloned by limiting dilution techniques. Briefly, the cells were cultured at dilutions varying from 10-1 cells per well in 96-well tissue culture plates (Costar, Cambridge, MA, #3596). Wells that contained only one colony were identified by microscopic examination, then tested for anti-PAC 605 activity by EIA. Positive clones were expanded onto 24-well tissue cultures plates (Costar, Cambridge, MA, #3524), recloned and retested by the same methods. A single clone, designated XMMPS-605, was found to stably secrete monoclonal antibody; the monoclonal antibody was determined to be of immunoglobulin class IgG_2B by radial immunodiffusion and radioimmunoassay, using standard methods.

New Zealand Black mice (Jackson Laboratories, Bar Harbor, ME) were used to culture the hybridomas interperitoneally. Approximately 3×10^6 hybridoma cells were injected intraperitoneally (i.p.) into mice that had been pretreated as follows: 1) injected i.p. one week earlier with 0.5 ml of pristane (Aldrich Chemical Co., Milwaukee, WI), and 2) injected i.p. one day earlier with 2 mg of cyclophosphamide (Adria Laboratories, Columbus, OH). The resultant ascites fluid, collected 11-15 days after injection of the hybridomas,

contained on average 5 mg/ml of the anti-PAC 605 antibody, XMMPS-605, as determined by radial-immunodiffusion (Meloy, Radial Immunodiffusion, Springfield, VA, Plate #J-307), performed according to the method given in Meloy instruction sheet entitled "Quantitative Immunodiffusion Plates for Mouse Immunoglobulins," which is incorporated by reference.

The antibody in ascites fluid was purified by using a protein-A Sepharose C1-4B column (Pharmacia, Inc., Piscataway, NJ) by methods well-known to those skilled in the art, as described elsewhere (Ey, Immunochemistry (1978) 15:429-436). Determination of immunoglobulin subclass (IgG2B) was accomplished by immunodot assay, using subclass specific antibody conjugated to peroxidase (Southern Biotechnology Associates, Inc., Birmingham, AL).

B. Enzyme-Linked Immunoassay (EIA) Using Whole Bacteria

Thirty gram negative bacteria strains were tested to determine the extent of cross-reactivity of XMMPS-605 monoclonal antibody. The sources of these strains were as follows: 1) Salmonella minnesota R595, chemotype Re, strain KNV, was acquired from Dr. Otto Westphal (Max Planck Institute fur Immunobiologie, Freiburg, Federal Republic of West Germany); 2) the international types of Pseudomonas aeruginosa, E. coli serotypes 055:55 and 026:B6 were purchased from the American Type Culture Collection (A.T.C.C.); 3) P. aeruginosa PAC 1R, PAC 557, PAC 605, from Pauline Meadow, (University College at London); 4) P. aeruginosa Fisher types 1-7 were donated from Dr. Matthew Pollack (Uniformed Services University, Bethesda, MD); 5) E. coli 014:K7 was obtained from Dr. Erwin Neter and Dr. H.Y. Whang (Children's Hospital of Buffalo, New York); 6) E. coli 085:H9 was obtained from the Center for Disease Control (Atlanta, GA); 7) the Escherichia coli J5 rough mutant

was obtained from Dr. Abraham Braude (University of California, San Diego); 8) the remaining strains were clinical isolated #7711, #3632, #4194 from the UCLA Medical Center.

These bacteria were first cultured on trypticase-soy agar (TSA) plates. Bacterial cells were harvested and their concentration adjusted to 1.5 x 10^8 cells/ml in normal saline. Concentrations were determined by the measured absorbance (optical density) of the bacterial suspensions at 570 nanometers (nm). Fifty µl aliquots of these suspensions were coated onto wells of flat-bottom polyvinyl plates (Falcon LabWare, Oxnard, CA, #3912), then centrifuged for 30 minutes at 2000 x G. Pelleted cells were fixed by the addition of 150 µl/ well of 0.25% gluteraldehyde/phosphate buffered saline 0.01M, pH 7.4 (hereinafter PBS) for 15 minutes at room temperature. Supernatants were discarded and the plates washed with PBS, and then blocked overnight at room temperature with 0.1% gelatin/PBS.

The remainder of the assay was identical to that described for EIA using LPS in Example III of EP 0217527, supra. The plates were washed and 100 µl/well of affinity-purified XMMPS-605 monoclonal antibody (1 µg/ml) was allowed to react with the coated antigens for one hour at room temperature. Following a PBS wash, 100 µl/well of goat anti-mouse IgG-peroxidase conjugate was added and allowed to react for one hour at room temperature. After a final wash, 2-2'azino-di(3-athyl-benzthiazolin-sulfonate)(6) (ABTS) (Boehringer Mannheim Biochemicals, Indianapolis, IN), substrate was added (100 µl/well); color development proceeded for 15 minutes, at which point the absorbance at 405 nm was recorded as previously described.

The results of these assays are presented in Table 1. All strains of Pseudomonas aeruginosa and P. maltophilia exhibited significant absorbance at 405 nm (range 0.37 to 1.60 O.D.). Of the other strains of

gram negative bacteria tested, none showed significant reactivity. The antigenic determinant of lipopolysaccharide with which the monoclonal antibody is reactive is therefore shown to be common to all strains of P. aeruginosa and to P. maltophilia, but is not present on other gram negative and gram positive bacteria.

Table 1 Enzyme-Linked Immunosorbent Assay (EIA) Of XMMPS-605 Binding To Whole Bacteria

Bacteria	Absorbance At 405 nm
Pseudomonas aeruginosa PAC 1R	0.75 O.D.
Pseudomonas aeruginosa PAC 557	1.60 O.D.
Pseudomonas aeruginosa PAC 605	1.60 O.D.
Pseudomonas aeruginosa Fisher Type 1	0.73 O.D.
Pseudomonas aeruginosa Fisher Type 2	0.76 O.D.
Pseudomonas aeruginosa Fisher Type 3	0.75 O.D.
Pseudomonas aeruginosa Fisher Type 4	1.52 O.D.
Pseudomonas aeruginosa Fisher Type 5	1.53 O.D.
Pseudomonas aeruginosa Fisher Type 6	0.41 O.D.
Pseudomonas aeruginosa Fisher Type 7	0.37 O.D.
Pseudomonas aeruginosa Intern´l Type 3	1.57 O.D.
Pseudomonas aeruginosa Intern´l Type 4	1.55 O.D.
Pseudomonas aeruginosa Intern´l Type 9	1.53 O.D.
Pseudomonas aeruginosa Intern´l Type 11	1.47 O.D.
Pseudomonas aeruginosa Intern´l Type 12	1.54 O.D.
Pseudomonas aeruginosa Intern´l Type 13	1.60 O.D.
Pseudomonas aeruginosa Intern´l Type 14	1.58 O.D.
Pseudomonas aeruginosa Intern´l Type 15	1.57 O.D.
Pseudomonas cepacia	0.03 O.D.
Pseudomonas pickettii	0.03 O.D.
Pseudomonas putida	0.03 O.D.
Pseudomonas maltophilia	1.34 O.D.
Salmonella minnesota R595	0.02 O.D.
Escherichia coli J5	0.02 O.D.
Escherichia coli 014:K7	0.01 O.D.
Escherichia coli 014:H31	0.02 O.D.
Escherichia coli 085:H9	0.00 O.D.
Enterobacter cloaceae	0.02 O.D.
Serratia marcescens	0.00 O.D.
Klebsiella pneumoniae	0.01 O.D.

C. Enzyme-Linked Immunosorbent Assay (EIA) Using Purified Lipopolysaccharide (LPS)

A total of 24 different purified lipopolysaccharide extracts from the bacteria listed on Table 2 were used to determine the extent of cross-reactivity of the anti-PAC 605 monoclonal antibody, XMMPS-605. The LPS of P. aeruginosa PAC 605 and that of Acinetobacter calcoaceticus (UCLA clinical isolate #7471) were extracted from the bacterial outer membranes according to published methods (Darveau, et al., Jour. of Bacteriology (1983) 155:831 which is incorporated by reference). Purified LPS from P. aeruginosa Fisher types 1-7 were obtained from Parke-Davis & Co. (Detroit, MI), and the remaining purified antigens were purchased from List Biologicals (Campbell, CA).

Table 2. Lipopolysaccharide Extracts from Bacteria

Antigen
Salmonella minnesota R60 (Ra) LPS
Salmonella minnesota R345 (Rb) LPS
Escherichia coli J5 (Rc) LPS
Salmonella minnesota R7 (Rd) LPS
Salmonella minnesota R595 (Re) LPS
Pseudomonas aeruginosa PAC 605 LPS
Pseudomonas aeruginosa Fisher types 1-7
Escherichia coli J5 boiled cells
Escherichia coli 014:K7 boiled cells
Pseudomonas aeruginosa PAC 605 boiled cells
Pseudomonas aeruginosa Fisher 2 boiled cells
Acinetobacter calcoaceticus boiled cells

Preparations of these purified antigens were diluted to 25 µg/ml in water contained 0.5% triethylamine. One hundred µl/well of these antigens were coated onto 96-well EIA plates (Costar, Cambridge, MA, #3590). Following overnight incubation at room temperature, the plates were blocked with 0.1% reagent grade gelatin (Difco, Inc., Detroit, MI) in PBS for two hours at 37°C. This step was essential to prevent nonspecific binding of the antibody to the polystyrene wells. The plates were then washed once with PBS.

Monoclonal antibody XMMPS-605 which had been affinity purified was diluted to 5 µg/ml in 0.1% gelatin/PBS and allowed to react with the coated antigen for one hour at room temperature. After three washings with PBS, 100 µl of the second antibody (goat anti-mouse IgG-peroxidase conjugate (Cappel, Malvern, PA, #0600-3161)) was added to the reaction wells, and left to react at room temperature for one hour. The plates were again washed three times with PBS. Positive reactions were detected by adding ABTS (Boehringer Mannheim Biochemicals,

Indianapolis, IN). This substrate was prepared by first making a stock solution of 20 mg/ml ABTS in 0.1M citrate buffer (pH 4.5). The solution was then diluted 1:50 in citrate buffer and to it was added a 1:1000 dilution of 30% hydrogen peroxide. Color development was allowed to proceed for 15 minutes, and the absorbance was read at 405 nm in a Titertek Elisa Reader (Flow Labs, McLean, VA). The results of these assays showed that the XMMPS-605 antibodies bind only to LPS from P. aeruginosa and P. maltophilia after testing against LPS from a wide range of gram negative bacteria. The LPS of all seven of the Fisher strains of Pseudomonas aeruginosa and PAC 605 showed absorbance (range 0.78 to 1.06 O.D.). None of the LPS from the remaining gram negative bacteria tested showed significant absorbance.

Results of these assays are presented in Table 3. As can be seen therein, XMMPS-605 binds to all P. aeruginosa representing all Fisher groupings, as indicated by the relatively high levels of absorbance. None of the LPS from other species tested, including preparations of purified core region LPS, exhibited significant absorption.

Table 3 XMMPS-605 Binding Activity To Lipopolysaccharide (LPS) As Determined By Enzyme-Linked Immunoabsorbent Assay (EIA)

Antigen	Absorbance At 405 nm
Pseudomonas aeruginosa PAC 605*	0.78 O.D.
Pseudomonas aeruginosa Fisher 1	1.01 O.D.
Pseudomonas aeruginosa Fisher 2	1.05 O.D.
Pseudomonas aeruginosa Fisher 3	1.06 O.D.
Pseudomonas aeruginosa Fisher 4	0.95 O.D.
Pseudomonas aeruginosa Fisher 5	0.86 O.D.
Pseudomonas aeruginosa Fisher 6	0.86 O.D.
Pseudomonas aeruginosa Fisher 7	0.95 O.D.
Salmonella minnesota Wild Type	0.02 O.D.
Salmonella minnesota R60 (Ra)*	0.02 O.D.
Salmonella minnesota R345 (Rb)*	0.08 O.D.
Salmonella minnesota R5 (Rc)*	0.01 O.D.
Salmonella minnesota R7 (Rd)*	0.03 O.D.
Salmonella minnesota R595 (Re)*	0.01 O.D.
Escherichia coli J5 (Rc)*	0.05 O.D.
Escherichia coli K235	0.00 O.D.
Escherichia coli 011:B4	0.00 O.D.
Escherichia coli 055:B5	0.00 O.D.
Escherichia coli 026:B6	0.00 O.D.
Escherichia coli 0127:B8	0.00 O.D.
Escherichia coli K12	0.01 O.D.
Klebsiella pneumoniae	0.04 O.D.
Serratia marcescens	0.01 O.D.
Acinetobacter calcoaceticus	0.00 O.D.

* LPS used in assay was a preparation of the core region isolated from mutants which do not express O-side chains.

D. Immunodot Assay Binding To Purified Lipopolysaccharide (LPS)

Purified LPS (100 µg/ml, PBS) was applied to cellulose nitrate membrane (0.20 µm, Sartorius, Hayward, CA, #11307) as 1 µl "dots" and allowed to air dry for several minutes. The membranes were then blocked in 1.0% reagent grade gelatin/PBS for 30 minutes at room temperature. The remainder of the rapid, room temperature assay was accomplished as follows: Immersion of the antigen treated membranes in culture supernatant (containing XMMPS-605 monoclonal antibody) for 30 minutes, followed by a PBS wash; immersion in goat anti-mouse IgG-peroxidase conjugate for 30 minutes, followed by a PBS wash; immersion in 4-chloro-1-naphthol (Sigma Chemical Co., St. Louis, MO), substrate for color development of purple, positive "dots." Positive reactions usually occur within 5 minutes. Preparation of substrate: 4-chloro-1-naphthol was prepared as a 0.3% stock solution (w/v) in methanol, then diluted 1:5 in 0.01M PBS; to which was added a 1:1000 dilution of 30% hydrogen peroxide.

The dots were scored visually for positive reaction, using a scale of 4+ for strong color to 1+ for weak color. As indicated by the data presented in Table 4, LPS from all Fisher serotypes of P. aeruginosa gave a positive reaction (2+ or 3+) indicating binding by XMMPS-605, while no samples of LPS from other bacteria presented discernible color, indicating no binding by XMMPS-605.

Table 4 Immunodot Assay of XMMPS-605 Binding To Purified Lipopolysaccharides

Antigen	Reaction
Pseudomonas aeruginosa PAC 605*	2+
Pseudomonas aeruginosa Fisher 1	3+
Pseudomonas aeruginosa Fisher 2	3+
Pseudomonas aeruginosa Fisher 3	3+
Pseudomonas aeruginosa Fisher 4	3+
Pseudomonas aeruginosa Fisher 5	3+
Pseudomonas aeruginosa Fisher 6	3+
Pseudomonas aeruginosa Fisher 7	3+
Salmonella minnesota R60 (Ra)*	--
Salmonella minnesota R345 (Rb)*	--
Salmonella minnesota R7 (Rd)*	--
Salmonella minnesota R595 (Re)*	--
Escherichia coli J5 (Rc)*	--
Escherichia coli 055:B5	--
Klebsiella pneumoniae	--
Acinetobacter calcoaceticus	--

* LPS used in assay was a preparation of the core region isolated from mutants which do not express O-side chains.

E. Electrophoretic Transfer And Western Immunoblotting

The electrophoretic transfer of separated lysed whole cells and lipopolysaccharide components from SDS-PAGE gels to nitrocellulose sheet (Sartorius membranes) was adapted from the method of Towbin et al., Proc. Nat. Acad. Sci. U.S.A. (1979) 76:430-4354. The gels were placed in the Bio-Rad transblot electrophoretic transfer cell (Bio-Rad, Richmond, CA) containing 25 mM Tris, 19 mM glycine pH 8.3, 20% v/v methanol and 0.2% w/v/ SDS. The nitrocellulose sheet was placed on gel toward the anode in order to transfer the image from the gel to the sheet. Electrotransfer using electrophoresis constant power supply (Pharmacia, Inc., Piscataway, NJ) was carried out at 300 mA for 18 hours. After electrobotting, the nitrocellulose was washed in 0.01 M phosphate buffered saline (PBS) at room temperature for 1 hour. The nitrocellulose sheet or strips were washed 3 times. Next, the sheet or strip was incubated for 1 hour with XMMPS-605 monoclonal antibody at a concentration of 20 µg XMMPS-605/ml in 0.01 M PBS pH 7.2. The nitrocellulose sheet was washed twice for 10 minutes each and then incubated in a 1:400 goat anti-mouse IgG-peroxidase conjugate (Cappel Laboratories, Malvern, PA) (Fc fragment specific) for 1 hour. The nitrocellulose sheet was again washed 3 times as described above and the bound peroxidase conjugated second antibody was detected by soaking the nitrocellulose in the substrate-color-reagent 4-chloro-1-naphthol and hydrogen peroxide.

The standards used in the SDS-PAGE were: Cytochrome C (12.4 migration KD); Cytochrome C Dimer (24.8 migration KD); Cytochrone C Trimer (37.2 migration KD); Cytochrome C Tetramer (49.6 migration KD); and Cytochrome C HCX (74.4 migration KD). Molecular weights of bound components were determined relative to these standards.

Fig. 1 shows sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of whole cell extracts of Pseudomonas aeruginosa binding with XMMPS-605 by Western Immunoblot. The samples are as follows:

Lane	P. Aeruginosa Serotype
1	Fisher 1
2	Fisher 2
3	Fisher 3
4	Fisher 4
5	Fisher 5
6	Fisher 6
7	PAC 605

For each sample, binding was shown to a low molecular weight component of the cell lysate which migrated to approximately 12 kilodaltons (kD).

Fig. 2 shows SDS-PAGE analysis of P. aeruginosa lipopolysaccharide binding with XMMPS-605 by Western Immunoblot. The samples are as follows:

Lane	P. Aeruginosa Serotype
1	PAC 605 LPS
2	Fisher 1 LPS
3	Fisher 2 LPS
4	Fisher 4 LPS

Fig. 2 shows a binding pattern similar to that obtained with whole cell lysates, with binding to a low molecular weight component in the region of 12 kD. There is thus shown to be for all Fisher serotypes tested a common LPS component to which XMMPS-605 selectively binds. Lane 7 of Fig. 1 and Lane 1 of Fig. 2 also show binding of XMMPS-605 to a component of PAC 605 which migrates equivalently to a component of the Fisher strains, thus indicating that the antigenic determinant of the 12 kD component is in the core lipopolysaccharide rather than the O-specific side chains as PAC 605 does not express the O-specific side chains.

F. Polyacrylamide Gel Electrophoresis (SDS-PAGE) And Silver Staining

Sodium dodecyl sulfate gel electrophoresis was run using a modification of the method of Laemmli, Nature (1970) 227:680. The slab gels consisted of 10-20% linear acrylamide gradient with 1.6% bis in 3M Tris-HCl resolving buffer pH 8.8 and 4% acrylamide stacking gel in 0.5M Tris-HCl pH 6.8.

Washed live bacteria (1 x 10^{10} cells/ml) suspended in saline, and purified lipopolysaccharide samples in water (1 mg/ml) were mixed with equal parts of sample buffer containing 0.1 M Tris-HCl, 2% w/v SDS, 10% v/v glycerol, 1% v/v 2-mercaptoethanol and 0.01% bromophenol blue. The mixtures were heated in a 100°C water bath for 10 minutes and thirty microliters of sample were applied to slab gels. Electrophoresis was run at 35 mA per gel in Tris-glycine buffer pH 8.8 until the tracking dye exited the gels.

The sensitive silver stain method of Tsai and Frasch, Analyt. Biochem. (1982) 119:115-119, was used to detect lipopolysaccharide in polyacrylamide gels. Electrophoretically resolved denatured whole cell components were stained according to the silver stain method of Morrissey, Anal. Biochem. (1981) 117:307.

G. Enhanced Opsonophagocytosis Of Bacteria In Presence Of Antibody

All seven Fisher serotypes of Pseudomonas aeruginosa were tested in vitro to determine their effect on certain aspects of the immune response with which a host would counteract a bacterial infection, such as opsonization and phagocytosis of invading cells. All bacteria were grown overnight on blood agar. After a visual check for purity, the isolates were transferred to Brain Heart Infusion Broth (BBL Microbiology Systems, Cockeysville, MD) and grown for 4 hours at 37°C with constant shaking. The bacteria were washed twice

with Hanks Balanced Salt Solution (HBSS), and then brought to a concentration of 1 x 10^8 organisms/ml by comparing the turbidity to an appropriate McFarland standard.

Opsonophagocytosis was measured in a chemiluminescence assay using a Beckman LS-250 liquid scintillation spectrophotometer (Beckman Instruments, Inc., Fullerton, CA). Chemiluminescence is the light released by a white cell after it has engulfed a bacteria. The reaction was performed in the dark, using previously dark-adapted polypropylene scintillation vials. The vials contained 0.9 ml of HBSS, 0.1 ml of luminol (2 x 10^{-5}M) and 0.075 ml of diluted human whole blood. The background was equilibrated for about 15 minutes, to reach approximately 15,000 CPM.

Heparinized blood was obtained from healthy donors. To 5 ml of blood, 1 ml of dextran (6%) was added. The erythrocytes were allowed to sediment for 60 minutes. The upper layer, containing the neutrophils, was centrifuged at 1000 x G for 10 minutes. To lyse the remaining erythrocytes, the pellet was exposed to hypotonic saline (0.22%) for 30 seconds. Then, an equal volume of 1.54% saline was added to restore isotonicity. The neutrophils were suspended in HBSS and the final cell suspension adjusted to 2 x 10^6 cells/ml.

For each strain tested with XMMPS-605, a graph of counts per minutes (CPM) vs. time elapsed (minutes) was prepared. Numbers shown on Table 5 indicate maximum CPM values for each strain tested. The time at which maximum counts occurred varied according to strain, ranging from 30-80 minutes. These data indicate that for all serotypes except Fisher 1, phagocytosis is enhanced by the addition of XMMPS-605 above that obtained in the control sample containing only HBSS and in the samples containing only pooled human serum.

Table 5 Chemiluminescent Determination Of Opsonophagocytosis

XMMPS-605 Activity vs. Pseudomonas aeruginosa

Bacteria	Peak Counts x 10^3 HBSS (Control)	PHS	PHS + PCB5
P. aeruginosa PAC 605	38	36	135
P. aeruginosa Fisher type 1	38	36	36
P. aeruginosa Fisher type 2	45	85	170
P. aeruginosa Fisher type 3	22	60	98
P. aeruginosa Fisher type 4	22	30	120
P. aeruginosa Fisher type 5	38	82	120
P. aeruginosa Fisher type 6	35	45	110
P. aeruginosa Fisher type 7	40	50	135

HBSS = Hank's Balanced Salt Solution
PHS = Pooled Human Serum
XMMPS-605 MoAb: Concentration = 2 µg/ml

As a confirmatory method of determining levels of cell death due to opsonization and phagocytosis, direct measurements of bacterial mortality were made. A test tube containing appropriate medium was innoculated with 2 x 10^6 polymorphonuclear cells/ml and 1.5 x 10^7 bacteria/ml (ratio 1:10). The tubes were incubated at 37°C in a water bath. At 0, 60, and 120 minutes, dilutions were made in sterilized water and the number of colony forming units (CFU) growing on Trypticase-Soy Agar (TSA) plates were determined by counting microscopically. The results confirmed the opsonic activity of XMMPS-605 as demonstrated in the chemiluminescent assays. These results are summarized in Table 6.

Table 6 Measurement of Opsonophagocytosis By Cell Mortality Determination

PCB5 MoAb Activity vs. Pseudomonas aeruginosa

Bacteria	Colony forming units/ml x 10^3* Bacteria Only	PHS	PCB5 + PHS
P. aeruginosa Fisher type 1	78,000	6,800	6,800
P. aeruginosa Fisher type 2	74,000	6,100	480
P. aeruginosa Fisher type 3	74,000	6,400	530
P. aeruginosa Fisher type 4	76,000	6,900	49
P. aeruginosa Fisher type 5	78,000	6,400	550
P. aeruginosa Fisher type 6	76,000	6,300	46
P. aeruginosa Fisher type 7	75,000	590	49

* Counts of bacteria performed on tubes which were incubated for 60 minutes in a 37°C water bath.

PHS = Pooled Human Serum

XMMPS-605 : Concentration = 2 µg/ml

H. Use Of XMMPS-605 Monoclonal Antibody For Prophylaxis

150 µg of affinity purified XMMPS-605 monoclonal antibody was injected i.p. into 4-week-old CD-1 female mice (Charles River Breeding Labs, Inc., Wilmington, MA). Live bacteria were prepared as follows: strains of P. aeruginosa were grown in Brain Heart Infusion broth (BBL Microbiology Systems, Cockeysville, MD) overnight at 37°C. The bacterial cells were washed twice with sterile saline, then adjusted to approximately 1 x 10^9 cells/ml saline, by comparing the optical density of the cell suspension to a standard curve relating absorbance to viable cell numbers. Approximately 18 hours after XMMPS-605 monoclonal antibody was injected, an approximate LD_{100} dose of Pseudomonas aeruginosa cells was injected i.p. into experimental mice and control mice (which received the same protocol, but no XMMPS-605 monoclonal antibody). A previous dose-response study determined the LD_{100}, which was defined as the lowest dose that would kill 100% of the mice. Survivors were recorded at 48 hours. The results for individuals receiving Fisher 3 and 4 inoculation at a dosage of about 1 x 10^8 cells/individual indicate that pretreatment with XMMPS-605 conferred enhanced ability to survive the bacterial infection. These results are summarized in Table 7.

Table 7 Animal Studies: Prophylactic Treatment Of Pseudomonas Aeruginosa Infection With XMMPS-605

Organism	Survivors: XMMPS-605 Pretreatment	Survivors: No Pretreatment
P. aeruginosa Fisher 1 (Dose = 1 x 10^8 bacteria/individual)	2/19	1/19
P. aeruginosa Fisher 2 (Dose = 2.5 x 10^8 bacteria/individual)	4/29	3/29
P. aeruginosa Fisher 3 (Dose = 1 x 10^8 bacteria/individual)	8/27(30%)	4/27(15%)
P. aeruginosa Fisher 4 (Dose = 1 x 10^8 bacteria/individual)	13/27(48%)	2/27(11%)

I. "Checkerboard" ELISA Assessment of the XMMPS-605 Determined Epitope

To clearly define that antibody XMMPS-605 recognizes a core LPS determinant which is distinguishable from serotype-defined LPS structures, a series of experiments was conducted. These included "checkerboard" ELISA and Western blot assessment of the XMMPS-605-determined epitope expressed on Fisher 1 and PAC 605 LPS molecules. These results were supported by competitive ELISA assays using solid-phase immobilized Fisher 1 LPS molecules.

One hundred µl of an aqueous solution (25 µg/ml) of Fisher 1, E.coli J5 (both from List Biological Laboratories, Inc., Campbell, CA) or PAC 605 (described above) lipopolysaccharides were incubated overnight at room temperature (RT) in wells of either Falcon 3912 MicroTest III poylvinyl chloride (Becton Dickenson Labware, Oxnard, CA) or Immulon 2 polystyrene (Dynatech Laboratories, Inc., Chantilly, VA) 96-well

flat-bottomed plates. Fisher 1 and J5 LPS solutions contained 0.05% triethylamine (TEA), while the PAC 605 LPS solution contained 0.5% TEA in distilled water. After washing twice with 0.01 M PBS with 0.02 M $MgCl_2$ (PBS), pH 7.2, wells were blocked with PBS containing 1% gelatin (Bacto, Difco Laboratories, Detroit, MI) (PBS/gelatin) for 2 hours at 37°C. After two washes with PBS, 100 µl of primary monoclonal antibodies XMMPS-OP1 (PIC9, Stoll et al., Infect. Immun. (1986)53:656-62) or XMMPS-605 diluted to 5 µg/ml in PBS or PBS containing 0.05% Tween-20 (Sigma Chemical Co., St. Louis, MO) (PBS/Tween) were incubated at RT for 90 min. Controls consisted of gelatin-blocked wells without LPS antigen incubated with primary monoclonal antibodies, and wells with PBS or PBS/Tween diluents incubated in place of monoclonal antibody. The values shown below in Table 8 are corrected values relative to PBS or PBS/Tween incubations with no LPS controls. Wells were then washed three times with 1% gelatin in either PBS (PBS/gelatin) or PBS/Tween (PBS/Tween/gelatin), and incubated 1 h at RT with horseradish peroxidase-conjugated goat IgG fraction anti-mouse immunoglobulins (IgA, IgG and IgM, heavy and light chain specific, Cappel Laboratories, Cochranville, PA) diluted 1:1000 in PBS/gelatin or PBS/Tween/gelatin. After washing four times with PBS/gelatin or PBS/Tween/gelatin, 100 µl of ABTS (2,2´-azino-di-[3-ethylbenzthiiazonline sulfonate (6)], Boehringer Mannheim Biochemicals, Indianapolis, IN) substrate was added. This was prepared directly before use by making a 1:50 dilution of a 20 mg/ml stock solution in citrate buffer, pH 4.5, and adding 30% H_2O_2 to make a 1:1000 dilution. Color development was allowed to proceed for 20 min and absorbance was read at 405 nm (EIA Autoreader Model EL310, Bio-Tek Instruments, Inc., Burlington, VT). Results are expressed as mean absorbance values from duplicate wells. The results shown in Table 8 clearly indicate that monoclonals XMMPS-605 and XMMPS-OP1

recognize separate epitopes on the Fisher 1 LPS molecule and that the XMMPS-605-determined epitope is shared by the PAC 605 LPS molecule and the Fisher 1 LPS molecule.

Table 8. "Checkerboard" ELISA

Corrected OD_{405} Values

Primary antibody	Plate	Tween	LPS Type J5	PAC 605	Fisher 1
XMMPS-OP1	Falcon	-	0.03	0.01	1.50
		+	0.11	0.10	2.46
	Dynatech	-	----*	0.04	1.42
		+	----	0.01	>3.0
XMMPS-605	Falcon	-	0.22	0.66	0.95
		+	----	0.98	0.56
	Dynatech	-	0.10	0.41	0.57
		+	----	0.69	0.21

* Indicates 0.00 or negative value when control background values are subtracted.

J. "Checkerboard" Immunodot Assessment of the XMMPS-605-Determined Epitope.

An immunodot assay was performed in a checkerboard fashion to further define that the XMMPS-605- and XMMPS-OP1-determined epitopes on the Fisher 1 LPS molecules are distinct entities. The results are summarized in Table 9. Antibody XMMEN-OE5 was included as a negative control in addition to the PBS and no LPS controls.

Purified LPS was applied to nitrocellulose paper (Trans-Blot Transfer Medium, 0.45 µM, Bio-Rad, Richmond, CA) as 1 µl "dots" at positions corresponding to the wells of a 96 well vacuum filter manifold (Hybri-Dot Manifold, Bethesda Research Laboratories, Gaithersburg, MD). LPS was prepared in PBS at a concentration

of 100 µg/ml for Fisher 1 LPS (0.1 µg/dot) and 500 µg/ml for PAC 605 LPS (0.5 µg/dot). The paper was air dried for 15 minutes at RT and excess binding sites were blocked with PBS/gelatin for 30 minutes at RT. The paper was washed by addition of 200 µl PBS/well, and monoclonal antibodies were added (50 µl/well) at a concentration of 10 µg/ml in PBS. The paper was washed three times with washing buffer (0.1% gelatin, 0.05% Tween-20 in PBS, 100 µl/well/wash). Dots were developed with a 1:500 dilution of affinity-purified, peroxidose-conjugated rabbit anti-mouse IgG and IgM (H+2, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in washing buffer at 50 µl/well. The paper was washed four times with washing buffer (100 µl/well/wash) and bound peroxidase-conjugated second antibody was detected using the substrate 4-chloro-1-naphthol. Blots were scored for intensity as described above. The results are again consistent with the recognition of distinct epitopes on the Fisher 1 LPS molecule by XMMPS-OP1 and XMMPS-605.

Table 9. "Checkerboard" Immunodot

Immunodot Intensity			
Primary	LPS type		
antibody	no LPS	PAC 605	Fisher 1
none*	0	0	0
XMMEN-0E5	0	0	0
XMMPS-605	0	2	2
XMMPS-OP1	0	0	4

* PBS Control

K. Western Blot Assessment of Fisher 1 LPS Molecular Heterogeneity and the Distinct LPS and Common LPS Molecules Recognized by XMMPS-605 and XMMPS-OP1

Molecular heterogeneity can be detected when smooth LPS preparations are assessed by SDS-polyacrylamide gel electrophoresis. The lowest molecular weight species on such gels are lipid A-core oligosaccharide molecules which differ in the number of core oligosaccharide components. The high molecular weight species are molecules with a complete core component and covalently linked oligosaccharide side chain. The species bearing oligosaccharide side chains can be differentiated from complete core and partial core components by Western blot analysis with a serotype-specific (oligosaccharide side chain-specific) antiserum or monoclonal antibody.

Although our results indicate that the XMMPS-605-determined epitope is represented in LPS preparations from a wide variety of Pseudomonas isolates, as described above had not determined that this epitope is intact or accessible on molecules with oligosaccharide side chain. That is, XMMPS-605 could be reactive with an oligosaccharide side chain-deficient subpopulation of LPS molecules (core) from any given smooth strain. Therefore Western blot analysis (described below) was performed to determine the intactness and accessibility of the epitope.

Gel electrophoresis and electrophoretic transfer to nitrocellulose paper (blotting) were performed by the techniques of Sidberry et al., J. Immunol. Methods (1985)76:299-305, with minor modifications. Briefly, electrophoresis was carried out using a 4.5% acrlamide stacking gel and a 15% running gel. Transfer to 0.2µM nitocellulose paper (Sartorius-Membranfilter GmbH, Goettingen, West Germany) was achieved by electroblotting overnight at 100 mA using a Trans-Blot cell (Bio-Rad, Richmond, CA). Excess nitrocellulose binding sites were blocked using 5% non-fat dry milk, 0.01% antifoam

A in PBS. LPS blots were visualized by development with antibody XMMPS-605 or antibody XMMPS-OP1 (both used at 5 µg/ml in PBS with 1% gelatin) followed by affinity-purified, peroxidase-conjugated rabbit anti-mouse IgG and IgM (H + L, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) diluted 1:500 in washing buffer (PBS with 0.05% Tween). Bound peroxidase-conjugated second antibody was detected using the substrate 4-chloro-1-naphthol.

The Western blot shown in Fig. 3 indicates that antibody XMMPS-605 is reactive with higher molecular weight forms of Fisher 1 LPS, core LPS from Fisher 1, and core LPS from PAC 605. Antibody XMMPS-OP1 is reactive only with high molecular weight Fisher 1 LPS molecules which bear oligosaccharide side chains. (Fig. 5 legend- A: molecular weight standards. B: blot developed with PBB/gelatin (control). Lane 1, Fisher 1 LPS (List Biological); Lane 2, PAC 605 LPS; Lane 3, Fisher 1 LPS (Ribi Immunochem). C: blot developed with XMMPS-OP1. Lane contents as in B. D: blot developed with XMMPS-605. Lane contents as in B. The reactivity of XMMPS-605 with PAC 605 LPS can not be clearly seen in the photograph. The PAC 605 LPS preparation used here was clearly resolved into a major low molecular weight component following SDS-PAGE electrophoresis (see Fig. 4). The blot developed using XMMPS-605 and PAC 605 LPS clearly corresponded to this low molecular weight component. In Fig. 4 the gel used for the Western blot described in Fig. 3 was silver stained by the procedure of Sidberry et al. after electrophoretic transfer. A similar gel which was stained without electrophoretic transfer (not shown) confirmed that the PAC 605 LPS preparation consisted primarily of a single low molecular weight component.

The Western blot shown in Fig. 5 confirms these observations and further indicates that the antibody produced by the XMMPS-605 hybridoma line is

reactive with higher molecular weight forms of Fisher 2 LPS and core LPS, while antibody produced by the XMMPS-OP1 hybridoma line is not reactive with Fisher 2 LPS. In Fig. 5 gel electrophoresis and electrophoretic transfer were performed as described in Fig. 3 except that the running gel was a 10-15% acrylamide gradient. (Fig. 5 legend- A: molecular weight standards. B: blot developed with XMMPS-605. Lane 1, Fisher 1 LPS (List Biological); Lane 2, PAC 605 LPS; Lane 3, Fisher 2 LPS (List Biological); Lane 2, PAC 605 LPS; Lane 3, Fisher 2 LPS (List Biological). C: blot developed with PBS/gelatin (control). Lane contents as in B. D: blot developed with XMMPS-OP1. Lane contents as in B.) These results prove that the XMMPS-605-determined epitope is intact and accessible on SDS-dissociated LPS molecules with oligosaccharide side chains.

EXAMPLE II - A HUMAN MONOCLONAL ANTIBODY WHICH REACTS WITH THE XMMPS-605-DETERMINED EPITOPE

A. Description of the Screening Assay.

A competitive inhibition ELISA was established to screen for human monoclonal antibodies which recognize the XMMPS-605-determined epitope associated with Pseudomonas core LPS. One hundred µl of 25µg/ml solution of monoclonal antibody XMMPS-OP1 in 0.01 M PBS with 0.02 M $MgCl_2$, pH 7.2 (PBS) was applied to wells of Immulon 2 polystyrene 96-well flat-bottomed plates (Dynatech Laboratories, Inc., Chantilly, VA) and incubated overnight at RT. After washing three times with PBS, wells were blocked with PBS containing 1.0% gelatin (PBS/gelatin) for 2 h at 37°C. After two additional washes with PBS, 100 µl of a 25 µg/ml solution of P. aeruginosa Fisher 1 LPS (List Biological Laboratories, Campbell, CA) in PBS was incubated for 90 min at RT. Control wells consisted of (1) gelatin-blocked wells with neither XMMPS-OP1 nor LPS antigen applied, (2) wells incubated with XMMPS-OP1, gelatin-blocked, but without the

addition of Fisher 1 LPS, and (3) wells incubated overnight with PBS alone, gelatin-blocked, and incubated with Fisher 1 LPS. After LPS incubation, wells were washed four times with PBS/gelatin containing 0.05% Tween-20 (PBS/Tween/gelatin). Next, 100 µl of either a 25 µg/ml solution of XMMPS-605 (competitor) in PBS/Tween/gelatin or PBS/Tween/gelatin alone (control) was incubated at RT for 90 min. Wells were then washed four times with PBS/Tween/gelatin, incubated at RT for 90 min with 100 µl of 5.0 µg/ml of alkaline phosphatase-conjugated XMMPS-605 (XMMPS-605-AP, (conjugated according to the method of Dertzbaugh et al. J. Immunol. Meth. (1985)83:169-77, with minor modifications)) in PBS/Tween/gelatin, and washed four times with PBS/Tween/gelatin. XMMPS-605-AP bound to the LPS, with or without competing XMMPS-605, was quantitated by the addition of 100 µl of 1.0 mg/ml p-nitrophenyl phosphate substrate (Sigma 104 phosphatase substrate, Sigma Chemical Co., St. Louis, MO) in 0.1 M glycine buffer, pH 10.4, containing 1 mM $MgCl_2$ and 1 mM $ZnCl_2$. Absorbance at 405 nm was read using an EIA Autoreader (model EL310, Bio-Tek Instruments, Inc., Burlington, VT). The results are presented as mean absorbance and are summarized in Table 10. Using this assay, a clear ELISA signal is generated by the binding of XMMPS-605-AP to immobilized Pseudomonas LPS, and this signal is inhibited by competitor XMMPS-605 antibody.

Table 10. Competitive Inhibition ELISA

Corrected OD_{405} Values

XMMPS-605 competitor	Solid phase XMMPS-OP1	Fisher 1 LPS	OD405
-*	+	-	0.02
-	-	+	----[b]
-	+	+	1.64
+	+	-	0.04
+	-	+	0.08
+	+	+	0.53

* indicates PBS control.

[b] indicates 0.00 or negative value when control background values are subtracted.

In this particular assay, the Fisher 1 LPS antigen was solid phase-immobilized using antibody XMMPS-OP1. Because LPS in aqueous solutions consists of aggregates rather than dispersed molecules, solid phase immobilized XMMPS-605 could be used in place of XMMPS-OP1. If the solid-phase antibody used was XMMPS-605, then other Pseudomonas LPS types, including PAC 605 LPS, could be used in place of Fisher 1 LPS. In the assay described here XMMPS-OP1 was solid-phase immobilized by passive adsorption to plastic. As will be apparent to those skilled in the art the antibody could be biotinylated and solid phase-immobilized using avidin-coated plastic. Similarly, antigen adsorption could be achieved using a variety of serotype-specific monoclonal or polyclonal antibodies or other molecules with demonstrated affinity for lipopolysaccharides. In addition, certain Pseudomonas lipopolysaccharide antigens could be directly adsorbed onto the solid phase.

B. Production of Human Monoclonal Antibodies

Peripheral blood mononuclear cells are isolated from the blood of patients who have been exposed to gram negative bacteria, such as cystic fibrosis patients, by centrifugation of the buffy coat cells over Ficoll Hypaque. The T lymphocytes are depleted from the peripheral blood mononuclear cells by rosetting with AET (2-amino-ethyl-isothiouronium)-treated sheep red blood cells (SRBC). B lymphocytes are isolated by centrifuging the rosetted peripheral blood mononuclear cells over Ficoll Hypaque and incubating the non-rosetted cells at 37°C for 2 h on plastic to deplete monocytes. The subsequent non-adherent B lymphocyte population is harvested. Alternate sources for obtaining human lymphocytes include tonsils, spleen or lymph nodes, using similar isolation techniques.

The harvested B lymphocytes are incubated for 2 hours at 37°C at 2 X 10^6 cells/ml with a 1:4 dilution of supernatant from B95-8 cells (A.T.C.C. No. CRL 1612), an Epstein-Barr virus (EBV) transformed marmoset lymphoma cell line which releases high titers of transforming EBV. This B95-8 supernatant is collected 4 days after initiation to culture of 5 X 10^5 cells/ml in RPMI 1640 with L glutamine, 15% fetal bovine serum, penicillin-streptomycin, nonessential amino acids, and pyruvate. (Other techniques of EBV transformation can be used, as described previously). The B cells are then centrifuged out of the B95-8 supernatant and plated in 96-well plates at 10^4 cells/well in a total volume of 100 µl/well. The B cells may be cultured in the presence or absence of irradiated normal human peripheral blood buffy coat cells (i.e., "feeder cells"). These cultures of EBV transformed cells are allowed to proliferate for a given period of time, for example 14 days, or until they display marked proliferation. In order to isolate cells producing human MoAb having XMMPS-605-like activity, the supernatant from the cultures are harvested and

screened on P. aeruginosa LPS in the presence or absence of XMMPS-605, using standard ELISA techniques previously described. The plates are developed with enzyme-conjugated anti-human IgG antiserum which detects both heavy and light chains, and which has little or no cross-reactivity with mouse immunoglobulin ("Rabbit α human IgG (H + L), minimum cross reactivity with mouse serum proteins" or "Mouse α human IgG (H + L), minimum cross-reactivity with mouse serum proteins" (both by Jackson Immunoresearch Labs Inc., West Grove, PA). The B cells from those wells showing positive reactions with LPS which has not been preincubated with XMMPS-605 and showing reduced reactions with LPS which has been preincubated with XMMPS-605 are expanded and cloned using limiting dilution techniques. EBV transformants may be expanded directly without fusion. Alternately, clonal populations or enriched (by limiting dilution techniques) populations of specific antibody secreting cells are then fused with a suitable enzyme-deficient myeloma, lymphoblastoid cell line or heteromyeloma, such as, respectively, NS-1 (A.T.C.C. No. TIB 18), WIL2-NS (A.T.C.C. No. CRL 8155), or SHM-D33 (A.T.C.C. No. CRL 1668).

EXAMPLE III - HYBRIDOMA XMMPS-OP1

A. Production of XMMPS-OP1 Hybridomas

Balb/c mice (Charles Rivers, Wilmington, MA) were immunized with 1 x 10^8 boiled cells of P. aeruginosa Fisher Type 1. After primary immunization, the mice were boosted with an intraperitoneal injection of 1 x 10^8 boiled cells at one month intervals, for two months.

Four days following the last antigen boost, spleen cells from an immunized mouse were aseptically removed. Following procedures as outlined elsewhere (St. Groth, J. Immuno. Meth. (1980) 35:1), 5 x 10^7 spleen cells were fused with an equal number of SP2/0-Ag14, as previously described, using polyethylene glycol 4000

(Merck and Co., Inc., Rahway, NJ). Hybrid cells were placed into 96-well culture plates (Costar, Cambridge, MA #3596) on medium which had been pre-incubated with a feeder layer of normal Balb/c thymocytes (1 x 10^5 cells per well, one day before fusion). Cells were cultured at 37°C, in a 10% CO_2 atmosphere, in the following medium for the first two weeks. Dulbecco's Modified Eagle's Medium, with glutamine, and glucose at 4.5 g/l (Gibco, Santa Clara, CA, #320-1965), Fetal Bovine Serum (10%) (Microbiological Associates, Walkerville, MD), sodium pyruvate (1 mM) (Gibco, Santa Clara, CA, #320-1360), penicillin (50 µ/ml) -- Streptomycin (50 µ/ml) (Gibco, Santa Clara, CA, #600-5070), and hypoxanthine-aminopterin-thymidine (HAT) which was prepared by using hypoxanthine (10 mM) thymidine (1.6 mM) combined with 0.04 mM aminopterin (Sigma Chemical Co., St. Louis, MO). Medium for regular maintenance (past two-weeks fusion date) was identical to the above, except that no aminopterin was included in the medium (HT medium).

Between two to four weeks post-fusion, cultures of hybrid cells were tested for antibody binding to purified LPS by EIA. Cultures that were positive on repeated testing were then cloned by limiting dilution techniques. Briefly, the cells were cultured at a concentration of between 10-1 cells per well in 96-well tissue culture plates (Costar, Cambridge, MA, #3596). Wells that contained only one colony were identified by microscopic examination, then tested for anti-Fisher Type 1 activity by EIA. Positive clones were expanded onto 24-well tissue cultures plates (Costar, Cambridge, MA #3524), recloned and retested by the same methods. A clone, designated XMMPS-OP1, was found to stably secrete monoclonal antibody; the monoclonal antibody was determined to be of immunoglobulin class IgG1 by radial immunodiffusion and EIA using standard methods.

Balb/c mice (Charles River) were used to culture the hybridomas intraperitoneally. Approximately 3

x 10^6 hybridoma cells were injected intraperitoneally (i.p.) into mice that had been pretreated as follows: injected i.p. one week earlier with 0.5 ml of pristane (Aldrich Chemical Co., Milwaukee, WI). The resultant ascites fluid, collected 11-15 days after injection of the hybridomas, contained on average 5-20 mg/ml of the antibody, as determined by radial-immunodiffusion (Meloy, Radial Immunodiffusion, Springfield, VA, Plate #J-304).

The antibody in ascites fluid was purified by using a Protein A Sepharose Cl-4B column (Pharmacia, Inc., Piscataway, NJ) by methods well-known to those skilled in the art, as described elsewhere (Ey, Immunochemistry (1978) 15:429-436). Determination of immunoglobulin subclass (IgG1) was accomplished by EIA using rabbit anti-mouse subclass antibody (Miles Labs, Naperville, IL) and anti-rabbit Ig conjugated to peroxidase (Cappel, Malvern, PA).

Hybridoma cell line XMMPS-OP1 was deposited with the American Type Culture Collection (A.T.C.C.) on April 2, 1987 and given A.T.C.C. Accession NO. HB 9384.

EXAMPLE IV - DIAGNOSTIC EIA

Monoclonal antibodies of the present invention, or their functional equivalents, are utilized in an immunoassay using standard and well-known methods (for example, Methods in Immunodiagnosis, 2nd Edition, Rose and Bigazzi, eds., John Wiley and Sons, 1980). Such assay may be, for example, of direct format (labelled first antibody reactive with the antigen), an indirect format (a labelled second antibody reactive with the first antibody), a competitive format (for example, addition of labelled antigen), or a sandwich format (both labelled and unlabelled antibody), as well as other formats well-known in the art.

In one such embodiment, a tissue extract from a patient suspected of having a bacterial infection is applied to an insoluble matrix or solid substrate, such

as cellulose strips, agarose or other particles so as to produce a bacteria-substrate complex. Alternatively, the substrate may have attached thereto an antibody so as to effect attachment of bacteria from a solution onto the substrate. The substrate is then washed, preferably with PBS, to remove unbound materials.

EXAMPLE V - THERAPEUTIC APPLICATIONS

A number of examples of in vivo application for the murine monoclonals are provided. The parameters for the human monoclonals are essentially identical to those described for the murine monoclonal antibodies.

Early data from the Phase I clinical trial of monoclonal antibodies produced by hybridoma XMMEN-OE5 are in anecdotal form as reported below.

1. The first patient, F.R., was a 60 year old male with suspected gram negative sepsis. He received 0.1 mg/kg (total dose 8.5 mg) of XMMEN-OE5 i.v. over one hour. He experienced no adverse effects. Blood cultures subsequently demonstrated that he did not have gram negative bacteremia, but instead had a raging fungemia due to Torulopsis. The fungal abscess was surgically drained and he remained in stable condition three weeks after the administration of antibody.

2. The second patient, T.G., was a 57 year old female with documented gram negative bacteremia and pyelonephritis secondary to ureteral obstruction. She underwent surgical nephrostomy placement and received 0.5 mg/kg (total dose 36 mg) of XMMEN-OE5 i.v. over 1½ hours. She experienced no adverse effects, rapidly defervesced, and remained afebrile 12 hours after infusion. The prompt resolution of her fever is possibly due to the administration of antibody.

3. The third patient, K.S., was a 60 year old female with severe coronary artery disease who was admitted for unstable angina. She underwent coronary artery bypass surgery which was complicated by a

transient episode of acute renal failure. Post-operatively, she developed a fever of 102.8° and subsequently had Acinetobacter cultured from her blood. Antibiotics were started, and ten days post surgery, she received XMMEN-OE5 0.5 mg/kg (total dose 42 mg) i.v. over 1½ hours. Following antibiotic and anti-endotoxin antibody treatment, blood cultures became negative and her condition steadily improved. She remained in stable condition one week after administration of antibody.

4. The fourth patient, S.D., was a 60 year old female with persistent gram negative bacteremia (Klebsiella) of unknown etiology. An extensive workup revealed no source of infection, but urinary abnormalities made pyelonephritis the most likely cause. On her third hospital day, despite antibiotic therapy, her blood cultures again grew Klebsiella organisms. She experienced spiking fevers up to 104.5°. An emergency CT scan of her body was performed with the hope of locating an abscess which could be drained in an attempt to improve her clinical condition. No abscess or other site of infection was found. She was then treated with XMMEN-OE5 monoclonal antibody 2 mg/kg (total dose 157 mg) i.v. over 2½ hours. Twelve hours after infusion, her temperature was below 100°, and her clinical condition had improved markedly. Blood cultures were repeated and continued to grow gram negative organisms. Therefore, despite continued gram negative sepsis, her clinical condition improved markedly following the administration of monoclonal antibody. Her antibiotic regimen was then changed to a different combination of drugs, and her blood cultures subsequently became negative. She remained in improved condition 4 days after antibody therapy.

EXAMPLE VI - IMAGING OF LOCALIZED BACTERIAL INFECTION

Monoclonal antibodies which react selectively with an antigenic determinant common to strains of infecting bacteria were utilized to determine the location and extent of a localized bacterial infection, especially those caused by gram negative bacteria, by methods well-known in the art, for example, Larson, et al., Journal of Clinical Investigation (1983) 72:2101, which is incorporated by reference. Monoclonal antibodies are preferably radiolabelled by radioiodination or by other radiolabelling techniques well-known in the art, such as chelation using a chelating agent such as diethylenetriaminepenta-acetic acid (DTPA); or are otherwise labelled, such as with agents having paramagnetic properties, with chemiluminescent substrates, or with components of an enzymatic reaction. The radiolabelled monoclonal antibodies are purified and formulated for pharmaceutical use. A solution of the labelled monoclonal antibodies in a carrier, for example in phosphate buffered saline, is injected intravenously into a host. The appropriate dose is in the range of about 100 µg to 50 mg. Time is permitted for the antibodies to migrate to regions of the body having concentrations of cells with antigenic determinants reactive therewith. Concentrations of radioisotopes in certain tissues are determined or may be mapped either by techniques of whole body imaging which are well-known in the art (See, for example, Rainsbury, et al., Lancet October 22, 1983, 934 which is incorporated by reference), or by evaluating biopsied tissue or extracted body fluid using a scintillation counter. Where non-radioactive labels are used, other appropriate monitoring means are employed, such as a detector of nuclear magnetic resonance or a spectrophotometer. Areas of high radiation levels are indicative of the presence of localized bacterial infection.

Although the present invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the amended claims.

WHAT IS CLAIMED IS:

1. A method for producing human monoclonal antibodies capable of binding an epitope on gram negative bacterial core lipopolysaccharide comprising the steps of (1) screening monoclonal antibodies secreted from transformed human cell lines by employing a competitive assay against non-human monoclonal antibodies capable of binding said epitope and (2) selecting human cell lines which secrete monoclonal antibodies competitive with said non-human monoclonal antibodies.

2. The method according to Claim 1, wherein the human cell line is derived from human B lymphocytes from a patient hosting an infection of gram negative bacteria.

3. The method according to Claim 1, wherein the human cell line has been transformed with Epstein-Barr Virus.

4. The method according to Claim 3, wherein the Epstein-Barr virus is released by B95-8 marmoset lymphoma cells designated A.T.C.C. Accession No. CRL 1612.

5. The method according to Claim 1, wherein the non-human monoclonal antibodies are secreted by a hybridoma cell line selected from the group consisting of the cell lines designated A.T.C.C. Accession No. HB 8909, A.T.C.C. Accession No. HB 9081, A.T.C.C. Accession No. HB 9082, and A.T.C.C. Accession No. HB 9083.

6. A human monoclonal antibody produced by the method of Claim 1.

7. The monoclonal antibody of Claim 6 wherein said monoclonal antibody is attached to a detectable label.

8. A human cell line selected by the method of Claim 1.

9. A diagnostic method for detecting the presence of bacterial cells in a solution suspected of harboring said cells, comprising the steps of:

a) attaching said cells in said solution to a solid support;

b) contacting said solid support with labelled monoclonal antibodies according to Claim 7; and

c) monitoring said solid support for the presence of said labelled antibody.

10. The diagnostic method of Claim 9, wherein said detectable label is capable of emitting radiation.

11. The method of Claim 10 wherein the in vivo detectable label is a radioisotope.

12. A method for detecting a localized bacterial infection in a human patient comprising the steps of:

a) administering to said patient monoclonal antibodies according to Claim 7 wherein said detectable label is an in vivo detectable label;

b) permitting said labeled monoclonal antibodies to accumulate in said localized infection; and

c) monitoring said patient so as to determine the site of said localized infection.

13. A method of prophylactically treating human patients at risk for bacterial infection, comprising administering to said patient a prophylactically

effective amount of monoclonal antibody according to Claim 6, whereby the risk for infection is reduced.

14. A method of therapeutically treating a human patient infected with bacterial cells comprising administering to said patient a therapeutically effective amount of monoclonal antibody according to Claim 6 whereby said infection is alleviated.

15. A kit for use in a method for detecting, treating or preventing gram negative infection in a human patient comprising a monoclonal antibody according to Claim 7.

16. A kit according to Claim 13 wherein said detectable label is a radioisotope.

Fig. 1

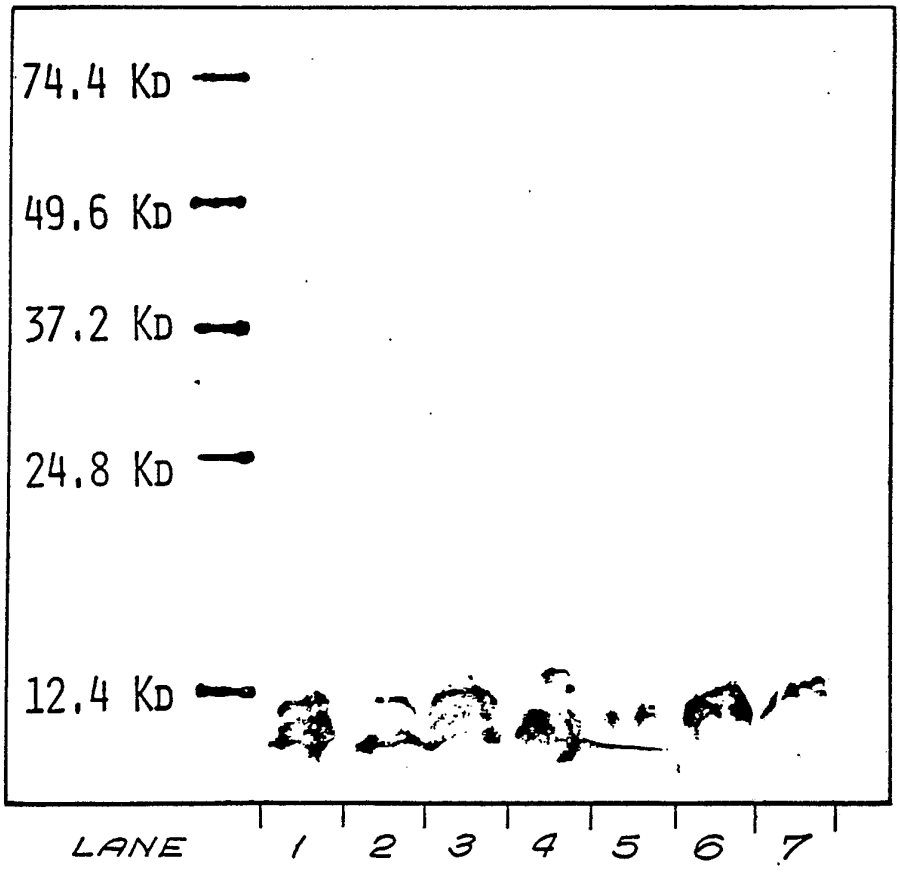
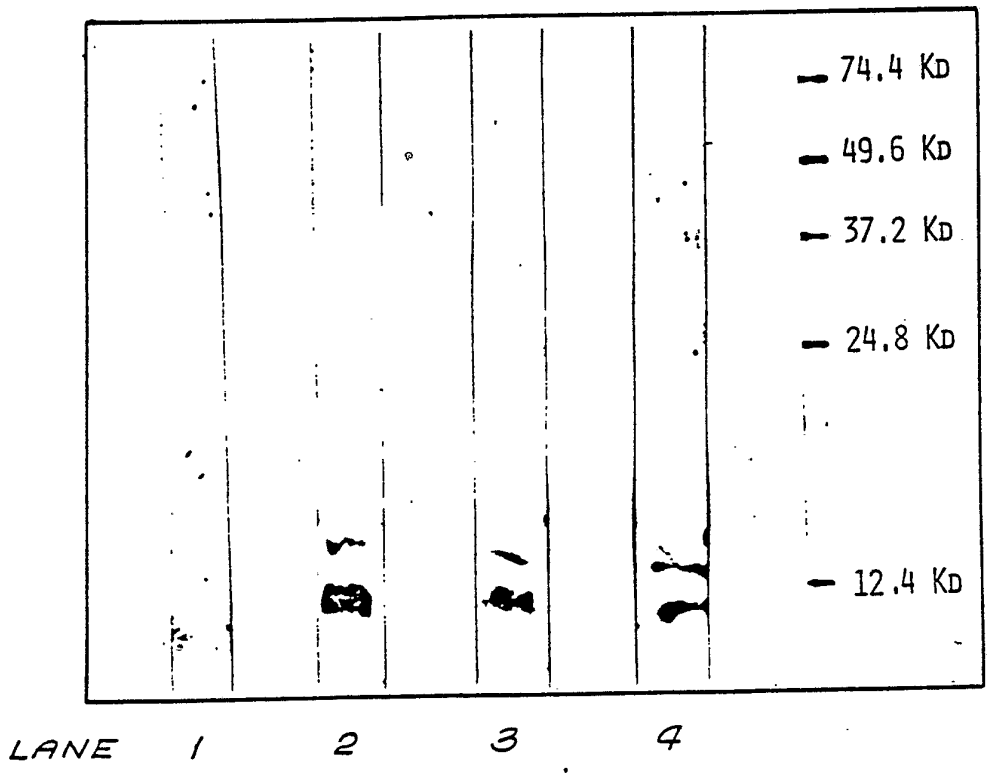


Fig. 2



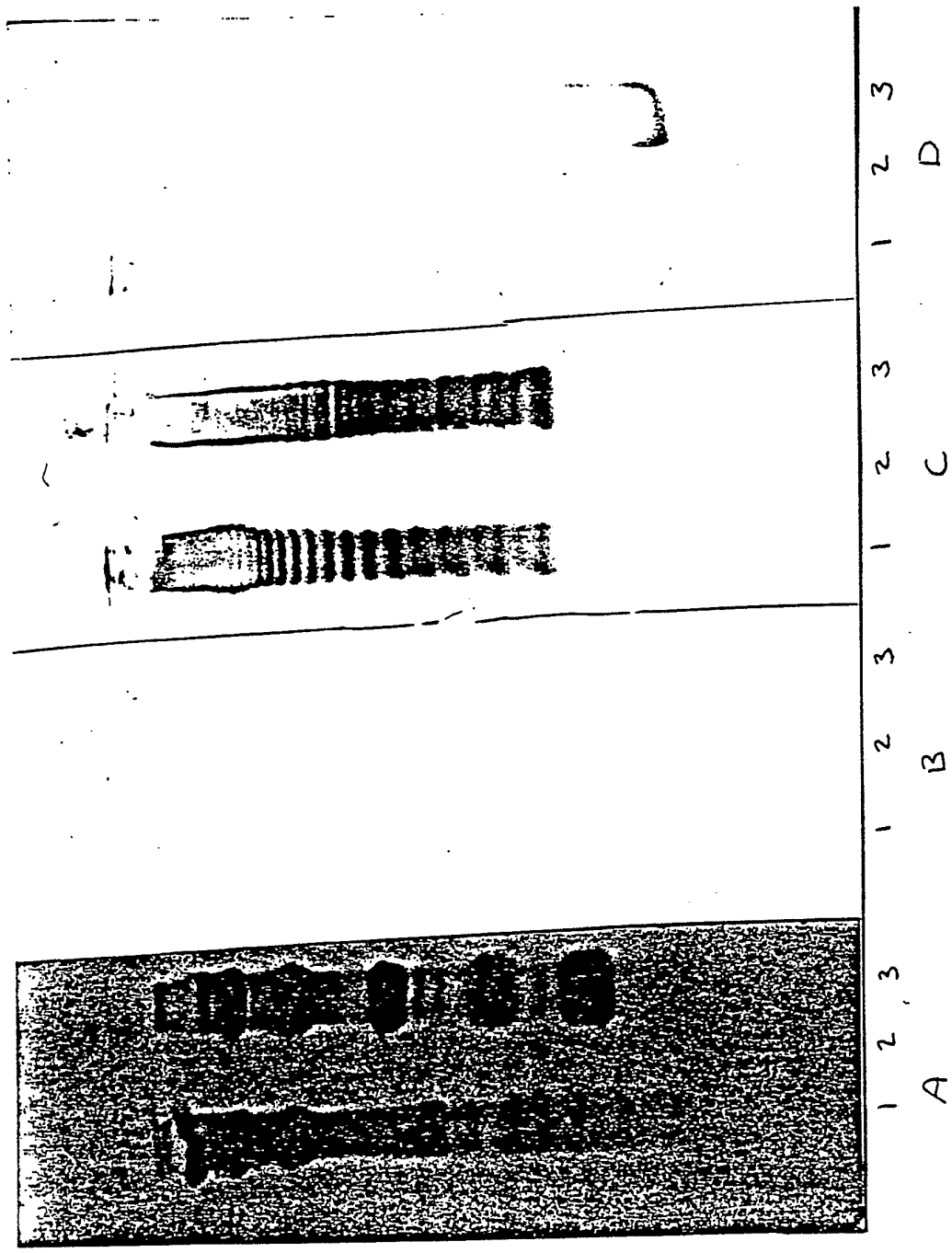


FIG. 3

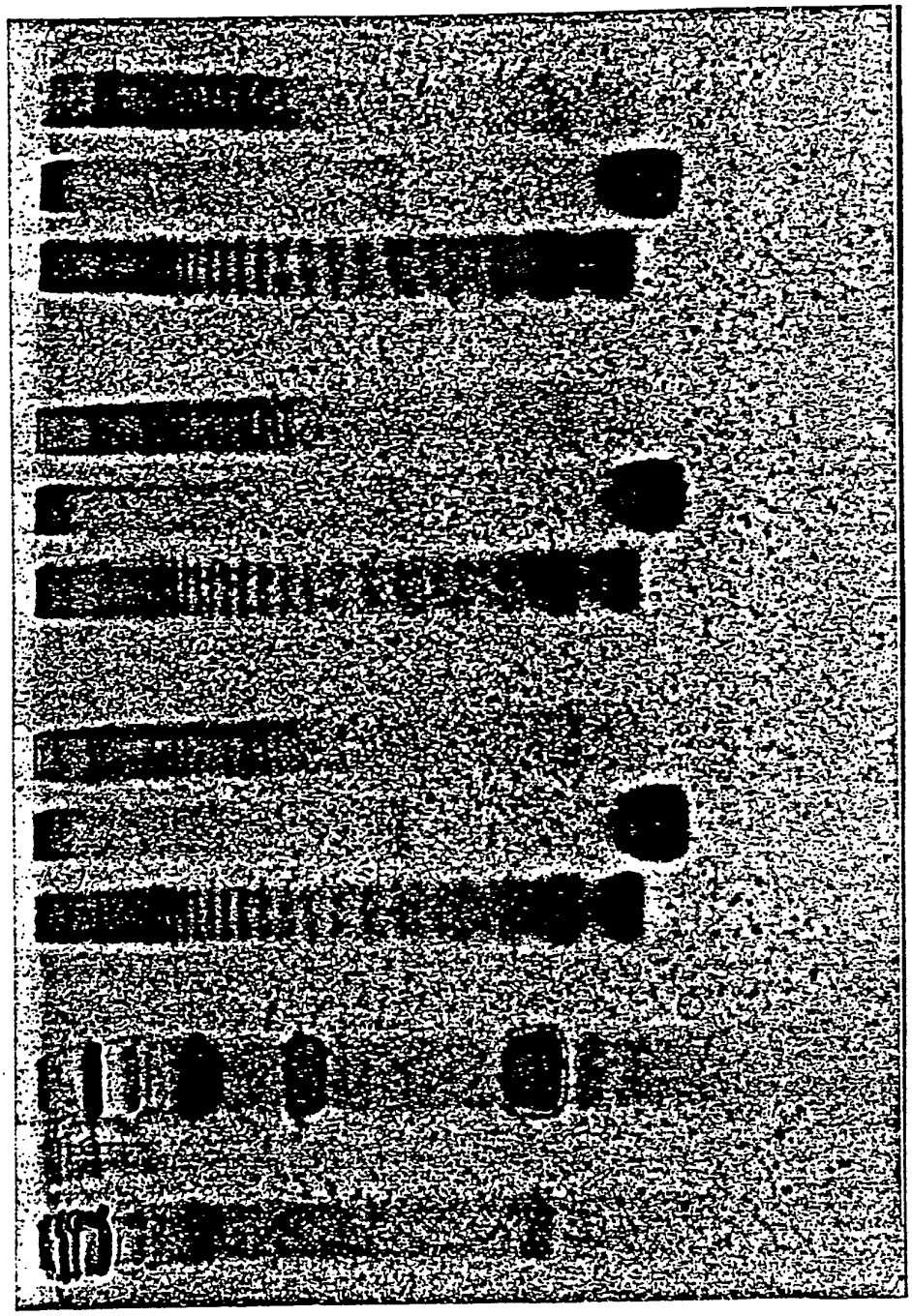


FIG. 4

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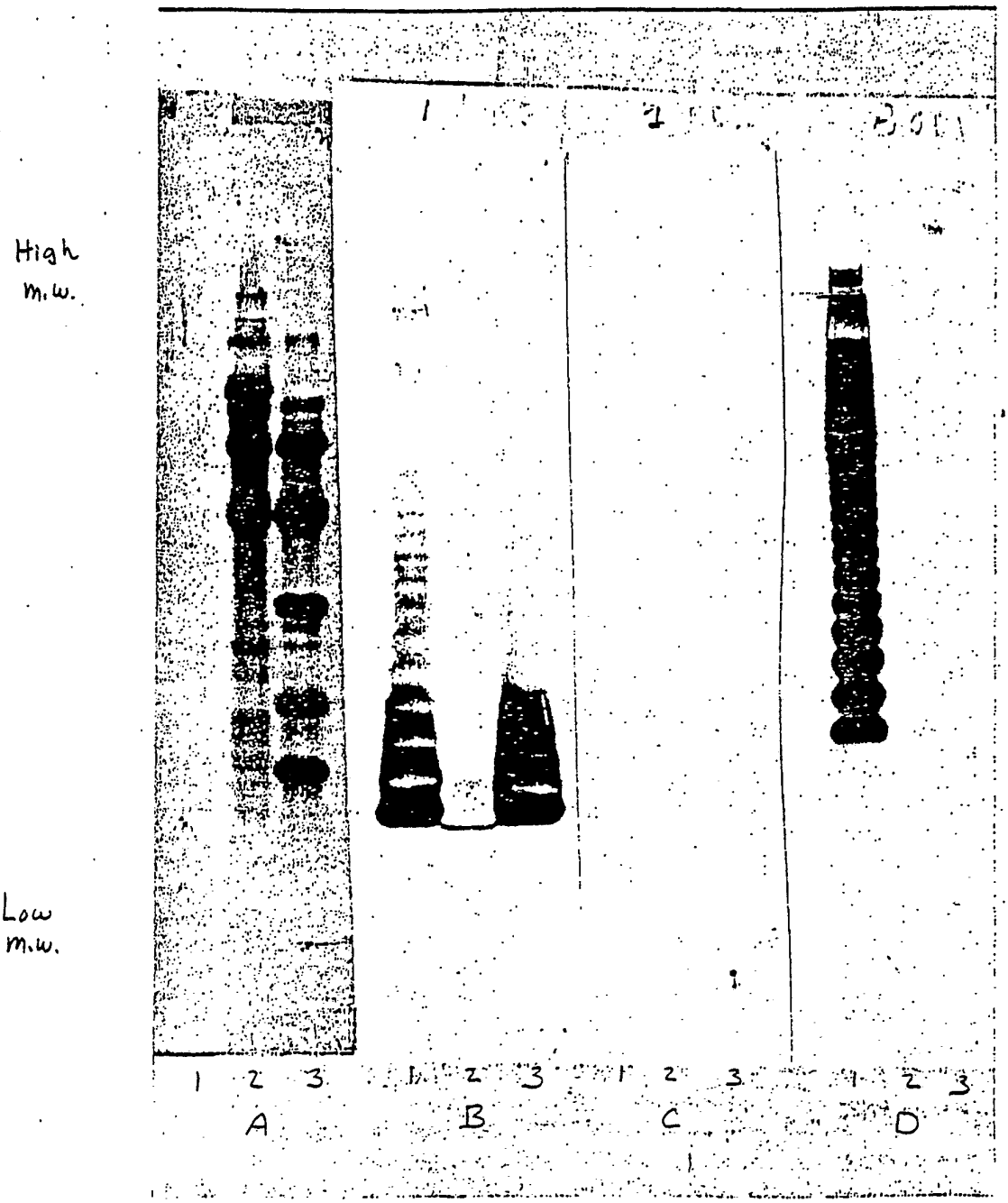
FIG. 5



INTERNATIONAL SEARCH REPORT

International Application No. PCT/US88/01047

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (4): G01N 33/535; C12N 5/00; C07K 15/00

U.S. Cl.: 435/7, 240.1; 424/1.1, 87; 530/387

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	424/1.1, 87 935/92,96,100,107,110 435/7,68,240.1,240.27,810 530/387

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

Database: Chemical Abstracts (File Ca, 1967-1988).
Search Terms: Applicant's names.

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y,P	Chemical Abstracts, Volume 107, Number 5, issued August 1987 (Columbus, Ohio), L.S. YOUNG, "Monoclonal antibodies binding determinants of gram negative bacteria," Eur. Pat. Appl. EP 217,527 published 08 April 1987. See Abstract number 37948U.	1-16
Y	Journal of Immunological Methods, Volume 76, Issued 11 February 1985, H. SIDBERRY ET AL, "Immunoenzymatic analysis by monoclonal antibodies of bacterial lipopolysaccharides after transfer to nitrocellulose," see pages 299-305, especially pages 301 and 304.	1-16
X Y	EP, 0,163,493 (Genetic Systems Corporation) 12 April 1985. See entire document.	6-16 1-5

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
04 August 1988	07 SEP 1988
International Searching Authority	Signature of Authorized Officer
ISA/US	MARGARET MOSKOWITZ PRIMARY EXAMINER

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	European Journal of Biochemistry. Volume 132, issued 1983, P.S.N. ROWE ET AL, "Structure of the core oligosaccharide from the lipopolysaccharide of Pseudomonas aeruginosa PAC1R and its defective mutants," see pages 329-337.	1-16

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers , because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.