PRODUCTION OF A MONOCLONAL ANTIBODY TO *PASTEURELLA HAEMOLYTICA*

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CHAPTER I

INTRODUCTION

Bovine pneumonic pasteurellosis is an economically important disease to the livestock industry (Jensen, 1968). *Pasteurella haemolytica* biotype A serotype 1 is a frequently isolated bacteria from the lungs of cattle with fibrinous pneumonia and is considered the major pathogenic bacterial species of the disease (Jubb, Kennedy and Palmer, 1985). The pathogenesis of the disease is still unknown, but currently many investigators are separating and defining the antigenic components of the bacterial products in an attempt to understand immunity to *P. haemolytica*.

The leukotoxin produced by *P. haemolytica* has been studied and partially characterized by a number of investigators (Baluyut, et al., 1981; Mosier, et al., 1983; Maheswaran, et al., 1980; Chang, et al., 1986a; Shewen and Wilkie, 1982). The gene producing the leukotoxin has been cloned by Lo, et al. (1985). Other antigenic preparations have been studied. These preparations include outer membrane proteins (Squire, Smiley and Croskell, 1984), and capsular-rich antigens (Gilmour, et al., 1979; Gentry, Corstvet and Panciera, 1982; Yates, Stockdale and Babiuk, 1983; Lessley, et al., 1985; Confer, et al., 1985b; McKinney, et al., 1985; Donachie, et al., 1984).

Monoclonal antibody technology has given many new insights into disease processes at the immunological and molecular level (Goding,

1983). It is a technology in which splenic lymphocytes from hyper-immunized mice are fused with myeloma cells to produce hybrid cells. These cells secrete antibody of a given specificity.

With the combination of P. haemolytica antigen preparations and monoclonal antibody technology, the exploration into the pathogenesis of P. haemolytica can now be extended to the molecular level. In this study, monoclonal antibodies to a component of the bacterial cell have been produced and characterized.

CHAPTER II

LITERATURE REVIEW

Bovine pneumonic pasteurellosis, also called bovine shipping fever, frequently occurs soon after such stressful situations as transport, dietary adaptation, and grouping in new, and many times, crowded environments (Dyer, 1982; Jubb, Kennedy and Palmer, 1985). It is a disease that is a severe economic liability to the beef industry (Jensen, 1968) despite management practices implemented to decrease stressful factors that may predispose to this disease (Dyer, 1982). Although many factors and etiologies may contribute to disease development, it is unclear as to the relationship of stress, bacteria, and viruses as etiological agents (Thomson, et al., 1975). Nevertheless, this disease complex is most often associated with pathogenic strains of Pasteurella haemolytica biotype A serotype 1 (Jubb, Kennedy and Palmer, 1985).

The characteristic lesions of pneumonic pasteurellosis are fulminating fibrinous pleuropneumonia with interstitial and alveolar edema, interstitial lymphatic thrombosis, fibrinous pleuritis, and alveolar wall necrosis. Primarily involved is the anterioventral portion of the lung, particularly in the cranial and middle lobes. There may also be an associated serofibrinous pericarditis and the presence of a yellow pleural effusion (Rehmtulla and Thomson, 1981). Microscopically, large numbers of the small, rod-shaped, encapsulated, gram-negative bacterium

are present adjacent to compacted debris of inflammatory cells (Jubb, Kennedy and Palmer, 1985; Jensen and Mackey, 1979). The locally released bacterial endotoxin and cytotoxin (leukotoxin) as well as the capillary thrombosis are thought to cause the characteristic coagulation necrosis associated with the pneumonic lesions (Jubb, Kennedy and Palmer, 1985). The alveoli are usually filled with fibrin, macrophages, and neutrophils. Lesions from experimentally-induced disease are similar to those present in the natural disease (Rehmtulla and Thomson, 1981).

Pasteurella haemolytica is frequently present in the upper respiratory tract of healthy animals. The lungs are probably exposed to a small number of bacteria during normal breathing (Hamdy and Trappy, 1967; Thompson and Gilka, 1974). Thomson, Benson and Savan (1969) reported that high accumulations of bacteria, P. haemolytica in particular, in the nasal passages eventually resulted in substantial lung exposure and subsequent pneumonia. They also found that P. haemolytica was in higher concentrations in the nasal flora of sick animals compared to well animals. This indicated that the bacteria proliferated greater in the sick animal, thereby allowing for greater opportunity to gain access to the lungs (Thomson, et al., 1975). Slocombe, Derksen and Robinson (1984b) found that deep breathing after cold exposure may increase the number of aerosol particles and enhance depositions of these particles in the lung parenchyma.

The experiments of Slocombe, et al. (1984a) revealed that one hour after exposure to P. haemolytica there was a decrease in the arterial oxygen tension. This hypoxia was thought to be due to a ventilation perfusion mismatch or a diffusion impairment resulting in the persistent

perfusion of poorly ventilated pneumonic regions of the lung. The hypoxia of the pulmonary tissue was found to continue and by 12 hours postinfection there was an increase in pulmonary resistance resulting from obstruction of peripheral airways with purulent exudate and edema. This increase in resistance was also accompanied by extensive areas of atelectasis, exudative pneumonia, and obstructive suppurative bronchiolitis.

The role of other contributing factors in the pathogenesis of the disease is unclear. Following viral infection, the normal pulmonary defense mechanisms are compromised or destroyed (Dyer, 1982). Included in these mechanisms is the mucociliary apparatus and intrapulmonary phagocytic defenses. Viral infections have been shown to markedly impair the clearance of inhaled bacteria if viral infection occurs one to two weeks prior to shipment of the cattle (Thompson and Gilka, 1974). Viral agents have disrupted the mucous flow out of the lung as well as the bactericidal activity of alveolar macrophages (Allan and Msolla, 1980; Jakob, 1981). Parainfluenza-3 virus, infectious bovine rhinotracheitis, and bovine virus diarrhea have been shown to destroy the pulmonary macrophage (Rossi and Kiesel, 1977). These viruses clearly can inhibit the normal protective mechanisms of the lung, yet, no direct cause and effect relationship was found between the parainfluenza-3 virus (PI_3) and P. haemolytica pneumonia (Thomson, Benson and Savan, 1969). Houghton and Gourlay (1983) reported a synergistic effect between P. haemolytica and Mycoplasma bovis in producing pneumonia in gnotobiotic calves. While simultaneous intranasal or intratracheal innoculation of M. bovis and 18-hour cultures of P. haemolytica did not induce severe disease or extensive pneumonic consolidation, the combination of M.

bovis and a 6-hour culture of P. haemolytica did increase the severity of illness and pulmonary lesions. The most severe disease was produced by intranasal and intratracheal inoculation of 6-hour cultures of P. haemolytica one day after intranasal inoculation with M. bovis (Gourlay and Houghton, 1985).

Although alveolar macrophages appear to be inefficient in phagocytizing and killing of P. haemolytica, they are able to control small numbers of the organism. Once the organisms are present in large numbers, the bacteria may be able to further impair macrophage phagocytosis through the production of leukotoxin (Markham and Wilkie, 1980; Shewen and Wilkie, 1982). Maheswaran, et al. (1980) determined that P. haemolytica, in the presence of opsonins found in normal serum of adult cattle or antiserum, are avidly phagocytized by alveolar macrophages. Furthermore, heat stable opsonins (antibodies) seem to be more important than heat labile opsonins (complement) in the phagocytosis of P. haemolytica.

Both live P. haemolytica and culture supernatant are toxic to alveolar macrophages (Markham and Wilkie, 1980; Benson, Thomson and Valli, 1978; Kaehler, et al., 1980b; Shewen and Wilkie, 1982). Cell-free culture supernatant impairs phagocytosis at low concentrations and is lethal for bovine leukocytes at higher concentrations. Cytotoxicity was related to the number of cells taken up by the macrophage (Markham and Wilkie, 1980). Wilkie (1982) suggested that high opsonizing antibody responses may not only enhance the phagocytosis of the organism but also enhance cytotoxic effects on the alveolar macrophages. Sterile culture supernatants of P. haemolytica caused inhibition of the luminal dependent chemiluminescence (LDCL) assay response of phagocytically

stimulated neutrophil preparations from ruminants but not from nonruminants. The LDCL response was higher for opsonized than nonopsonized bacteria and remained increased longer for opsonized bacteria (Chang, et al., 1986a). Yet, with the presence of opsonizing antibody, phagocytosis and degradation of most bacteria by alveolar macrophages did not prevent the cytotoxic effects on the alveolar macrophages (Maheswaran, et al., 1980).

The leukotoxin, present in culture supernatants, is a heat-labile exotoxin. Optimum leukotoxic production occurs during the logarithmic growth phase (Baluyut et al., 1981; Kaehler, et al., 1980b; Chang, Renshaw and Richards, 1986b; Berggren, et al., 1981). It is oxygen stable, susceptible to pH extremes, and kills bovine neutrophils and mononuclear leukocytes. It is inactivated by trypsin, contains no detectable endotoxin, and contains carbohydrate and protein moieties (Baluyut, et al., 1981; Kaehler, et al., 1980b; Chang, et al., 1986b). The leukotoxin is nontoxic to equine, feline, rabbit, canine, porcine, and human blood lymphocytes or bovine erythrocytes, bovine kidney cell cultures, or bovine primary spleen cell cultures (Lo, et al., 1985; Chang, et al., 1986a; Kaehler, et al., 1980a).

Leukotoxin neutralizing activity has been demonstrated with bovine IgG_2 purified from pooled seropositive sera (Cho, et al., 1984). Bovine IgG_2 is the predominant immunoglobulin in the bovine lung and the neutralizating antibody may be a protective immunological response that occurs in pneumonic pasteurellosis (Cho, et al., 1984; Butler, 1981; Gentry, et al., 1985). The antigen specificity of the antibody potentially plays an important role in protection against the disease. It has been postulated, though not proven, that the IgG class of

antisomatic antibody is potentially hazardous whereas the IgG class of antitoxic antibody may benefit in the prevention of fibrinous pneumonia (Cho, et al., 1984).

Capsular somatic antigen preparations have been extracted from P. haemolytica using potassium thiocyanate, sodium salicylate, and sodium chloride. Immunization with those extracts enhanced resistance of cattle and lambs against experimental challenge with the bacterium (McKinney, et al., 1985; Gilmour, et al., 1983; Yates, et al., 1983). Corstvet, et al. (1982) demonstrated the cell-associated capsular material is produced in greatest quantity during the early logarithmic growth phase corresponding to 2-6 hour of culture growth. Cell-associated capsular material diminished with culture age. Organisms from cultures 16 hours old or greater were found to have little capsular material remaining on the organism. Adlam, et al. (1984) found that capsular polysaccharide had two component sugars (2-acetamido-2-deoxymannopyranose and 2-acetamido-2-deoxymannosyranosyluronic acid) localized on the periphery of the bacterial cell wall. The purified polysaccharide was found to be specific to the A1 serotype of P. haemolytica, immunogenic to sheep but not rabbits, and periodate treatment did not significantly alter its precipitability with immune serum. Antiserum to the purified polymer reacted with sodium salicylate and sodium chloride extracts of P. haemolytica. McKinney, et al. (1985) purified saline extracted capsular material using preparative isoelectrofocusing. The extract contained proteins ranging from 33 kDa to greater than 80 kDa. Calves vaccinated with this extract had greater resistance to experimental pneumonic pasteurellosis than did nonvaccinated calves. Later, Lessley, et al. (1985) purified saline extracted capsular antigens using

chromatofocusing. Six antigenic fractions were collected and identified as glycoproteins. One of the fractions contained lipopolysaccharide. These antigenic fractions were found to cross react with the antigenic fractions isolated earlier by McKinney, et al. (1985).

The lipopolysaccharide from the organism has been isolated (Confer and Simons, 1986; Brogden, et al., 1986) and was evaluated with respect to effect on bovine leukocytes. It was found to be non-toxic to polymorphonuclear leukocytes, increased the migration of peripheral blood leukocytes, and was blastogenic to bovine lymphocytes in moderate concentrations while suppressive in high concentrations. Antibodies to lipopolysaccharide did not correlate with protection against experimental bovine pneumonic pasteurellosis.

Two major outer membrane proteins (30 kDa and 42 kDa) have been isolated by Squire, Smiley and Croskell (1984). Because these proteins are involved in transport mechanisms and adhesion to host cells, antibodies to these proteins have been suggested to kill these organisms through inhibition of protein function. Antigenicity of these proteins was not determined in these studies.

Following vaccination with P. haemolytica bacterins, not only were cattle not protected against experimental pneumonic pasteurellosis or from natural disease (Martin, 1983; Confer, et al., 1985a), but enhancement of experimental and natural disease has been shown to occur (Friend, et al., 1977; Wilkie, Markham, and Shewen, 1980). Panciera, et al. (1984) recently induced antibody titers to somatic antigens by vaccinating with live Pasteurella sp. Calves vaccinated with 6-hour cultures showed greater resistance to the challenge exposure than those innoculated with 20-hour cultures (Confer, et al., 1984). There was a

correlation between high antibody titer and low pulmonary lesion scores regardless of the age of culture used as the immunogen (Panciera, et al., 1984; Confer, et al., 1984).

Many antigenic preparations, as discussed above, have been isolated in an attempt to study the immunological significance and the importance of such antigens in the protection from pneumonic pasteurellosis.

A technology currently used in an attempt to uncover the complex pathogenesis in many disease processes is the production of mononclonal antibody (Mab) hybridomas. Monoclonal antibodies have been used as probes for fine structure of proteins, histocompatibility, tumor localization and classification, purification of molecules by affinity chromatography, and identification of previously undefined antigens on the surface of lymphocytes (Goding, 1980).

Hybridomas are the result of fusion of antigenically stimulated lymphocytes with myeloma cell lines. The rationale behind cell fusion is covered in various literature reviews (Goding, 1980; Paul, 1984). It is based on the formation of heterokaryons as a result of fusion of the plasma membranes. At the next cell division, a hybrid cell results. After repeated cloning and passage, a hybrid cell line is developed and these are referred to as hybridomas.

Several procedures are required to allow for the replication of these hybrid cells. Aminopterin, a folic acid antagonist, blocks the main biosynthetic pathways for purines and pyrimidines. Mammalian cells have salvage pathways in which preformed nucleotides are recycled. These salvage pathways depend on thymidine kinase (TK) and hypoxanthine guanine phosphoribosyl transferase (HGPRT). If the cell is provided with thymidine and hypoxanthine and the enzymes TK and HGPRT are present, DNA

synthesis can still occur. If spleen cells processing TK and HGPRT are fused with myeloma cells lacking TK and HGPRT, only the hybrid will grow in media containing hypoxanthine, aminopterin, and thymidine.

The myeloma cell lines currently used for hybridoma production vary in several characteristics. Many of the early cell lines had the capacity to continually synthesize and secrete sets of both light and heavy immunoglobulin chains from the fusion partners. Recently a variety of genetic defects resulting in failure of immunoglobulin production or secretion by the myeloma cell line have been described (Wallach, et al., 1982). The X63-Ag.8.653 has irreversibly lost the ability to express γ_1 or K chains (Kearney, et al., 1979). This line is a subclone of the mouse myeloma cell line P3-X63-Ag8 and is derived from the original HAT sensitive myeloma cell line variant of MOPC-21. MOPC-21 is the cell line in which Cotton and Milstein (Kearney, 1984) first constructed a continuous antibody secreting cell line. The serum of myeloma or hybridoma-bearing mice contain large amounts of homogeneous antibodies. The advantages of having a cell line such as X63-Ag.8.653 is that of obtaining a high fusion frequency and a large number of hybridomas producing only the specific antibody of the desired antigen stimulated spleen cells (Kearney, et al., 1979).

Screening the hybridomas for specific antibody production is time consuming and laborious because it is frequently performed on many preparations. The screening procedures used should be simple, rapid, convenient, and environmentally safe (Paul, 1984). Other considerations include the ultimate use of the monoclonal and the limitations of the screening technique with respect to the antigen used.

There are frequently used procedures for screening hybridomas for

specific antibody production such as solid-phase radioimmunossay, enzyme-liked immunosorbent assay (ELISA), immunoflorescence, cytotoxicity assays, rosetting assays, assays to inhibition of activity, and polyacrylamide electrophoresis with Western immunoblotting. Western blotting, also called protein blotting, is reviewed by Towbin and Gordon (1984). It is based on the transferring proteins from gel electrophorograms to a solid phase which allows the access of information regarding the immunoreactions to the antigen in question. Diffusional transfer of proteins to nitrocellulose was reported by Renart, Reiser, and Stark (1979), followed by Towbin, Staehelin and Gordon (1979) in which proteins were transferred in an electric field with noncovalent binding to nitrocellulose. Once the desired antigen is blotted onto a solid-phase nitrocellulose sheet, it may be reacted with immunoglobulin to determine the specific protein in question. Several detection systems are used to identify reactive proteins, of which peroxidase is the most widely used enzyme label. Whether or not desired sensitivity can be achieved with blotted proteins depends on the degree of antigen renaturation, suppression of background staining, and sufficient concentration, affinity, and incubation times of the immune reagents (Towbin and Gordon, 1984).

Once hybridomas are replicating in cell culture and found to be secreting antibodies, the cells must be cloned to assure that the antibody produced is a monoclonal antibody, i.e., derived from one cell. There are three methods of cloning hybridoma cells. Soft agar cloning uses two layers of agar, a firm underlayer and a second soft agar layer containing the cells to be cloned (Goding, 1983). The method of

limiting dilution is such that growth follows the Poisson distribution expressed by the equation

$$P_{(\lambda)} = \frac{\mu\lambda}{\mu} e^{-\mu}$$

(Lefkovits and Waldmann, 1979). The dilutions are made serially to achieve a statistical probability of having only one cell per well (Snedecor and Cochran, 1980). The third cloning method is one in which the Fluorescence-activated Cell Sorter (FACS) is used (Parks, Bryanum and Herzenberg, 1979). Although the equipment is expensive, the main advantage is it allows for selection of cells of desired antigen-binding specificity (Goding, 1983). Once the desired hybridoma has been selected and cloned, large scale production, purification, and characterization of the monoclonal antibody may proceed.

Monoclonal antibodies can potentially further the purification and characterization of P. haemolytica antigenic preparations available today and allow for the isolation of antigenic determinants to the organism. As new tools of techniques become available, so come the opportunities of unraveling the complex pathogenesis in disease entities such as Bovine Pneumonic Pasteurellosis. The purpose of the studies described herein were to attempt the production of a monoclonal antibody to the leukotoxin of P. haemolytica.

CHAPTER III

MATERIALS AND METHODS

Microorganism

The Pasteurella haemolytica biotype A serotype 1 used in these experiments were originally isolated from the trachea of a feedlot calf (Corstvet, et al, 1973). Cultures were grown on either brain-heart infusion agar (BHI) or enriched BHI agar at 37°C. The cultures were used directly or resuspended in BHI broth or in RPMI 1640 tissue culture medium (Gibco, Grand Island, New York) depending on the intended use, as described below.

Pasteurella Leukotoxin Immunizing Antigen Preparation

The preparation of the semicrude leukotoxin antigen and the partially purified cytotoxic preparation were as described by Mosier, et al. (1986).

Antigen Screening Preparations

Lactoferrin Cytotoxin

Leukotoxin was prepared using lactoferrin as the protein source in RPMI 1640 medium as described by Gentry, et al. (1986).

RPMI-Leukotoxin

Leukotoxin was produced as described by Baluyut, et al. (1981). Lyophilized P. haemolytica was grown on enriched BHI agar plates for 18 hours. Fifteen colonies were placed in 100 ml of BHI broth in 250 ml flasks and grown for 6 hours at 37°C. The cell suspensions were centrifuged at 13,500 x g in a Sorvall Superspeed RC2-B centrifuge (DuPont, Wilmington, Delware) for 30 minutes at 10°C. The cell pellet was resuspended in 200 ml of RPMI 1640 medium and placed in a 500 ml flask and incubated at 37°C for four hours in a Lab-Line^{R} incubator-shaker (Lab-Line Instruments, Melrose Park, Illinois). Afterwards, the suspensions were centrifuged, as above, and the supernatants sterilized by passage through consecutive 0.45 um and 0.22 um Nalgene filters. The supernatants were concentrated in dialysis tubing (>8,000 mw exclusion) using Aquacide II-A (Calbiochem, San Diego, California). The leukotoxin supernatants were dialyzed in 0.05 M phosphate buffered saline (PBS) (pH 7.2). The protein concentration was determined by a dye-binding method using commercially prepared reagents (Bio-Rad Laboratories, Richmond, California) and found to be 11 ug/ml.

Six-Hour Sonicated Antigen

Lyophilized P. haemolytica was reconstituted in 1 ml sterile distilled water and grown on BHI agar plates for 18 hours. Fifteen colonies were placed in 100 ml of RPMI 1640 medium in a 250 ml flask and grown at 37°C in a Lab-Line^{R} incubator-shaker. Cell growth was allowed to occur for 6 hours. The cells were centrifuged at 13,500 x g for 30 minutes in the Sorvall centrifuge. The cells were washed three times in cold PBS and resuspended in 30 ml of 0.5 M PBS. The cell suspensions

were sonicated on ice with the Sonifier^{R} Model 200 (Branson Sonic Power Company) at maximum power for 60 seconds and then centrifuged for 10 minutes in an Eppendorf Microcentrifuge.

Six-Hour Sodium-Dodecyl Sulfate Antigen

Lyophilized P. haemolytica were resuspended in 1 ml sterile distilled water and grown on BHI agar plates for 18 hours. Fifteen colonies were grown in 250 ml of RPMI 1640 medium in 250 ml flasks at 37°C in a Lab-Line incubator-shaker for 6 hours. The cell suspensions were centrifuged at 13,500 x g for 30 minutes in the Sorvall centrifuge. The cells were washed three times in cold PBS and resuspended in 30 mls of 5% sodium-docecyl sulfate (SDS).

Saline-Extract Antigen

The P. haemolytica saline extract antigen, rich in capsular polysaccharide, was that described by Lessley, et al. (1985).

Outer Membrane Proteins

Outer membrane protein antigens of P. haemolytica were provided and described by Squire, Smiley and Croskell (1984).

Antisera

Six to eight week old, female, BALB/C mice were immunized intraperitoneally with a 50 ug per mouse dosage of the semicrude leukotoxin preparation in 0.01 ml of Freund's complete adjuvant followed by three weekly 50 ug doses in 0.01 ml of Freund's incomplete adjuvant. The mice were boosted intraperitoneally every 4 to 6 weeks with a 1:1 ratio of

Freund's incomplete adjuvant and a 50 ug per mouse dose of a partially-purified cytotoxin preparation. The mice were periodically bled from the tail vein. The blood was pooled, allowed to clot, and then centrifuged for 10 minutes in an Eppendorf centrifuge. The serum was removed and stored at -70°C.

Negative control sera was collected similarly from pooled blood samples obtained from age and sex-matched BALB/C control mice.

Enzyme-Linked Immunosorbent Assay

The enzyme-linked immunosorbent assay (ELISA) was used to screen the hybridoma cells as described by Voller, Bidwell and Bartlet (1979). All antigen preparations were diluted in carbonate-bicarbonate buffer (pH 9.6). Preparations of 1 ug/ml or 10 ug/ml of antigen were placed on Immulon II plates (Dynatech Laboratories, Alexandria, Virginia) at 50 ul per well and allowed to coat overnight at 4°C. Control serum dilutions of 1:100 were prepared in 0.05 M PBS containing 0.05% Tween 20 solution (PBS-Tween 20 solution) and 1% bovine serum albumin (BSA) or 1% ovalbumin. Volumes of 50 ul of serum or hybridoma culture supernatants were allowed to incubate for 1 or 2 hours. Plates were washed five times with PBS-Tween 20 solution. Alkaline phosphatase-conjugated goat anti-mouse polyvalent whole molecule antibody (Sigma Chemical Company, St. Louis, Missouri) was mixed with PBS-Tween 20 solution and 1% ovalbumin at a dilution of 1:200 and allowed to incubate for 1 to 2 hours. The plates were washed five times with PBS-Tween 20 solution. The substrate, containing p-nitrophenyl phosphate in diethanolamine buffer, was added to the plate and allowed to incubate. After 30 minutes, the reaction was stopped by the addition of 50 ul of 3N sodium hydroxide per well. The

optical density of the plates was read at 405 nm (OD_{405}) in a Bio-Tek EIA reader Model EL307 (Bio-Tek Instruments Inc., Burlington, Vermont).

Evaluation of the Immunologic Schedule

To determine the optimal immunization dosage and schedule, murine splenic mononuclear leukocytes were isolated and the lymphocytic blastogenic response to various P. haemolytica antigen preparations was evaluated. In a method adapted from Baldwin, Antczak and Winter (1985), 12 mice, divided into 6 groups of 2 mice per group, were intraperitoneally injected with crude, semicrude, or a partially-purified leukotoxin in doses of 50 and 100 ug per mouse or 200 and 400 ug per mouse. The immunization schedule was similar to that described by Stahli, et al. (1980). The partially-purified antigen preparation was injected at weekly intervals for four consecutive weeks. Five days before recovering the lymphocytes, the mice were injected with either 50 or 200 ug of one of the three antigen preparations. For the next four days, the mice received 100 or 400 ug, respectively, of the same antigen previously injected. Serum was collected and the antibody response to the partially-purified antigen was determined by the ELISA. Subsequently, each mouse was killed by cervical dislocation and spleens were aseptically recovered and minced with a glass tissue homogenizer. The splenic mononuclear leukocytes were recovered by underlaying the spleen suspension with Ficoll-paque (Pharmacia Fine Chemicals, Piscataway, New Jersey) followed by centrifugation at 800 x g for 20 minutes at 20°C. The cells obtained from the Ficoll-plasma interface were washed three times in PBS, centrifuged at 700 x g for 10 minutes and were resuspended in 1.1 ml of RPMI 1640 medium containing 0.1% gentamycin. The cells

were counted and adjusted to a concentration of 2 x 10^6 cells/ml in RPMI 1640 medium. RPMI 1640 medium containing 0.1% gentamycin and 20% fetal bovine serum (FBS) was placed in a 96-well microtiter plate (Costar, Cambridge, Massachusetts) at 100 ul per well. An equal volume of each cell suspension was added to wells in the plate at 100 ul/well. All assays were carried out in duplicates. After a 3 day incubation at 37°C in a humid 5% CO_2 atmosphere, 20 ul of a 1:25 dilution of ^{3}H-Thymidine was added to each well. Eighteen hours after labeling, the cells were harvested on filter paper using a semiautomatic cell harvester (Skatron, Lier, Norway). The filter papers were dried for 2 hours at 60°C and added to a toluene-base scintillation cocktail (PPO-Bis MSB, ICN Biomedicals, Irvine, California). Counts per minute (CPM) were determined in a Packard Tri-Carb^R 300 refrigerated scintillation counter (Packard, Downers Grove, Illinois). The average of each group was taken and a stimulation index (SI) calculated:

$$SI = \frac{\text{Avg CPM for cells from injected mice}}{\text{Avg CPM for cells from control mice}}$$

Murine Monoclonal Antibody Production

Monoclonal antibodies were produced as described by Goding (1983) and Cunningham and Russell (1983). Six to eight week old, female, BALB/C mice were immunized intraperitoneally four times at weekly intervals with 0.02 ml of a emulsion containing equal volumes of Freund's incomplete adjuvant and 50 ug of a semicrude leukotoxin. Five days before fusion, the mice were injected intraperitoneally with 200 ug of a partially-purified cytotoxin preparation in sterile PBS. This was

followed by an injection of 400 ug of the same preparation for the next four days.

The mice were killed by cervical dislocation. Spleens were aseptically removed and minced through a sterile 100-mesh screen. The spleen cells were rinsed in Iscove's Modified Dulbecco medium (IMDM) (Gibco) containing 0.1% gentamycin and pelleted by centrifugation at 400 x g for 7 minutes. The cells were resuspended in 10 ml of IMDM containing 0.1% gentamycin.

Murine myeloma cells (x63-Ag8.6.5.3) were counted, mixed at a 1:1 ratio with the suspended spleen cells, and pelleted by centrifugation at 400 x g for 7 minutes. The supernatant was decanted and 3 ml of 35% sterile polyethylene glycol (pH 8.5) was added. The pellet was centrifuged at 400 x g for 2 minutes at 25°C, then allowed to remain undisturbed for 6 minutes at 30°C. To stop the fusion reaction, 40 mls of IMDM and 0.1% gentamycin were added to the cells. The cells were centrifuged at 400 x g for 8 minutes and the supernatant discarded. The cells were placed in 40 ml of IMDM containing 20% FBS (Hyclone, Logan, Utah) in 75 cm^2 flasks, and incubated overnight at 37°C and 8% CO_2. The following day, cells were placed into wells of 96-well microtiter plates (Nunc, Hazelton Research Products, Denver, Pennsylvania) at 200 per well. Fresh IMDM containing 0.1% gentamycin, 20% FBS, 5×10^{-3} M hypoxanthine, 2×10^{-5} M aminopterin, and 8×10^{-4} M thymidine was used to replace spent medium every other day for two weeks. At the end of two weeks, medium was replaced with IMDM containing 0.1% gentamycin, 20% FBS, 5×10^{-3} M hypoxanthine, and 8×10^{-4} M thymidine. During that time, all acidic culture fluids were tested for antibody to the cytotoxin preparations by the ELISA method.

Hybridoma cells in positive wells in 96-well plates were resuspended in 2.0 ml volumes in 24-well plates (Nunc). When cells became confluent, the culture fluids were tested by the ELISA and the cells were frozen in IMDM containing 0.1% gentamycin, 10% DMSO, and 20% FBS. The cells were cloned three times by limiting dilution at 0 to 4 cells per well. Each time the cells were cloned, they were regrown in 24-well plates and frozen. The limiting dilution method of cloning is one in which follows the Poisson distribution, thereby allowing a method of obtaining a reasonable probability that wells which show growth contain single clones (Goding, 1980). All cloning was carried out at least two dilutions that were ten fold. During the cloning, all positive wells subsequently used were ones giving the greatest probability of having a single clone per well. The probability of the hybridomas being monoclonal was calculated using information from Lietzke and Unsicker (1985).

Polyacrylamide Gel Electrophoresis and Immunoblot Techniques

Discontinuous sodium-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as described by Laemmli (1970) with some modifications, was used to differentiate the protein bands of the various antigen preparations. Antigen preparations were boiled for 90 seconds in 0.125 M Tris-Cl (pH 6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and bromophenol blue. Molecular weight marker proteins (Sigma Chemical Company), diluted 1:20, were used as standards on each gel.

Thirty ul of a prepared sample antigen were added to each well in a 4% acrylamide stacking gel containing 0.125 m Tris-Cl (pH 6.8) and 0.1%

SDS. This overlayed a 10% acrylamide resolving gel containing 0.375 M Tris-Cl (pH 8.8) and 0.1% SDS. The electrode buffer consisted of 0.025 M Tris (pH 8.3), 0.192 M glycine, and 0.1% SDS. Electrophoresis was conducted using a Mighty Small minature slab gel electrophoresis unit (SE200, Hoefer Scientific Instruments, San Francisco, California) at a constant current of 35 mA for 1.5 to 2 hours. Immunoblotting was performed as described by Towbin, Staehelin and Gordon (1979), with modifications described by Nielsen, et al. (1982). The gel was removed from the electrophoresis unit and washed three times for 10 minutes each in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, and 0.01% SDS, pH 8.3). The protein transfer from the gel onto nitrocellulose paper was done using the Mini Transphor (TE22, Hoefer Scientific Instruments) at a constant current of 250 mA for 1.5 hours. A portion of the nitrocellulose paper was stained with a mixture of 0.1% amido black 10B (BioRad Laboratories, Richmond, California), 45% methanol, and 10% acetic acid for 3 minutes and destained for 20 minutes in a mixture of 80% methanol, 3.5% acetic acid, and 16% distilled water. The remainder of the blot was quenched with 3% ovalbumin (Sigma Chemical Company) in PBS-0.05% Tween 20 solution for one hour at 37°C. The membrane was washed five times at 5 minute durations with PBS (pH 7.5) containing PBS-0.1% Tween 20 solution. The membrane was cut into strips and incubated overnight at room temperature with antisera controls or monoclonal antibody supernatant. The strips were washed five times at 5 minute durations with PBS-0.1% Tween 20 solution. Strips incubated with mouse serum or culture supernatant were further incubated with biotinylated anti-mouse species specific whole immunoglobulin molecule, whereas strips incubated with rabbit or bovine sera were incubated with

biotinylated Protein A (Amersham, Arlington Heights, Illinois). The biotinylated compounds were diluted 1:300 in PBS-Tween 20 solution containing 1:300 in 1% ovalbumin. Incubation was for 1 hour at room temperature followed by five washes in PBS-0.1% Tween 20 solution. Each nitrocellulose strip was then incubated with a 1:400 dilution of streptavidin-biotinylated enzyme preformed complex in 1% ovalbumin for 30 minutes. Antigenic bands were seen after incubation for 10 - 15 minutes in a mixture of 0.5 mg/ml 4-chloro-1-naphthol in PBS containing 0.03% H_2O_2. Blots were rinsed in PBS and allowed to air dry.

Detection of Carbohydrate Epitopes Using Periodate Oxidation

The method described by Woodward, Young and Bloodgood (1985) was modified slightly and used to determine if the mononclonal antibody was directed against antigenic determinants with a carbohydrate moiety. Two Immunlon II (Dynatech Laboratories) 96-well microtiter plates were coated overnight at 4°C with 50 ul per well of the partially-purified leukotoxin, the 6-hour sonicated antigen, and the capsular extract antigen at 10 ug of protein/ml in carbonate-bicarbonate buffer (pH 9.6). After coating, one plate was used as a control, the other was treated with sodium m-periodate. The plates were then washed five times with PBS-Tween 20 solution and patted dry. The periodate-treated plate was flooded two times with 50 mM sodium acetate buffer (pH 4.5) and patted dry. This plate was incubated for 1 hour at room temperature in the dark with 10 mM sodium m-periodate in 50 mM sodium acetate buffer (pH 4.5), then rinsed two times in 50 mM sodium acetate buffer, and patted dry. A 50 mM sodium borohydride solution in PBS was added to the wells

for 30 minutes at 25°C, followed by five washes in PBS-Tween 20 solution. The assay was completed similarly for both the control and periodate-treated microtiter plates. Monoclonal culture supernatant, positive control mouse serum, or FBS control media were added to the 96-well microtiter plate and allowed to incubate for 1 hour. The plates were washed five times with PBS-Tween 20 solution. Alkaline phosphatase conjugated goat anti-mouse polyvalent whole molecule antibody (Sigma Chemical Company), diluted 1:200 in 1% ovalbumin, was added to each well and incubated for 1 hour. The plates were washed five times with PBS-Tween 20 solution. Substrate [p-nitrophenyl phosphate in 10% diethanolamine buffer (1 mg/ml)] was added to each well and incubated for 30 minutes. The enzymatic reaction was stopped by the addition of 50 ul of 3 N sodium hydroxide per well. The OD_{405} values were determined.

Determination of Immunoglobulin Isotype

The monoclonal antibody was subisotyped by the ELISA method using a mouse mononclonal subisotyping kit containing rabbit anti-mouse IgG, IgG_{2a}, IgG_{2b}, IgG_3, IgM, and IgA (Hyclone Laboratories).

Quantitation of Immunoglobulin

The immunoglobulin concentration of the hybridoma supernatants were quantified by the method of Schoenfeld, et al. (1983). Affinity purified goat anti-mouse IgM (u chain specific) (Cooper Biomedical Inc., Malvern, Pennsylvania) was placed in wells a 96-well microtiter Immulon II plates at 10 ug/ml diluted in PBS (pH 8.5). The plates were incubated at 4°C overnight. The plates were washed three times with PBS-Tween 20 solution. Two-fold dilutions of each monoclonal antibody supernatant

and the standard mouse immunoblobulin (Meloy Laboratories Inc., Springfield, Virginia), at ten-fold dilutions, were placed on the respective plates. The plates were incubated for 1 hour then washed three times in PBS-Tween 20 solution. Alkaline phosphatase goat anti-mouse polyvalent antiserum was incubated at a 1:200 dilution at 4°C overnight. A color reaction was quantitated as described above. Concentrations of IgM present in the supernatants were calculated from standard curves constructed of the reaction of the immunoglobulin standards.

Indirect Fluorescent Staining of Various Bacteria

Pasteurella haemolytica A1, Pseudomonas aeroginosa, Pasteurella multocida, Hemophilus influenza, Klebsiella pneumoniae, and Escherichia coli were grown for 18 hours on enriched BHI agar plates. Each culture was removed from the plate with a cotton swab and emulsified in PBS. The cells were washed three times in PBS and centrifuged at 800 x g for 10 minutes. Cells were reconstituted to 0.5 McFarland standard and 20 ul of each cell suspension was placed on a clear glass microscope slide. The slides were air dried then fixed in acetone for 10 minutes. Slides containing each bacterium were covered with the mononclonal antibody supernatant or IMDM with 15% FBS negative control and placed in a humidified chamber at 37°C for 1 hour. All slides were washed five times in PBS with agitation at 5 minute intervals. A 1:5 dilution fluorescein-conjugated goat anti-mouse IgM immunoglobulin (Meloy Laboratories Inc., Springfield, Virginia) was incubated on the slides at 37°C for 1 hour. The slides were washed five times at 5 minute intervals in PBS and

briefly rinsed in distilled water. A coverslip was mounted on each slide with glycerol and viewed using a fluorescent microscope.

^{51}Chromium-Release Assay

The ability of the monoclonal antibody to neutralize the leukotoxic effects of P. haemolytica were tested by the ^{51}chromium-release assay. The leukotoxin was prepared as described by Lo, et al. (1985). Mixed peripheral blood leukocytes were collected from healthy donor calves. Leukocytes were separated from the blood by flash lysis of the erythrocytes with hypotonic distilled water, resuspensed to isotonicity and centrifugation of the cell pellet. The cells were washed twice with physiologic PBS and resuspended to a final concentration of 1 x 10^7 cells/ml in RPMI 1640 medium. The cells were labeled with 50 uCi of Na_2 [^{51}Cr] O_4 (ICN Pharmaceuticals Inc., Irvine, California) per ml of cells. The cells were incubated for 1 hour at 37°C in a shaker bath. The cells were washed three times with PBS, resuspended in RPMI 1640 medium, recounted, and the final concentration was determined to be 6.9 x 10^6 cells/ml.

The monoclonal supernatant immunoglobulin and FBS-IMDM negative control culture fluid were precipitated with 50% ammonium sulfate. The precipitates were centrifuged at 800 x g for 15 minutes at 0°C. The retrieved pellets were resuspended in 5 ml of PBS and dialyzed in dialysis tubing (>8,000 mw exclusion) in PBS. The undiluted precipitated mononclonal antibody and FBS-IMDM negative control were added directly to 96-well tissue culture plates (Corning Glass Works, Corning, New York). Likewise, 60 ul of each RPMI 1640 medium, PBS, NaOH, or hyperimmune bovine serum were added directly to the plate as test

controls. Leukotoxin dilutions (1:4, 1:16; 1:64) in the amount of 120 ul was added to the corresponding wells. The plate was allowed to incubate for 10 minutes at room temperature. The addition of 90 ul per well of ^{51}Cr labelled cells were placed in the microtiter wells and the plate was incubated for 1 hour at 37°C in 5% CO_2. The plate was centrifuged for 10 minutes at 200 x g, after which 150 ul of supernatant was transferred to a plastic test tube for counting in an automated gamma counter (Searle Analytical Co.). Counts per minute (CPM) were determined and percent killed was calculated:

$$\% \text{ Killing} = \frac{\text{Avg CPM test sample - Avg CPM of PBS negative control x 100}}{\text{CPM positive control - CPM of RPMI negative control}}$$

Electron Microscopic Analysis

Lyophilized P. haemolytica were reconstituted in 1 ml sterile distilled water and grown on BHI agar plates for 18 hours. Fifteen colonies were placed in 100 ml of RPMI 1640 medium in a 250 ml flask and grown at 37°C for 6 hours. The cells were centrifuged at 800 x g for 15 minutes. The cells were washed in 1 ml of 1%-PBS and centrifuged in an Eppendorf microcentrifuge for 2 minutes. They were then washed three times in PBS. The cells were resuspended in 500 ul of Mab E11 in culture supernatant or IMDM with 15% FBS and allowed to incubate for 15 minutes at 37°C. The cells were microcentrifuged for 2 minutes and washed three times in 0.05% PBS-Tween 20 solution. A 1:5 dilution of goat anti-mouse IgM was conjugated with colloidal gold (Jansen, Belgium Germany) and incubated for 15 minutes at 37°C. The cells were microcentrifuged for 2 minutes and washed three times in 0.05% PBS-Tween 20

solution. The cells were resuspended in 500 ul of PBS and placed on nitrocellulose coated carbon-copper grids. A 1% solution of neutralizing phosphotunstic acid was used to coat the bacterial coated grids. The grids were read using a Hitachi transmission electron microscope.

CHAPTER IV

RESULTS

Evaluation of the Immunization Schedule

The mean stimulation index for the two mice immunized with various Pasteurella antigen preparations in each of the six groups was 8 to 18 times greater than that of the unimmunized control mice. Because of the large variation of individuals within groups using tritiated thymidine incorporation, the differences among the groups were not thought to be of practical consideration. The OD_{405} of the ELISA, measuring antibody production to the partially-purified leukotoxin, was found to vary approximately 0.208 between the high and low value. These data suggest that although the vaccination schedule tested produced a large increase in the stimulation index over the negative control mice, there was no practical significance in whether the mice received the more pure or the crude leukotoxin preparations. The immunizing preparation, likewise, did not produce a noticeable difference in antibody production in the individual mice.

Testing for Antibody Production by Hybridomas

After fusion of spleen cells to myeloma tumor cells, all wells of the 96-well microtiter plate having hybridoma cell growth were screened for antibody to the partially-purified leukotoxin preparation (Table I).

TABLE I

RESULTS FROM SCREENING THE INITIAL CELL CULTURE HYBRIDOMAS FROM FUSIONS 1 AND 2*

	OD Reading of		
Hybridoma No.	Hybridoma	Positive Mouse Sera	Media Control
2.5	0.639	1.370	0.013
2.31	0.430	1.370	0.013
2.21	0.565	1.370	0.013
1.22	1.999E	1.410	0.000
1.34	0.651	1.410	0.000
2.20	0.350	1.370	0.013
1.29	0.246	0.472	0.008
2.57	0.177	0.604	0.002
2.58	0.146	0.604	0.002
1.104	1.999E	1.999E	0.007
1.213	1.999E	0.726	0.024
1.227	0.547	0.726	0.024
1.131	0.714	0.844	0.021
2.106	0.593	0.573	0.019
1.183	0.814	1.031	0.059
2.350	0.889	1.150	0.021

*All hybridomas were screened using the ELISA method against the partially-purified cytotoxic preparation described by Mosier, et al. (1986). Optical densities were read at 405 nm. Optical densities at 1.999 E were equivalent to a reading > 2.0 at 405 nm.

Because of antibody reactions with extraneous protein contaminating that antigenic preparation, as determined by preliminary Western immunoblots, hybridoma supernatants listed in Table I and hybridoma supernatants from two subsequent fusions were tested for antibodies to a lactoferrin leukotoxin (Table II). The final leukotoxin preparation used to screen for antibody production was that made with RPMI 1640 medium without added protein (RPMI-leukotoxin) due to the reactions to extraneous proteins found in the lactoferrin leukocytoxin (Table II).

TABLE II

RESULTS FROM SCREENING HYBRIDOMAS WITH A LACTOFERRIN-LEUKOTOXIN*

	OD Readings of			
Hybridoma No.	Hybridoma	Positive Mouse Sera	Media Control	Lactoferrin Control
4.6.F6	0.477	0.525	0.030	ND**
1.213.E12	1.569	0.525	0.030	1.550
1.213.D9	1.102	0.525	0.030	1.835
1.213.H2	0.916	0.525	0.030	1.065
4.6.D1	0.425	1.999E	0.107	ND
4.4.D11	0.216	1.999E	0.107	ND
3.8.D8	0.762	1.999E	0.488	ND

*Preparation described by Gentry, et al. (1986). Clone numbers containing 1.213 were retrieved from frozen cell cultures. All others were the initial cell culture hybridomas of subsequent fusions. All ELISA optical densities were read at 405 nm.

**ND=Not Done.

Figure 1 describes the origin of each of the four monoclonal antibodies subsequently characterized. Positive wells were chosen on the basis of high optical density readings (Tables I and II) and cloned three consecutive times. The probability of any one of the four chosen hybridomas being a monoclonal antibody was greater than 97% (Table III).

Characterization of the Antibody

After the third cloning, 50 ml volumes of supernatant were produced from 1.213 E12H2E11 (Mab E11), 1.213 G11H11B1 (Mab B1), 1.22 C23B6G9 (Mab G9), and 1.104 C10D10A10 (Mab A10) for characterization. All monoclonal antibody preparations were reacted to various antigen preparations in the ELISA (Table IV). Mab G9 and Mab A10 appeared to be reacting to the extraneous bovine serum proteins present in the partially-purified leukotoxin. Mab B1 was unreactive to all of the available antigen preparations. Mab E11 appeared to be the only antibody reacting to P. haemolytica antigens. Because Mab E11 reacted to all antigenic preparations, it indicates that a common epitope is present in each preparation.

By SDS-PAGE, the SDS P. haemolytica antigen preparation and sonicated P. haemolytica antigen preparation were identical in appearance (Figure 2). When the SDS-P. haemolytica was immunoblotted with Mab E11, there were seven protein bands recognized ranging in size from 40 kDa to larger than 116 K (Figure 3). The IMDM with 15% FBS negative control demonstrated one protein band seen at 66 kDa which was consistently seen in the immunoblots with negative and positive control mouse sera, and the hyperimmune bovine serum to P. haemolytica. This band was disregarded henceforth and thought to be due to reactions with

Figure 1. Flow chart of the origin and subsequent characterization of each of the four chosen monoclonal antibodies

[a]Mosier, et al. (1986).
[b]Gentry, et al. (1986).
[c]Baluyut, et al. (1981).

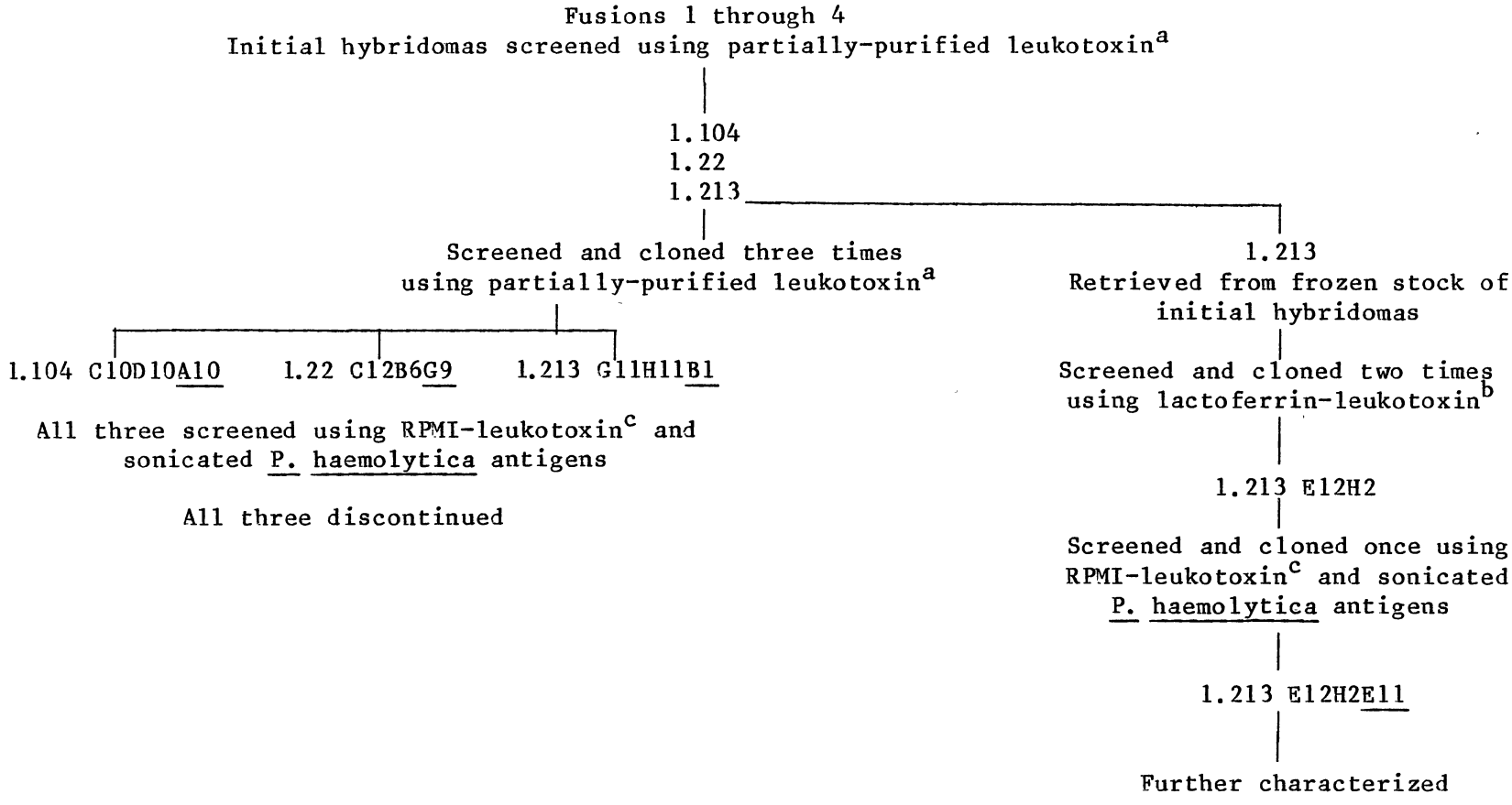

Fusions 1 through 4
Initial hybridomas screened using partially-purified leukotoxin[a]
1.104
1.22
1.213
Screened and cloned three times using partially-purified leukotoxin[a]
1.104 C10D10A10
1.22 C12B6G9
1.213 G11H11B1
All three screened using RPMI-leukotoxin[c] and sonicated P. haemolytica antigens
All three discontinued
1.213
Retrieved from frozen stock of initial hybridomas
Screened and cloned two times using lactoferrin-leukotoxin[b]
1.213 E12H2
Screened and cloned once using RPMI-leukotoxin[c] and sonicated P. haemolytica antigens
1.213 E12H2E11
Further characterized

TABLE III

LIMITED DILUTION CLONING*

Hybridoma No.	OD Reading of Hybridoma	OD Reading of Mouse Sera	OD Reading of Media Control	Antigen Preparation	Antigen Concentration	Limiting Dilution: Cells/Well	Limiting Dilution: Maximum Probability of More Than a Single Clone/Well	Limiting Dilution: Probability of Being Monoclonal (%)
1.213								
G11	1.999E	1.999E	0.057	partially purified cytotoxin[a]	10 ug/ml	0.50	0.09	
G11 H11	1.999E	1.096	0.052	partially purified cytotoxin[a]	10 ug/ml	1.50	0.59	98.6
G11 H11B1	1.716	0.653	0.012	partially purified cytotoxin[a]	10 ug/ml	0.60	0.26	
1.213								
E12	1.569	0.525	0.030	lactoferrin cytotoxin antigen[b]	10 ug/ml	0.08	0.0072	
E12 H2	1.999E	1.671	0.056	lactoferrin cytotoxin antigen[b]	10 ug/ml	0.08	0.0072	99.9
E12 H2E11	1.999E	1.628	0.062	RPMI-cytotoxin[c]	10 ug/ml	0.18	0.036	
1.22								
C12	0.869	1.478	0.031	partially purified cytotoxin[a]	10 ug/ml	0.50	0.09	
C12 B6	0.861	1.615	0.026	partially purified cytotoxin[a]	10 ug/ml	1.00	0.26	99.4
C12 B6G9	1.678	1.623	0.038	partially purified cytotoxin[a]	10 ug/ml	0.60	0.26	
1.104								
C10	0.980	1.293	0.081	partially purified cytotoxin[a]	10 ug/ml	0.50	0.09	
C10 D10	1.999E	1.615	0.076	partially purified cytotoxin[a]	10 ug/ml	4.00	0.91	97.9
C10 D10A10	1.730	1.623	0.062	partially purified cytotoxin[a]	10 ug/ml	1.00	0.26	

*All screening was accomplished using the ELISA. Optical densities were read at 405 nm.
[a]Mosier, et al. (in press).
[b]Gentry, et al. (in press).
[c]Baluyut, et al. (1981).

TABLE IV

SUMMARY OF THE REACTION OF THE VARIOUS ANTIGEN PREPARATIONS USED IN THE SCREENING AND CHARACTERIZATION OF EACH MONOCLONAL ANTIBODY

Preparation	Partially-Purified Leukotoxin[a]	6-Hour Sonicated P.Haemolytica	Saline Extract[b]	RPMI-Leukotoxin[c]	Outer Membrane[d]
E11	0.700	1.584	0.503	0.419	0.607
B1	0.038	0.100	0.034	0.032	0.082
G9	0.439	0.009	0.061	0.083	0.024
A10	0.548	0.017	0.078	0.058	0.027
15% FBS	0.030	0.081	0.062	0.076	0.025
+ Mouse serum	1.999E	0.596	1.396	0.660	0.839
- Mouse serum	0.057	0.197	0.266	0.200	0.519

*All screening was accomplished using the ELISA. Optical densities were read at 405 nm.
[a]Mosier, et al. (1986).
[b]Lessley, et al. (1985).
[c]Baluyut, et al. (1981).
[d]Squire, Smiley and Croskell (1984).

Figure 2. Sodium-Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis of the Various Antigen Preparations

A: SDS-PAGE Electrophoresis of Outer Membrane Protein Antigens obtained from Squire, Smiley and Croskell (1984).
B: SDS-PAGE Electrophoresis of sodium chloride extracted capsular antigens obtained from Lessley, et al. (1985).
C: SDS-PAGE Electrophoresis of sonicated P. haemolytica antigen preparation.
D: SDS-Page Electrophoresis of SDS-P. haemolytica antigen preparation.

Arrows indicate molecular weights of (top to bottom): 116 kDa, 66 kDa, 36 kDa, 29 kDa

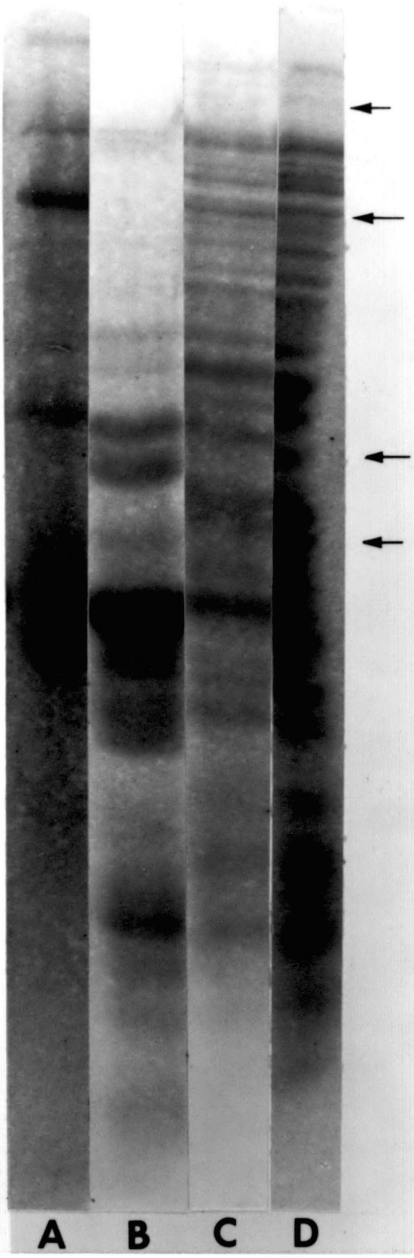
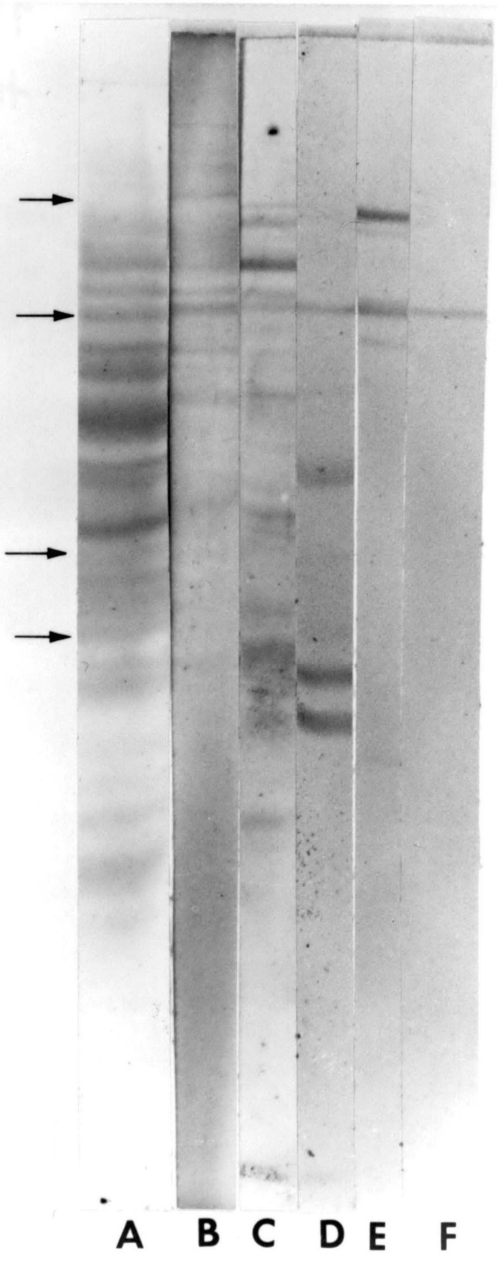

A
B
C
D

Figure 3. Western Immunoblots of the Sodium-Dodecyl Sulfate-*P. haemolytica* antigen preparations

A. Amido black stain of antigen.
B. Immunoblotted with Mab E11.
C. Immunoblotted with positive mouse serum.
D. Immunoblotted with negative mouse serum.
*Band not seen at approximately 100 kDa.
E. Immunoblotted with bovine hyperimmune serum.
F. Immunoblotted with 15% FBS in IMDM (negative control).

Arrows indicate molecular weights of (top to bottom):
116 kDa, 66 kDa, 36 kDa, and 20 kDa


A
B
C
D
E
F

antibodies in the IgG_1 subclass found in the FBS (data not shown). The hyperimmune bovine serum to P. haemolytica had three distinct bands to the SDS-P. haemolytica antigen. Two of the three bands were seen at approximately 110 kDa in the immunoblot with positive mouse serum. One band at approximately 60 kDa was seen in common with the immunoblot to Mab E11. Mab E11 had minimal reaction to the saline extract antigen and the outer membrane protein antigen. The immunoblot of Mab E11 to RPMI-leukotoxin (data not shown) was difficult to interpret due to the nonspecific binding of the streptavidin-biotinylated enzyme complex to the biotin found in the RPMI 1640 medium. Mab A10, Mab G9, and Mab B1 demonstrated only one band at 66 kDa (data not shown) similar to that demonstrated with IMDM with 15% FBS. These latter three monoclonal antibodies were not further characterized.

Reduction of the antigen preparations with sodium m-periodate prior to incubation with Mab E11 failed to substantially alter the OD_{405} values compared to the nonreduced antigen preparations (Table V). These results indicate the epitope seen by Mab E11 was not influenced by any carbohydrate moiety.

Clones of Mab E11 were characterized as IgM (Table VI). Quantitation of Mab E11 indicated that the monoclonal antibody supernatants contained approximately 13 ug of immunoglobulin per ml.

^{51}Chromium Release Assay

The results of the ^{51}Chromium (^{51}Cr) release assay are tabulated on Table VII. The hyperimmune bovine serum positive control showed neutralization of a 1:4, 1:16, and 1:64 dilution of the leukotoxin. Neither

TABLE V

TREATMENT OF THE VARIOUS ANTIGEN PREPARATIONS*

Antigen Preparation	Before Periodate Treatment			After Periodate Treatment		
	Saline Capsular Extract[a]	Antigen Preparation[b]	6-Hour Sonicated Antigen	Saline Capsular Extract[a]	Antigen Preparation[b]	6-Hour Sonicated Antigen
H2 E11	0.813	0.486	1.420	0.861	0.593	1.142
+ Mouse Serum	1.999E	1.999E	1.999E	1.999E	1.999E	1.999E
15% FBS	0.011	0.017	0.008	0.001	0.006	0.004

*Data is expressed as optical densities obtained by the ELISA method read at 405 nm before and after treatment with sodium m-periodate.

[a]Lessley, et al. (1985).

[b]Mosier, et al. (1986).

TABLE VI

ISOTYPING OF Mab E11*

Isotype	Mab E11	Optical Density Readings of Mouse Sera	PBS Negative Control
IgG_1	0.242	0.772	0.001
IgG_{2a}	0.172	1.269	0.024
IgG_{2b}	0.000	1.541	0.000
IgG_3	0.359	0.851	0.120
IgM	1.860	1.524	0.034
IgA	0.169	0.913	0.000

*Optical densities determined by ELISA read at 490 nm.

TABLE VII

^{51}CHROMIUM RELEASE ASSAY

Control Samples	Antibody Sample	Avg Specific Release (CPM)	Percent Killing
PBS - cells		1012.3	--
NaOH - cells		1312.3	--
RPMI - cells		1040.3	--
	E11-leukotoxin (1:4)	1308.3	100.0
	E11-leukotoxin (1:16)	1267.0	93.7
	E11-leukotoxin (1:64.5)	1262.3	92.0
E11-PBS		996.0	6.0
Hyperimmune bovine serum - Leukotoxin (1:4)		1051.0	14.2
Hyperimmune bovine serum - Leukotoxin (1:16)		1030.0	6.6
Hyperimmune bovine serum - Leukotoxin (1:64.5)		1042.0	10.9
FBS-leukotoxin (1:4)		1383.0	136.3
FBS-leukotoxin (1:16)		1258.0	90.4
FBS-Leukotoxin (1:64.5)		1343.3	121.7

the FBS negative control nor the Mab E11 neutralized any of the dilutions of leukotoxin.

Monoclonal Cross Reactivity to Other Gram Negative Organisms

MAb E11 was reactive to several other gram negative organisms using the immunofluorescence technique (Table VIII). Cross reactivity was seen very intensely with Haemophilus influenza and less intensely with Pasteurella multocida (Figure 4). Mab E11 reacted diffusely with the cell wall of P. haemolytica and more irregularly with the cell walls of the other two bacteria.

TABLE VIII

IMMUNOFLUORESCENCE OF MONCLONAL ANTIBODY E11 TO VARIOUS GRAM NEGATIVE BACTERIA*

Gram Negative Bacteria	Negative FBS Control	Monoclonal Antibody
Pseudomonas aeroginosa	-	-
Pasteurella multocida	-	+2
Hemophilus influenza	-	+3
Klebsiella pneumoniae	-	-
Escherichia coli	-	-
Pasteurella haemolytica	-	+3

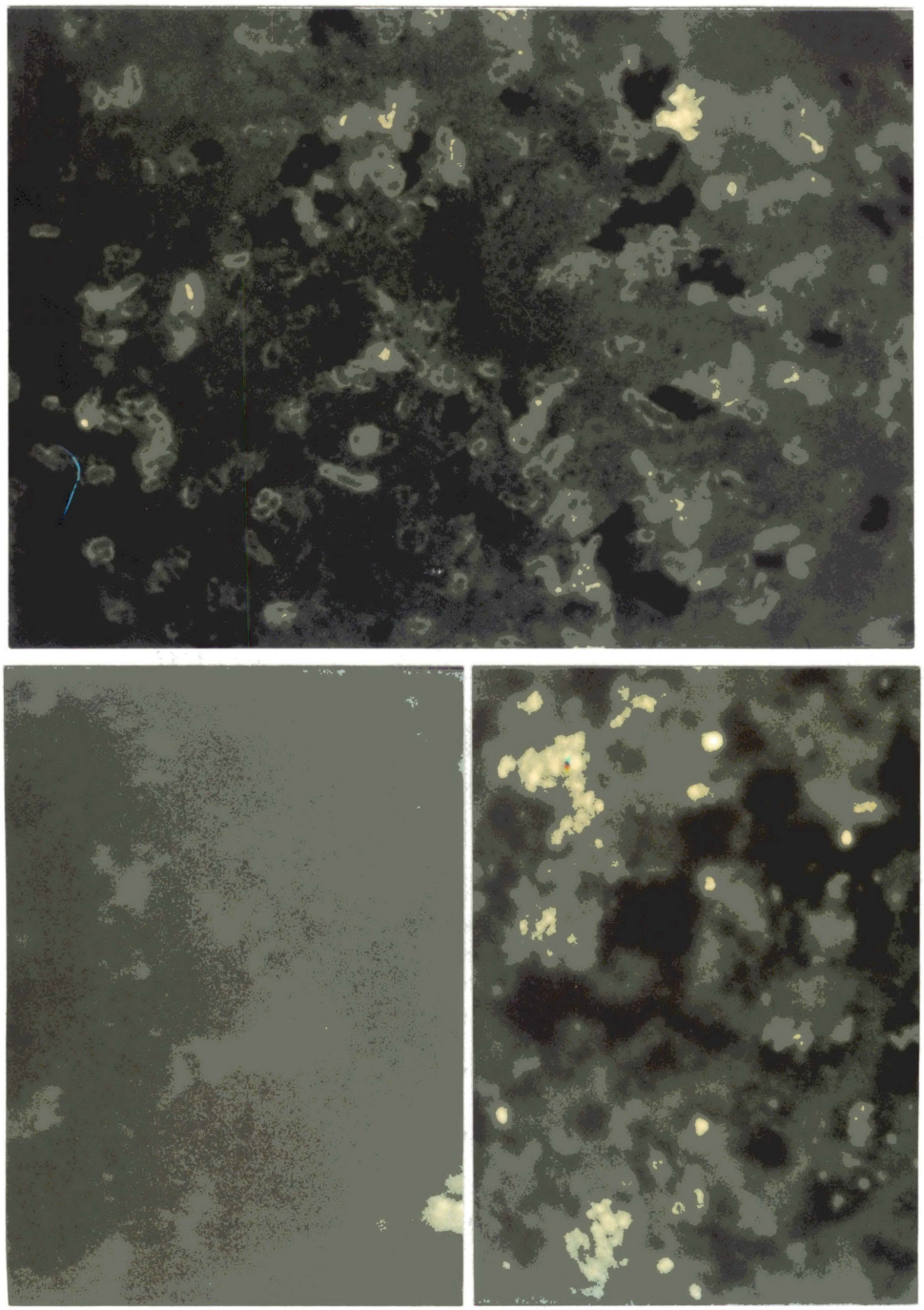
*The grading scale was similar to that used to grade groups A and B streptococci.(Manual of Clinical Microbiology, 1980).

Figure 4. Immunofluorescence of Monoclonal Antibody E11 against *P. haemolytica* and *Hemophilus influenza*

Top Photo: Immunofluorescence with *P. haemolytica*

Bottom Right Photo: Immunofluorescence with *Hemophilus influenza*

Bottom Left Photo: Immunofluorescence with 15% FBS IMDM (negative control)



Electron Microscopy of *P. haemolytica*

P. haemolytica was evaluated with Mab E11 by electron microscopy. Mab E11 did not bind to intact bacterial cell walls, but was seen within the cell boundaries when the membranes were disrupted (Figures 5 and 6). The pattern of antibody binding appeared in aggregations throughout the cytoplasm and not a diffuse pattern. The IMDM with 15% FBS negative control in Figure 7 revealed pores seen in an intact bacterial cell wall with disruption of the adjacent wall. There was no binding of colloid gold within any sections of the negative control examined.

Figure 5. Electron microscopy of Monoclonal Antibody E11 and P. haemolytica using colloid gold as the electron dense indicator. (Magnification 60,000 x; 1 mm = 0.017 u)

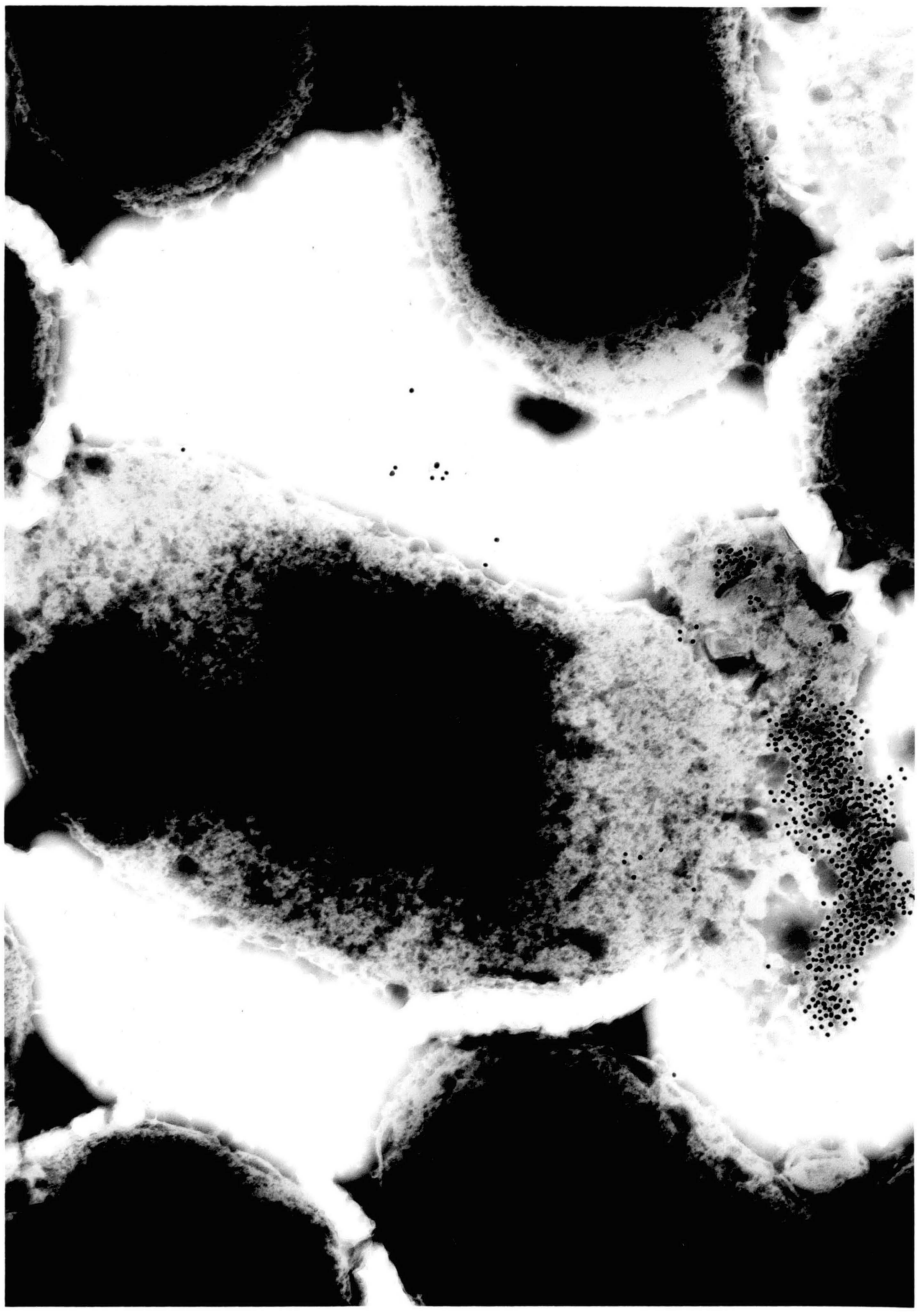


Figure 6. Electron microscopy of Monclonal Antibody E11 and *P. haemolytica* using colloid gold as the electron dense indicator. (Magnification 72,000 x; 1 mm = 0.014 u)

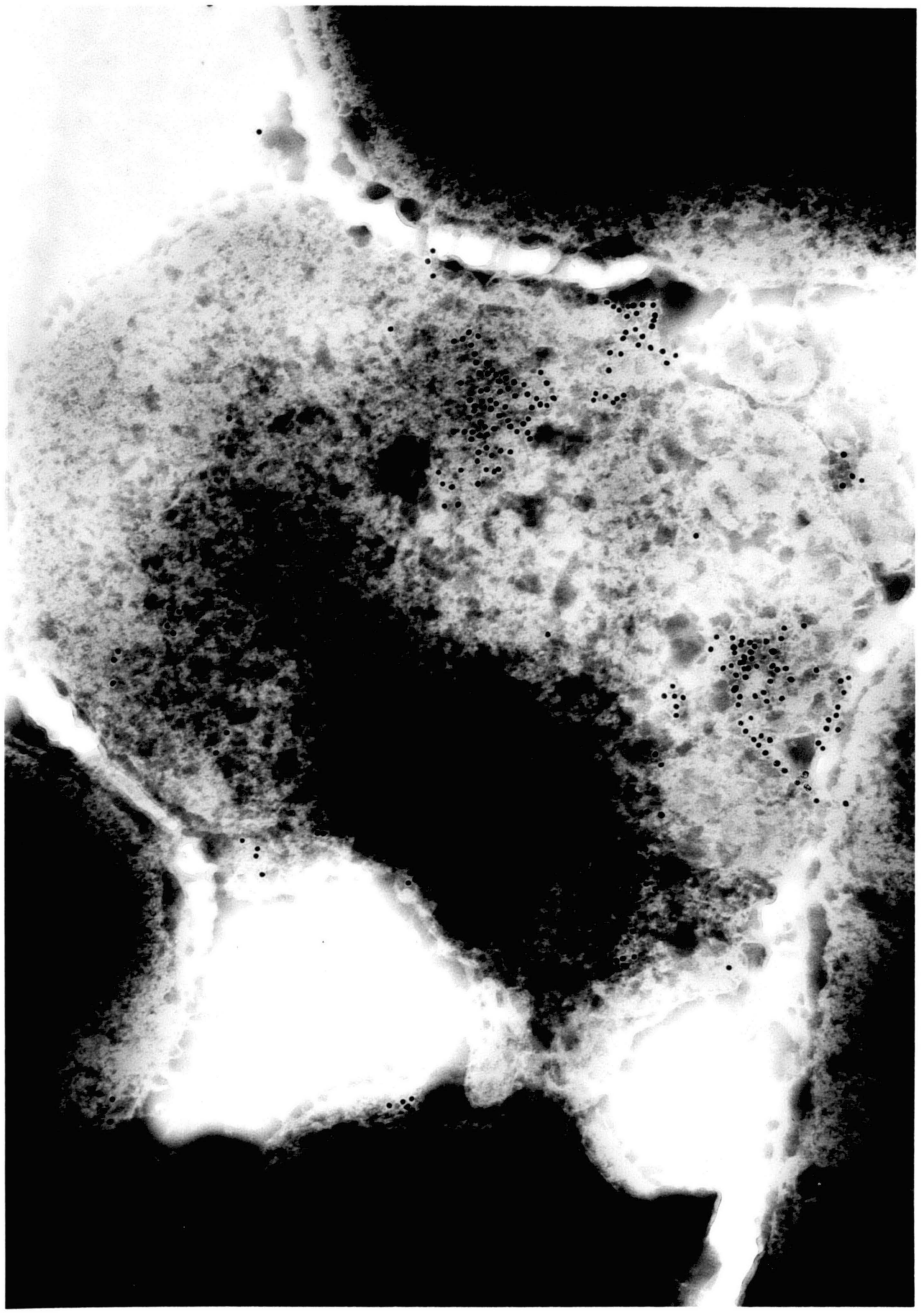


Figure 7. Electron microscopy of negative control fetal bovine serum media and P.haemolytica using colloid gold as the electron dense indicator. (Magnification 42,000 x; 1 mm = 0.024 u)

CHAPTER V

DISCUSSION

A monoclonal antibody (E11) P. haemolytica was produced. The ELISA was used for screening hybridomas for antibody to P. haemolytica. There are both advantages and disadvantages to this system. It is advantageous in that it is economical, efficient, and can screen large numbers of samples (Voller, 1979). The disadvantages included the presence of small amounts of extraneous serum proteins in the screening antigen preparations used in this study. Therefore, there was no ability to differentiate antibody responses to these proteins from responses to P. haemolytica antigens. In addition, it has been suggested that assays using antigen adsorbed to a solid support should not be used to screen monoclonal antibodies that will be used to detect soluble antigens, such as leukotoxin, because of conformational changes that can occur when solute antigens are adsorbed onto solid supports (Vaidya, Dietzler and Ladenson, 1985). Due to the difficulty in purifying the P. haemolytica leukotoxin antigen, the use of functional assays for screening would be more appropriate in detecting antibody of the desired specificity. The most appropriate functional assay is the detection of neutralizing antibodies against the leukotoxin.

Three of the four chosen hybridomas screened in this study seemed to produce antibodies that reacted with either nonbacterial proteins or proteins which cross react to bacterial antigens present in the

antigenic preparations. Three observations suggust this conclusion. The preliminary Western immunoblots using the polyclonal hybridoma fluid to react with BSA and partially-purified leukotoxin were nearly identical (data not shown), although this does not rule out the possibility that the leukotoxin co-migrated with a protein of the same molecular weight as BSA. Secondly, in ELISA using purified lactoferrin as the coating antigen and hybridoma fluids, there was a a high OD_{405} reading to lactoferrin. Thirdly, the ELISA there was a high OD_{405} reading to the lactoferrin and partially-purified leukotoxin prepara-tions but a low OD_{405} reading to the sonicated P. haemolytica or RPMI-leukotoxin preparations when reacted with the four monoclonal antibodies.

The rationale for using a lactoferrin-leukotoxin to screen the hybridomas was to avoid reactivity to BSA that was apparently present in the partially-purified leukotoxin used to immunize the mice. Cross reactions of the hybridoma findings with the lactoferrin protein in the lactoferrin-leukotoxin screening antigen may have occurred by two possible mechanisms (Goding, 1983). First, the monoclonal antibody may have recognized a structurally similar, nonidentical determinant which allows detectable binding, although possibly at a lower affinity. The second possible mechanism is that the monoclonal antibody may be multispecific and recognize two or more epitopes by binding to unrelated antigens with different affinities and types of interactions (i.e. electrostatic, hydrogen bonding, Van der Waals, or hydrophobic effects). Considering the large number of biological macromolecules, the specificity of a given monoclonal antibody is not expected to be absolute.

To aid in the characterization of Mab E11, an SDS-*P. haemolytica* extract, a saline extract, and outer membrane antigen preparations were used. Mab E11 recognized a noncarbohydrate epitope associated with seven protein bands in the SDS-*P. haemolytica* antigen preparation. The multiple number of protein bands is a result of a common recognition site on many differently sized protein molecules. One can only speculate as to their origin. It is possible the repeated epitope sites were to a common protein which the bacteria proteolytically auto-degraded. Although Mab E11 reacted significantly to both the outer membrane and saline extract antigen preparations in the ELISA, minimal reaction was detected to these two antigens on the Western immunoblot. Possible reasons for this difference in reactivity could relate to the nature of a monoclonal antibody and its relationship to an epitope. Conventional polyclonal antisera contains thousands of different antibody clones allowing the binding of antigenic determinants covering most or all of the external surface of the antigen. Therefore, small changes in the structure of the antigen due to denaturation usually has little effect on the binding of polyclonal antibodies (Goding, 1983). However, monoclonal antibodies usually bind a unique site on the protein molecule of the antigen. If this site is altered in any way, such as by SDS and heating, the antibody binding site may be destroyed. In the ELISA system, the native protein is in its three dimensional structure. With the Western immunoblot, the protein structure has undergone denaturation and may be in a linear configuration. Whether or not a conformational epitope can refold following heat and SDS treatment may change the reactivity of an antibody. If the antigenic sites depend on the three dimensional

conformation of the protein or epitope that is denatured and cannot refold, antibody binding cannot occur.

As seen by immunofluorescence, the fluorescence pattern with Mab E11 and P. haemolytica is suggestive of a structural membrane component of the bacteria. There is also a reaction of the Mab E11 with P. multocida and Hemophilus influenza. This suggests that there is a common epitope found among these three bacteria. By electron microscopy, antibody specificity appeared to be to an intracellular component. The differences between antibody patterns of the immunofluorescence and the electron microscopy are possibly due to the differences in bacterial fixation, behavior of the antigenic protein, or a concentration dependent aggregation of the colloidal gold. The formaldehyde concentrations used to fix the bacterial cells for electron microscopy could have potentially changed antigenic epitopes on the cell surface thereby changing the antibody binding site at the cell periphery. The antibody around the cell periphery with immunofluorescence could be either localized within the cell wall, periplasma, or cytoplasmic membrane. Further electron microscopic studies are needed to evaluate this discrepancy from the immunogluorescence studies.

Mab E11 has already been used, through the process of its own characterization, to, in turn, help characterize the P. haemolytica antigen preparations available. The noncarbohydrate epitope recognized was most likely a structural protein found in many P. haemolytica antigen preparations. The fact that several protein bands reacted with the antibody might be explained by proteolytic degradation of bacterial proteins in the antigen preparations. Mab E11 did not recognize an epitope when the saline extract and outer membrane protein antigens were

denatured but bound to antigenic epitopes in the nondenatured configuration. The epitope was one also found on H. influenza and P. multocida whole organisms. Mab E11 recognized the three dimensional conformation on the epitope sites on whole organisms as well as six protein bands on the denatured whole organism.

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