ABSTRACT

James E. Kendall Jr. THE RECOGNITION OF CROSS-REACTING NON-FLAGELLAR EPITOPES FROM SALMONELLA SPP. AND PASTEURELLA SPP.USING THE MONOCLONAL ANTIBODY, M467 AND POLYVALENT ANTISERA (Under the supervision of Dr. A. Mason Smith) Department of Biology December 1987.

The protein M467 produced by the mouse plasmacytoma MOPC 467 is an IgA class antibody that was shown in previous studies to bind Salmonella spp. flagellin, the monomeric protein of flagella. Further studies have demonstrated that M467 will precipitate antigens from heat extracts of Pasteurella pneumotropica indicating the presence of a common epitope shared by Salmonella flagellin and membrane components present in Pasteurella heat extract.

In the present study we have attempted to isolate and characterize the proteins carrying the epitopes present in Salmonella typhimurium and Pasteurella spp. using the M467 protein in double diffusion in agarose gels and various immunoblotting techniques. Bacterial protein patterns were evaluated by SDS-PAGE after Coomassie staining.

Isogenic strains of Salmonella typhimurium, ST 25 (flagellated) and ST 26 (non flagellated) were compared to determine if the epitope resided within the membrane of Salmonella. Extracts of Pasteurella multocida P. haemolytica, and P. gallinarium were also tested for activity with M467. During the course of this study it was determined that phenol extracts from the bacteria being investigated contained more available protein than did the heat extracts initially used. Phenol extracts were therefore used in immunoblotting and immunization preparations.

The results from immunoblotting provides evidence that in each of the bacteria shown to be bound by M467, there exists multiple molecular weight proteins that possess the epitope recognized by M467. It is suggested that the epitope present in these gram negative bacterial proteins is a highly conserved peptide sequence that for selective reasons has been retained in the genome of these bacteria throughout their evolution from a common ancestor.

It is believed that these bacteria which share a common epitope elicit cross-reacting antibodies that are able to recognize the epitopes of one another. To determine their immunogenic potential, the phenol extracts were used to immunize BALB/c mice. ST 26 and P. multocida phenol extracts were injected i.p. and i.m. using Freund's adjuvant. ELISA assays were performed to detect the production of polyvalent antibodies to the immunogens. Results from the ELISA indicate that the phenol extracts did induce antibody formation and that cross-reactivty was measured in reciprocal assays; ST 26 antisera reacted specifically with ST 26 phenol proteins and with the antigens from P. multocida phenol extract. P. multocida antisera reacted with P. multocida phenol extract and showed cross-reactivity with ST 26 phenol extract. Normal mouse serum was used as a negative control.

Western blots were performed on the ST 26 and P. multocida phenol extracts using the anti-ST 26 serum and anti-P. multocida serum. Results of these two blots further substantiates that there was cross-reactivity between the two phenol extracts of ST 26 and P. multocida. Cross-reacting bands appeared to be identical or very closely related by molecular weight estimation as well as antigenic properties.

THE RECOGNITION OF CROSS-REACTING, NON-FLAGELLAR EPITOPES FROM SALMONELLA SPP. AND PASTEURELLA SPP. USING THE MONOCLONAL ANTIBODY, M467, AND POLYVALENT ANTISERA

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Introduction

The bacterial cell surface and envelope components have been of medical interest for many reasons. There was early recognition that cell surface antigens played a role in the virulence of pathogens and that these antigens were useful for serologic identification and vaccine prophyllaxis. It has been determined that bacteria are probably the most important natural modulators of the host immune system and growing interests in this area have attempted to relate the structure of specific microbial products with their biological activities in a mammalian host. It is for these reasons that bacterial envelope components are being extensively studied.

Gram positive and gram negative bacteria differ fundamentally with respect to the structure and biochemical composition of their cell walls, the major difference being that gram negative cells contain an outer membrane, located at the outside of a monolayer of peptidoglycan. This outer membrane functions as a barrier between the external environment and the cell body. The outer membrane controls access of solutes and external materials to the cytoplasmic membrane. This dual role played by the outer membrane allows gram negative bacteria such as members of the Enterobacteriaceae family to inhabit the colon and the gut. The outer membrane protects the cytoplasmic membrane from the detergent-like action of bile salts, fatty acids, and glycerides in addition to the proteolytic and lipolytic enzymes produced in the gut (Lugtenburg and Van Alphea, 1983). Conjugative transfer, bacteriophage reception, bacteriocin specificity and selective uptake of nutrients are all processes that are facilitated

by the presence of structures associated with the outer membranes of gram negative bacteria (Osborne and Wu, 1980). The content of the outer membrane consists of phospholipids, lipopolysaccharides (LPS), and proteins. Phosphatidylethanolamine, phosphatidylglycerol, and diphosphatidylolycerol are the major phospholipids found in gram negative bacteria and are responsible for the lipid bilayer structure of the outer membrane. LPS, which is a characteristic of gram negative bacteria, is an amphipathic molecule with the lipid-A at the hydrophobic end and an oligosaccharide core with usually the O-antigen, a polymer of repeating carbohydrate units, at the hydrophilic end (Wheat, 1980). LPS is one of the most widely studied antigens of Salmonella and other gram negative bacteria. Within the bacterium, it functions as a selective permeability barrier of the cell and as a receptor for adsorption of some bacteriophages. For the host, various biological and pathophysiological effects have been attributed to the presence of LPS from experimental or natural infection. The beneficial effects include immunogenicity, enhanced non-specific resistance to bacterial infection, immunoadjuvancy, compliment activation, B-cell mitogenicity in mice, and tumor necrosis. Conversely, the detrimental effects from LPS exposure include local Schwartzman reaction, leukopenia, disseminated intravascular coagulation, endotoxin shock, and mortality (Elin and Wolff, 1976 and Morrison and Ryan, 1979).

The major proteins that are associated with the outer membrane are murein lipoproteins and matrix protein (porins). Matrix porins are a group of proteins that function as membrane pores to control

access of solutes to the periplasm and inner membrane which is accomplished by a battery of proteins which form transmembrane channels of varying degrees of specificity. The majority of studies of porin proteins have been with the Enterobacteriaceae family members, specifically E. coli and Salmonella typhimurium (Osborne and Wu, 1980; Lugtenburg and Van Alphen, 1983).

Three major proteins from S. typhimurium with 34,000, 35,000, and 36,000 molecular weights, have been identified and described as porin proteins. A fourth protein, with a molecular weight 33,000, has also been reported in S. typhimurium as a porin protein and is homologous to the OmpA protein of E. coli. Genetic mapping data indicate that the Salmonella porins of 35,000 and 36,000 MW, are genetically homologous to the E. coli proteins, OmpF and OmpC, respectively (Sato and Yura, 1979). The biologically active form of porin was reported by Nakae, Isaii and Torunaga (1979) to be a trimer made up of three individual porin proteins, each with the capacity to form a pore, and that the interaction of the three channels within a trimer is a highly cooperative phenomenon (NaKae, Ishii and Torunaga, 1979 and Tokunaga, Tokunage and Nakae, 1979). Additional information on porins of non-enteric organisms has begun to emerge recently. Proteus mirabilis, has two major outer membrane proteins with molecular weights of 39,000 and 36,000 and porin activity (Nixdorff et al., 1977). Pasteurella multocida has been described by Lugtenburg, Von Boxtel and Jong (1984) with H (heavy) and W (weak) major proteins with porin-like characteristics that are present in SDS-PAGE gels at molecular weights of 40,000 and 28,000.

The other major protein component of gram negative bacteria is associated with the lipid to form lipoproteins. Braun and investigators (1969) were the first investigators to purify an outer membrane lipoprotein, a lipoprotein from E. coli with a molecular weight of 7,200 and containing 58 amino acid residues which are covalently bound to the peptidoglycan layer. Braun's group also discovered that lipoprotein exists as twice as many copies in a free form (ie. not covalently attached to the peptidoglycan), than in the attached form. Lipoprotein is by far the most abundant protein in the gram negative cell and much progress has been made in purifying and sequencing both the free and attached forms of the lipoprotein of E. coli. It has been suggested that lipoprotein has several functions, such as determination or maintenance of the rod shape of the bacteria, stabilization of the outer membrane structure, and anchoring the outer membrane to the peptidoglycan layer (Braun and Rehn, 1969 and Sonntag et al., 1978).

The structure of the lipoprotein has been extremely well conserved in the Enterobacteriaceae (Nakemura, Pirtee and Inouye, 1979). Since lipoprotein may be obtained in pure form, the antisera produced against it can be use to detect the presence or absence of lipoprotein-related structures in these species (Braun, 1975). Antisera to lipoprotein from E. coli reacted with the lipoprotein from the envelopes of S. typhimurium, Shigella flexneri, Citrobacter spp. (Bosch, 1974) and Serratia marcescens (Halegoua, Hirashima and Inouye, 1974). The envelopes of other gram negative strains tested

(Aerobacter aerogenes, Klebsiella aerobacter, Proteus mirabilis, Pseudomonas aeroginosa) did not react with the E. coli anti-lipoprotein antibody (Bosch, 1974).

The use of bacterial products in prophylaxis of enterobacterial and other pathogenic gram negative bacteria diseases has proved difficult to achieve. In Salmonella for example, the only recognized vaccine still consists of whole killed bacteria. There are problems associated with this method, such as incomplete and sometimes short duration of protection, and the adverse effects due to toxicity of the lipopolysaccharide mentioned earlier (Ganfield, Rebers and Heddleston, 1976; Kuusi et al., 1979). It would be desirable to develope a vaccine based on a non-toxic component of these bacteria. It has been suggested that a logical component for immunization would most likely be the porin proteins found in the outer membrane of gram negative bacteria. The porins have been reported to be exposed on the surface of the bacteria because they serve as receptor sites for bacteriophages (Osborne and Wu, 1980) and also from evidence from radioactive iodine labelling (Lugtenberg et al., 1986). This evidence suggests that porins might be reached by antibodies and that infections due to these gram negative bacteria may be prevented with an effective vaccine that produces antiporin antibodies.

Studies conducted by Kuusi and colleagues (1979) demonstrated that immunization of White New Zealand rabbits and mice with a purified S. typhimurium porin complex was effective in producing antiporin antibodies, and that it had a highly significant protective capacity against intraperitoneal Salmonella infection in mice.

Supporting evidence comes from experiments conducted on P. multocida by Lugtenberg et al., (1986). It was found that the reaction between electrophoretically separated cell surface constituents with guinea pig and sow antisera showed that LPS as well as several membrane proteins were immunogenic. The outer membrane porin, protein H, resides on the surface of the outer membrane which makes it a logical candidate for vaccine analysis. Protein H possesses many of the physical properties of the porin proteins found in the family Enterobacteriaceae. Lugtenburg's group demonstrated that the H protein shares many properties with the pore proteins found in the Enterobacteriaceae, ie., insolubility in Triton X-100 in the presence of Mg^{2+}, resistance to degradation by trypsin, resistance to solubilization to free monomers in SDS at 37^{O}C, the formation of tight complexes with peptidoglycan, and localization at the cell surface. It may be predicted, according to Osborn and Wu (1980), that porin proteins must occur as major components in the outer membrane of all gram negative bacteria.

The constituents of gram negative bacterial outer membranes have been studied to determine the existance of common epitopes shared by these bacterial antigens and whether or not cross-reactivity may exist. Investigations by Hofstra and Dankert (1979) compared the major outer membrane proteins and their role as common antigens in the gram negative Enterobacteriaceae species. The results from their study indicated that of the twenty-three enterobacterial strains and five non-Enterobacteriaceae species investigated, all of the enterobacterial species cross-reacted with antisera generated against

E. coli outer membrane proteins. Cross-reactivity among the several E. coli serotypes showed a higher degree of binding than did the activity measured with S. typhimurium, K. pneumoniae, and P. vulgaris. The results of their experiments indicate that antigenic cross-reactivity of the major outer membrane proteins is a general phenomenon in the Enterobacteriaceae group and is independent of molecular weight variations among the different bacterial strains.

In the past, antisera had been the essential reagent of bacterial serology. Today, however, with the advances of hybridoma technology, the use of monoclonal antibodies to study bacteria is rapidly expanding. Monoclonal antibodies offer many advantages over polyclonal antibodies. A monoclonal antibody is monospecific in the sense that it recognizes a single antigenic determinant (epitope) or slight variations of it, whereas polyclonal antiserum is of undefinable complexity and the range of specificities cannot be ascertained. Since these monoclonal antibodies have a defined specificity for a single epitope, they will give lower non-specific binding and background in immunoassays. An additional advantage of monoclonal antibodies is that the hybridoma producing these antibodies is immortal and can be grown in large amounts in tissue culture or in ascites fluid and thus provide potentially inexhaustable supplies of antibodies. Also, monoclonal antibodies interact with their target antigens in a highly reproducible fashion, whereas polyclonal antisera may show variation from one batch to the next. These features of monoclonal antibodies make them the best possible reagents for

studying the serology and taxonomy of bacteria (Lam, Mutharia and Hancock, 1985).

Because of the importance of membrane protein structures and lipopolysaccharides in the study of immunity to bacterial diseases, monoclonal antibodies have become important reagents in modern studies (Mutharia, Lam and Hancock, 1985). Several studies have reported the use of monoclonal antibodies to study the cross-reactivity of LPS structures among gram negative bacteria. Extensive cross-reactivity between several gram negative bacteria were described by Mutharia's group when using monoclonal antibodies reactive to lipid A from E. coli and P. aeruginosa. Cross reactivity among the outer membrane and LPS preparations of 36 P. aeruginosa strains and 22 other gram negative bacteria from families Vibronaceae, Enterobacteriaceae, Rhizobiaceae, and Pseudomonadaceae were detected. It has been suggested that this extensive cross-reactivity detected by lipid A specific monoclonal antibodies may explain the protective effects of antisera to E. coli organisms against bacteremia caused by other gram negative bacteria (Braude, Ziegler and McCutcham, 1978). According to Mutharia, Lam and Hancock (1985), "The strong conservation of these antigenic determinants throughout gram negative bacteria suggest that lipid A has an important role in these bacteria and may indicate a common evolutionary lipid A molecule".

Highly specific monoclonal antibodies that react with common epitopes have been used to study the antigenic conservation of proteins and peptides among the Pseudomonads and other gram negative bacteria. In these studies, it was found that strains of the gram

negative bacterium, P. aeruginosa, all shared at least two separate, conserved outer membrane antigenic sites on proteins F and H2. Protein F is a porin protein and the protein H2 is a major outer membrane lipoprotein.

Cross-reactivity of the monoclonal antibody to P. aeruginosa lipoprotein H2 was seen in the outer membrane proteins of similar molecular weight in Western immunoblots from P. chlororapis, P. fluorescens, P. putida and P. syringae, and with higher molecular weight proteins in P. stutzeri, P. pseudomallei, P. anguilliseptica, and Azotobacter vinelandii. No cross-reactivity was detected in E. coli, P. acidovorans, P. maltophila or S. typhimurium. The results from these Western blots confirm data on taxononic studies from rRNA homology which suggest that A. vinelandii is related to the fluorescent pseudomonads, while P. acidovorans and P. maltophila are not (Mutharia, Lam and Hancock, 1985).

Similar antigenic conservation of the porin protein F from P. aeruginosa was detected in Western immunoblotting with all strains of P. aeruginosa and P. putida and P. syringae. No activity was seen in any of the other gram negative bacteria used (Lam, Mutharia and Hancock, 1985). These studies demonstrate the feasibility and application of monoclonal antibodies as tools to study the immunological and taxonomic relationship of bacterial outer membranes.

The production of monoclonal antibodies has also been described in mice as a result of oncogenic mechanisms. The most widely studied immunoglobulin-producing tumors in mice are the plasmacytomas which appear to arise from cells specialized to produce only a single

molecular type of immunoglobulin. It is generally accepted that normal plasma cells are similarly specialized and that the tumors by their great proliferation potential are amplifications of individual cell types within the immune system. Plasma cell tumors have been most useful in providing a source for large quantities of homogeneous immunoglobulins, since most of the tumors are relatively easy to transplant in syngeneic hosts. The transplants obtain massive size, approaching one-third of the body weight, and large quantities of immunoglobulin can be isolated from the serum, ascites fluid, or urine of mice. Tumor transplant lines are usually quite stable and maintain the continuous production of the characteristic immunoglobulin through many generations (Potter, 1972).

One such tumor is MOPC 467, a murine IgA protein producing plasmacytoma originally induced in a BALB/cAnN mouse. The MOPC 467 plasmacytoma was induced by intraperitoneal injections of mineral oil into BALB/c mice which are apparently genetically predisposed to tumor formation. Potter originally described the specificity of MOPC 467 to antigens isolated from P. pneumotropica and three different serotypes of Salmonella spp., as being cross-reactive and sharing lines of identity in Ouchterlony gels (Potter , 1971). MOPC 467 was shown to bind an antigen from over twelve species of Salmonella, Pasteurella and Herellea. The antigenic activity possessed by S. tranoroa was destroyed in the presence of trypsin and on this basis, it is thought that the antigens recognized by MOPC 467 were proteinaceous. Later studies (Smith and Potter, 1976) demonstrated that the purified IgA

protein from MOPC 467 ascites fluid, designated M467, precipitated S. milwaukee flagella, both monomeric (MON) and polymeric (POL) flagellin, and crude heat extract, but not with the lipopolysaccharide from S. milwaukee. However, these findings did not explain why M467 was able to bind P. pneumotropica because members of the Pasteurella genus are non-flagellated.

In the present study, the isolation and characterization of the antigens recognized by M467 in Salmonella spp. and Pasteurella spp. have been investigated. Two isogenic forms of S. typhimurium, ST 25 (flagellated) and ST 26 (non-flagellated), were compared to determine the presence of epitopes which possibly reside within the outer membrane. Four members of the Pasteurella genus were investigated to determine the existence of the antigens and to compare their relatedness by the presence of available epitopes. The immunogenic potential of outer membrane proteins from Salmonella spp. and Pasteurella spp. has also been studied. The importance of the findings of this study will be discussed in terms of modern health uses such as vaccine potential and prophylaxis against gram negative bacterial infections.

Materials and Methods

Mice

BALB/c mice were obtained from a breeding stock maintained at the E.C.U. School of Medicine. The mice were maintained in clean quarters and fed Wayne Mouse Chow and tap water ad libitum.

Bacterial Membrane Extractions and Purification

Pasteurella multocida (ATCC 15743), P. haemolytica (ATCC 33365), P. gallinarium (ATCC 13360), and Staphylococcus epidermidis (ATCC 155) were all obtained from American Type Culture Collection, Rockville, MD. Isogenic Salmonella typhimurium strains ST 25 (fla^+) and ST 26 (fla^-) were graciously provided by Dr. Michael Carsiotis of the Department of Microbiology, University of Cincinnati, Cincinnati, Ohio.

Salmonella and Pasteurella cultures were grown in 2 liter flasks containing 37g of brain-heart infusion (Becton-Dickinson and Co., Cockeyville, Md.) with 1% yeast extract dissolved in 1 l of dH_2O. The cultures were incubated at 37^oC for 48 h. The cultures were then centrifuged at 14,360 x g for 15 min on a Beckman Model J-21B centrifuge (Beckman, Fullerton, Ca.) and washed twice with sterile 0.15M NaCl. S. epidermidis was grown in Difco trypticase soy broth at 37^oC.

A. Purified Heat Extract (PHE) - Pelleted bacteria were suspended in one hundred ml of sterile 0.15M NaCl (saline) in a 250 ml Erlenmeyer flask. The flask was placed in a boiling water bath for 1 h. The suspension of cells were centrifuged an additional time at 14,360 x g and the supernatant was decanted. This supernatant was initially

referred to as heat extract (HE). The HE was further centrifuged at 40,000 x g and the supernatant collected. The supernatant was dialyzed in 3,500 MW cutoff dialysis tubing for two days with frequent dH_2O changes. This final preparation was referred to as purified heat extract (PHE) and was lyophilized following extensive dialysis.

B. Phenol Extract - A modified phenol/water extraction as described by Westphal and Jann (1965) was performed. An equal volume of 45% phenol was added (v/v) to the pelleted bacteria in a 250 ml Erlenmeyer flask and shaken in a 65^{O}C water bath for 30 min. The suspended cells were allowed to cool on ice for 15 min. The suspension was centrifuged at 9000 x g for 30 min after which the water phase was removed by aspiration. The phenol phase was placed into clean tubes and a re-extraction with an equal volume of dH_2O was performed by shaking the mixture well and centrifuging again at 9000 x g for 30 min. The aqueous phase was discarded. Proteins were precipitated by adding six volumes of 95% ethanol while gently stirring. The mixture was covered with parafilm and kept at -10^{O}C overnight.

The precipitate was washed three times in -10^{O}C ethanol. The pellet was resuspended in dH_2O and dialyzed extensively at 4^{O}C using Spectropore dialysis tubing (Thomas Scientific, Philadelphia, Pa.), M.W. cutoff 3,500 for 3 d with frequent changes of large volumes of dH_2O. The samples were shell frozen in liquid nitrogen and lyophilized .

C. Isolation of Outer Membrane Complexes - Outer membrane complexes were isolated as described by Poolman, Hopman and Zanen (1978). ST 26 bacteria were collected from brain-heart infusion broth by

centrifugation for 30 min at 7000 x g, 4°C. The pellet was washed twice in sterile saline. Following the final wash, the bacterial pellet was suspended in 50 ml of 0.2M LiCl, 0.01M EDTA (ethylenediamine tetraacetic acid, Sigma, St. Louis, Mo.), pH 7.0 and incubated for 2 h at 45°C in a water bath with agitation. The isolation of outer membrane complexes proceeded by differential centrifugation: 30 min at 20,000 x g, 4°C, followed by 60 min at 100,000 x g, 4°C. The latter pellet was suspended in 0.2 ml of 0.0625M Tris-HCl at pH 6.8 and stored at 4°C.

D. Boivin Endotoxin Extraction by TCA - Endotoxin protein was prepared from TCA-extracted endotoxin exactly as described by Sultzer and Goodman (1976). TCA-extracted endotoxin from S. typhimurium (lot#93F-4044), S. minnesota (lot#93F-4022), S. abortus equi (lot#83F-4003), were purchased from Sigma.

Mouse Immunizations

A. Phenol Soluble Polypeptide (PSP) Antigens - Phenol extracts of membrane antigens from P. multocida and ST 26 were used to immunize mice. Three mice were immunized on day 0 with 25 ug of P. multocida PSP extract and three others were injected with ST 26 PSP. The initial injection for each mouse contained 25 ug of PSP dissolved in 0.1 ml of 0.02M Tris, 0.15 M NaCl at pH 8.0 (TBS) and emulsified with an equal amount of Fruend's complete adjuvant (FCA). The first injection was given intraperitoneally (i.p.).

On day 7, the mice were given a second i.p. injection with 25 ug of PSP dissolved in 0.1 ml of TBS and emulsified with an equal amount of Freund's incomplete adjuvant (FIA). The mice were boosted on day

14 with 25 ug of PSP antigen in 0.1 ml of TBS. This injection was given subcutaneously in two locations, one behind the neck and the second on the hind-quarter. These mice were bled on day 19 by an intra-orbital bleeding method. Serum was collected by allowing the whole blood to clot on ice for 10 min and was then centrifuged at 500 x g and stored at $4^{O}C$.

B. Electroeluted Antigen Preparation and Injections

Five hundred ug of P. multocida PSP purified by electroelution was dissolved in 2 ml of sterile 0.15M NaCl. The 2 ml solution was added to the MPL and TDM Emulsion (Ribi, Hamilton, Montana) containing 0.5 mg monophosphoryl lipid A, 0.5 mg trehalose dimycolate, 0.04 ml squalene and 0.004 ml monooleate. The mixture was vigorously vortexed for 3 min to emulsify the solutions.

Five male mice were injected i.p. on day 0 with 0.2 ml of adjuvant containing 25 ug of the P. multocida antigen. An identical injection was given on day 7, and on day 14 all mice were bled and serum was collected as before.

Preparation of Affinity Gel and Purification of MOPC-467 Protein (M467).

A. Affinity Gel Preparation CH - Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, N.J.) was prepared as recommended by the manufacturers for coupling of proteins with carbodiimide as the coupling reagent. Solutions containing 10 to 20 mg POL/ml were depolymerized into monomeric form (MON) by adjusting the pH to 2.0 with 1.0 N HCl. The MON solution was mixed with an equal volume of the swollen gel and 1-ethyl-3-(3-dimethyl-aminoproyl)-carbodiimide HCl

(Sigma) was added to give a final concentration of 0.1M. The components were mixed on a gyratory platform for 18 h at room temperature. The gel was then centrifuged at 500 x g for 10 min. The supernatant was removed and the gel resuspended in 0.2M Tris-HCl, pH 8.0 for 2 h. The affinity gel (CH-MON) was washed repeatedly on a sintered glass filter with 0.2M glycine-HCl; pH 2.5 containing 1.0M NaCl. After a final wash the CH-MON was resuspended in TBS and stored at $4^{O}C$ in 0.01% NaN_3 until used (Smith, Slack and Potter, 1977).

B. Antibody Purification M467 monoclonal IgA antibody was collected from BALB/c mouse peritoneal ascites fluid carrying the MOPC 467 plasmacytoma. A 50% saturated ammonium sulfate precipitation was performed by adding 31.3g of ammonium sulfate (Sigma). The precipitated protein pellet was centrifuged at 3000 x g and the supernatant was decanted. The pellet was resuspended in sterile 0.15M NaCl and the 50% ammonium sulfate precipitation was repeated. After the pellet was again resuspended and pelleted, a 47% ammonium precipitation was done and the precipitate was pelleted by centrifugation at 3000 x g. The pellet was resuspended in 100 ml of 0.15M NaCl with 0.05M NaN_3 and stored at $4^{O}C$.

Immunoelectrophoresis

The purity of M-467 was tested by an immunoelectrophoresis assay. Gel Bond 85 mm X 100 mm immunoelectrophoresis gel plates were used. The hydrophobic/hydrophilic plates were cut in half lengthwise and placed on a leveling table with the hydrophilic side up. Agarose gel was prepared using 850 mg of Highest Electroendosmotic (HEEO) Agarose (Seakem, FMC Corp, Rockford, MD) added to 100 ml of 0.05 ionic

strength Oxoid Barbitone Acetate buffer (Oxoid Ltd., England). The agarose solution was placed in a boiling water bath until the agarose had completely dissolved. A 7.5 ml aliquot of 0.85% HEEO agarose was then poured onto the gel plates and allowed to solidify at room temperature. The gels were stored at 4°C in a humidity box. The immunoelectrophoresis pattern was made by using a template to cut two wells separated by a trough in the agarose gel. Excess agar was removed with suction. Capillary tubes were used to fill one well with purified M467 and clarified M467 ascites fluid in the other. The wells were filled several times allowing the sample to diffuse into the gel between additions. The gel was then electrophoresed at 38V for 50 min at 4°C using an Electrophoresis Continuous Power Supply (Pharmacia). After the 50 min electrophoresis, the gel was placed in a humidity box and the gel in the trough was removed with suction. The trough was then filled with goat anti whole mouse serum (Cooper Biomedical, Malvera, Pa.) and allowed to incubate overnight at 4°C.

Double Diffusion in Agar Gel Assay (Ouchterlony)

Gel plates were made using 850 mg of Ion Agar No. 2 (Colab Laboratories, Inc.) added to 100 ml TBS and placed in a boiling water bath until the agar had dissolved completely. A base coat solution of 0.1% French Agar was placed in the boiling water bath. Glass microscope slides (2.5 cm x 7.5 cm) were coated with the base coat and allowed to dry on a leveling table. 7.5 ml dissolved ion agar solution was added to the coated plates. The 0.85% Ouchterlony plates were allowd to solidify, placed in humidity boxes and stored at 4°C until used.

Reactivity of bacterial extracts with M467 was tested by dissolving the bacterial protein extracts in TBS (500 ug/100 ul) and placing aliquots from various species into each well. M467 filled the central well and the gel was incubated for 2-3 h at 37OC or at room temperature overnight.

SDS-PAGE Gel Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 14% acrylamide slabs (8 x 7 cm) as described by Laemmli (1979). Ten well SDS-PAGE gels were made with the multiple casting chamber, Model SE215, (Hoefer, San Francisco, Ca.). Electrophoresis of the gels was performed on the Hoefer Mighty small slab gel electrophoresis unit, model SE200 powered by an electrophoresis power supply (Pharmacia, Piscataway, NJ).

Stock 30% acrylamide (Bio-Rad) with 2.7% bis-acrylamide (Bio-Rad) were prepared and stored in a brown plastic bottle at 4^{O}C. The 14% resolving gel was prepared by adding 47 ml of stock bis-acrylamide to 25 ml of resolving gel buffer (1.5M Tris-HCR pH 8.8), 30 ul TEMED (N,N,N',N' tetramethylethylenediamine, Bio-Rad) and 27 ml dH_2^{O}. This mixture was degassed for 5 min and 0.5 ml of freshly made 10% ammonium persulfate (Sigma) was added to initiate polymerization of the gel. The unpolymerized mixture was gently poured into the casting chamber with the pre-arranged plates and 1.5 mm spacers. Air bubbles were avoided. A 0.5 ml aliquot of butanol was layered atop the resolving gel to provide a smooth interface for the stacking gel. This was allowed to sit at room temperature until the gel polymerized. The

butanol was poured off and 0.5 ml of resolving gel overlay solution (0.375M Tris-HCl, pH 8.8, 0.1% SDS) was added to the resolving gel until the stacking gel was prepared.

The stacking gel was prepared by adding 5.32 ml of stock bis-acrylamide to 10 ml of stacking gel buffer (0.5M Tris-HCl, pH 6.8), 24.8 ml of dH_2O, 20 ul of TEMED and degassed for 5 min. Two hundred ul of 10% ammonium persulfate was added, and the stacking gel was poured on top of each gel after the gel overlay buffer was removed. After enough stacking gel was poured to fill each gel cast, the polypropylene 10-well comb was inserted before polymerization occurred. This was allowed to sit at room temperature for 1 hr after polymerization of the gels. The casting unit was then disassembled and the gels were individualy wrapped in cellophane and stored at $4^{O}C$ for up to 1 week.

Protein samples were dissolved in treatment buffer (0.06M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercapto-ethanol) and heated in a boiling water bath for 5 min. Samples were prepared with 5 ug of protein sample per ul of sample buffer. Appropriate amounts were placed in each well. Laemmli (1979) tank buffer (0.025M Tris pH 8.3, 0.192M glycine, 0.1% SDS) was used to fill the gel electrophoresis system, and the gels were electrophoresed at 150V constant voltage until the smallest molecular weight marker had reached the bottom of the gel. Gels were passively stained in 0.125% Coomassie Blue R-250 (Bio-Rad) in 50% methanol and 10% acetic acid overnight. They were passively destained in 25% methanol and 10% acetic acid.

Isolation of Proteins from SDS-PAGE gels

The procedure for isolating proteins from acrylamide gels using the Model UEA Electroelution Unit (International Biotechnologies, Inc., New Haven, Conn.) was performed as described by the manufacturer.

The 14% SDS-PAGE gels were prepared as described above, except with one-half the amount of stacking gel and without combs for wells. This allowed for optimal use of the gel and provided the necessary trough for sample application. Five hundred ul of sample buffer (0.125 Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol) containing 2.5 mg of P. multocida phenol soluble polypeptides (PSP) was added to each gel. The gel was electrophoresed as described above in the Mighty Small Slab Gel Electrophoresis Unit for 50 min, after which the gel was removed from the gel plates and a 0.5 cm vertical strip of gel was sliced off with a scalpel. The remainder of the gel was placed in a small amount of tank buffer until the sample vertical slice could be stained in 0.125% Coomassie Blue R-250 (Bio-Rad, Richmond, Ca.), 50% methanol and 10% acetic acid and destained in 25% methanol and 10% acetic acid in order to locate the position of the bands to be excised. The electroelution unit was filled with 450 ml of tank buffer (25mM Tris, pH 8.3, 0.192M glycine, 0.1% SDS) and the V-channels of the unit were loaded with 125 ul each of 7.5M ammonium acetate (Sigma) salt cushion, using a 1.0 ml Hamilton syringe. Acrylamide gel slices containing the desired protein bands were excised and minced into small pieces and placed into the slots on the eluter platform. The protein was eluted for 45 min at 125V at room

temperature. After 45 min, the ammonium acetate containing the eluted proteins was removed without draining the tank buffer or removing gel samples. All gel samples were electroeluted twice. Six SDS-PAGE gels were electrophoresed to obtain the necessary quantity of purified bands. The eluted protein was placed in dialysis tubing (Spectropore, 3500 MW cutoff) and dialyzed extensively for two days. The samples were lyophilized overnight. Protein determinations were performed using the Bio-Rad protein micro-assay technique described by Bradford (1976).

Protein Electrotransfer (Protein Blotting)

Following SDS-PAGE as described above, a nitrocellulose membrane (0.45 uM, Hoefer, San Francisco, Ca.) was cut to the appropriate dimensions of the SDS-PAGE gel to be transferred. Both nitrocellulose membrane and gel were suspended in transfer buffer (0.192M glycine, 0.025M Tris, 20% (v/v) methanol, pH 8.3) to equilibrate for 20 min. The protein electrotransfer procedure was done on a Mini-Transphore TE-22 System (Hoefer, San Francisco, Ca.). One side of the TE-22 holding cassette and one foam sponge were placed into the tray of transfer buffer. All trapped air bubbles were pressed out. One sheet of blotter paper (Whatman #3 filter paper) was placed on the sponge and the gel was laid on top of the blotter paper avoiding the trapping of air bubbles under the gel. The nitrocellulose membrane was placed over the gel, again avoiding air bubbles. A second piece of blotting paper was placed atop the nitrocellulose followed by the other half of the holding cassette. The assembled holding cassette with the

nitrocellulose and gel were inserted into the transfer chamber with approximately 700 ml of transfer buffer. Transfers were carried out in the constant current mode at 200 MA for 45 min using an ISCO model 493 electrophoresis power supply (Isco, Lincoln, Neb.).

Detection of Transferred Antigens (Immunoblot)

Following electrotransfer of proteins, residual binding sites on the nitrocellulose membrane were blocked in order to prevent non-specific binding of antibodies. A nitrocellulose membrane was immersed in blocking medium (TBS with 5% BSA (bovine serum albumin) Sigma, St. Louis, Mo.) and incubated at $37^{O}C$ for 30 min, or at $4^{O}C$ overnight. The membrane was washed 2 times in TTBS (TBS plus 0.05% Tween-20, polyoxyethylene sorbitan monolaurate (Tween 20) Sigma) for 10 min with agitation and was then incubated for 2 h at room temperature with affinity column purified M467, diluted to provide 10 ug/ml in 1% BSA-TBS. The nitrocellulose membrane was again washed twice with TTBS for 10 min each wash. The membrane was incubated at room temperature with 10 ml of goat anti-mouse IgA conjugated with biotin (Sigma) diluted to 10 ug/ml in 1% BSA-TBS. Three 20 min washes with TTBS were performed followed by incubation at room temperature with 10 ml of avidin-peroxidase (Sigma) diluted to 10 mg/ml in 1% BSA-TBS. Next, two 10 min washes in TTBS were done with agitation. Substrate solution was prepared as follows: 6 mg of horse radish peroxidase (HRP) color developer reagent from Bio-Rad was dissolved in 2 ml of cold methanol. Six ul of cold 30% H_2O_2 were added to 10 ml of TBS and thoroughly mixed with the 2 ml of methanol-HRP solution. The substrate solution was overlaid on the nitrocellulose membrane and

incubated on a rocker until sufficient color had developed. The membrane was rinsed in TBS and stored at $4^{O}C$ in the dark until photographed.

Immunoblotting with mouse antiserum

Protein electrophoresis and transfer to a nitrocellulose membrane was done exactly as described above. For the immunoblot, 10 ml of mouse serum diluted 1:100 in BSA-TBS, was used as the primary antibody and 10 ml of biotin conjugated goat antimouse IgG, IgA, and IgM (10 ug/ml in BSA-TBS) from Cooper Biomedical (Malvera, Pa.) , was used as the secondary antibody. The immunoblot protocol was exactly as described above.

Protease Digestion of PSP

5 mg of P. multocida PSP was dissolved in 0.5 ml of TBS, pH 7.0. Two hundred and fifty ug of Pronase (Calbiochem, La Jolla, Ca.) was added to the protein solution and incubated overnight at $37^{O}C$. Following incubation, 0.5 ml of 2X SDS-PAGE sample buffer was added.

Transmission Electron Microscopy of Bacteria

Bacteria grown in broth cultures were washed twice in 0.15M NaCl and resuspended in 1 ml of saline solution. Two-fold dilutions were made in 100 ul in order to make a dilution sample that was not too dense for clarity. Several slotted grids (Ted Pel, Inc., Tustin, Ca.) were made for each dilution sample. A Bacterial suspension (one drop) was placed on top of the grid by the float method, for 2h. After this

time, the droplet was absorbed off with lens tissue, and the grids allowed to air dry for 30 min. The bacteria were stained with 1 drop of 0.2% uranyl acetate for 1 min and air dried. The grids were viewed in a model JEM 1200EX transmission electron microscope from JEOL (Tokyo, Japan).

Enzyme Immunosorbent Assay (EIA) for the Detection of Antibodies Against Bacterial Membrane Antigens

The enzyme immunosobent assay (EIA) was performed ;using a combination of the methods described by Klaassen, Bernard and DiGiacomo (1985) and Kendall, Ioneseu and Dreesman (1982).

A. Plate Preparation - Bacterial antigens used for the detection of antibodies were diluted to 1 ug/ml in coating buffer (15mM Na_2CO_3, 35mM $NaHCO_3$ pH 9.6, 0.05% NaN_3). A 100 ul aliquot of diluted antigen preparation was added to each well of a Cooke Microtiter System plate (Dynatech Laboratories, Inc., Alexandria, Va.), and stored overnight at $4^{O}C$. Unbound antigen was discarded, and the wells were washed three times with phosphate buffered saline containing 0.1% Tween-20 (TPBS). Unbound sites were blocked by the addition of 200 ul of 10% BSA in coating buffer followed by incubation at $37^{O}C$ for 1 h in a moist chamber. At the end of the incubation period, the plates were washed three times with TPBS. The coated plates could be stored at $4^{O}C$ for several days.

B. EIA Procedure - In all experiments, serial 2-fold dilutions of antisera in .1% BSA in PBS were prepared. A 100 ul aliquot of dilute, 0.1% BSA in PBS, was added to each well of a Linbro E.I.A. microtitration plate (Flow Laboratories, Inc. McLean, Va.). Fifty ul

of diluted serum was added to corresponding wells of the plates containing the coated antigens. The plates were incubated in a moist chamber at 37^{O}C for 2 h and, following incubation, the plates were washed again three times with TPBS. One hundred ul of biotin conjugated goat anti-mouse IgA, IgG, and IgM (Sigma) diluted 1:1000 with 0.1% BSA in PBS was added to each well and incubated for 1 h at 37^{O}C. The plates were again washed three times with TPBS, after which 100 ul of avidin conjugated alkaline phosphate (Cooper Biomedical) diluted 1:500 in BSA-PBS was added to each well and incubated for 1 h at 36^{O}C. The plates were given three 10 min washes in TPBS after which 100 ul of substrate, Sigma 104 Phosphatase (disodium p-nitrophenyl phosphate) dissolved in freshly prepared 10% diethanolamine was placed in each well. After the reactants incubated at 30 min at room temperature, absorbance was read at 405 nm on a Titertek Multiskan (Flow Laboratories). Each plate contained as controls no serum, and normal mouse serum diluted 1:100 (non-immunized mouse serum in PBS). Each experiment was performed in duplicate. The last dilution of antiserum producing O.D.$_{405}$ values greater than 2 times the O.D. value produced by non-immune serum was taken as the titer of that serum.

Isoelectric Focusing Gel

Isoelectric focusing was done on premade Isogel agarose IEF plates (FMC BioProducts, Rockland, Me.) with a 3-7 pH range. Power was supplied by the ISCO Model 493 electrophoresis power supply. Isogel (FMC) pI markers with a 3-7 pH range were used. PSP samples from ST 25, ST 26, and P. haemolytica were run on the gel at 640V, 10W

and 16.5 mA for 46 min. The anode solution used was 0.5M acetic acid, pH 2.6 and the cathode solution used was 0.1M L-histidine (free-base). The gel was run as described by the manufacturer. The gel was fixed in 36% (v/v) methanol, 10% trichloroacetic acid, and 3.5% sulfosalicylic acid for 10 min and rinsed 5 min with distilled water to remove excess fixative. The gel was then completely dried with warm air for 30 min and stained with 1% Coomassie Brilliant Blue R-250, 25% ethanol and 9% acetic acid for 30 min. The gel was soaked with 25% ethanol and 9% acetic acid solution until fully destained and then dried with a warm air flow ($60^{O}C$).

HPLC

High pressure liquid chromatography (HPLC) of PSP was done on a Waters system (Waters Chromatography Div., Milford, MA) using a DuPont GF250 column (DuPont, Wilmington, DE). PSP samples were dissolved in 0.3 M phosphate pH 7.5 buffer and chromatographed in the same buffer at a rate of 1 ml/min. Calibration of the GF250 column for molecular weight estimations was accomplished using the following proteins with their molecular weights: Myosin (205,000), phosphorylase B (97,400), pepsin (34,700), trypsinogen (25,000), myoglobin (16,950), myoglobin-CnBr fragment I+II (14,400), myoglobin CnBr Fragment I (8,160), myoglobin CnBr fragment II (6,210), and myoglobin CnBr fragment III (2,510).

Results

Precipitin Reactions Between M467 and Bacterial Antigens

As a preliminary investigation, the bacterial antigens present in the heat extract were tested against M467 in double diffusion in agar gel (Ouchterlony) to determine whether or not M467 would precipitate the antigens. Bacterial PHE from the following organisms were used: ST 25, ST 26, *P. multocida*, *P. haemolytica*, *S. epidermitis* and *E. coli*. Flagellin (MON) from *S. milwaukee* was used as a positive control. Precipitin lines of identity (Fig. 1) were detected between wells containing flagellin and ST 25. No lines of precipitation were seen in ST 26 *P. multocida*, *P. haemolytica*, *P. gallinarium*, *E. coli*, or *S. epidermidis*.

Bacterial phenol extracts were insoluble in TBS and therefore resisted movement into the agar. No lines of precipitation were detected using PSP extracts.

Immuno-Dot Blotting with Bacterial Antigens and M467

To detect M467 reactivity against bacterial antigens that were unable to produce precipitin lines, heat extracted antigens were immuno-dot blotted with M467 (Fig. 2). ST 25, ST 26, *P. multocida* and *P. haemolytica* all showed a positive reaction with M467. *S. epidermitis* was not bound by M467.

These results indicated that the bacterial strains that reacted positively with M467 possess common epitopes that were present in the heat extracts.

FIGURE 1. M467 Precipitin reaction with bacterial antigens in an Ouchterlony gel. Well A, M467; 1) S. milwaukee flagellin (MON); 2) ST 25 PHE; 3) ST 26 PHE; 4) MON; 5) P. multocida PHE; 6) P. haemolytica PHE. Well A'; M467; 7) MON; 8) E. coli PHE; 9) S. epidermitis PHE; 10) MON; 11) P. gallinarium PSP; 12) P. haemolytica PSP.

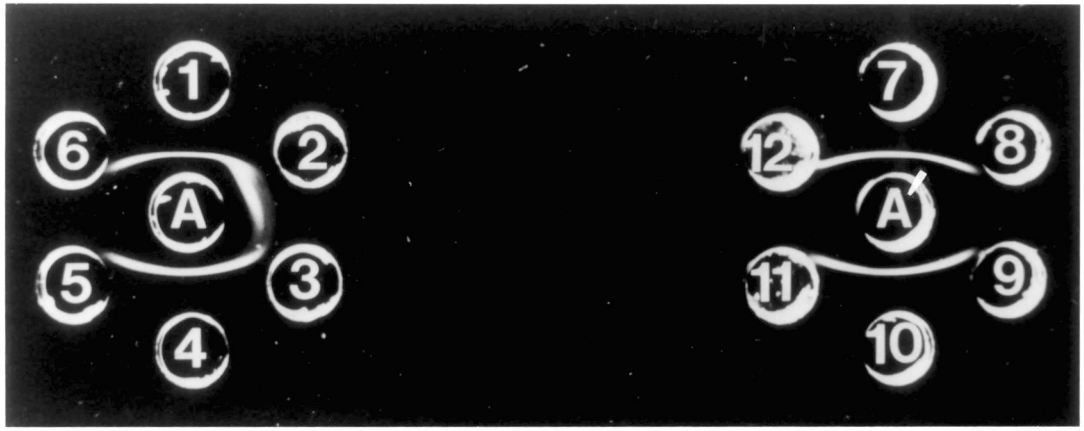
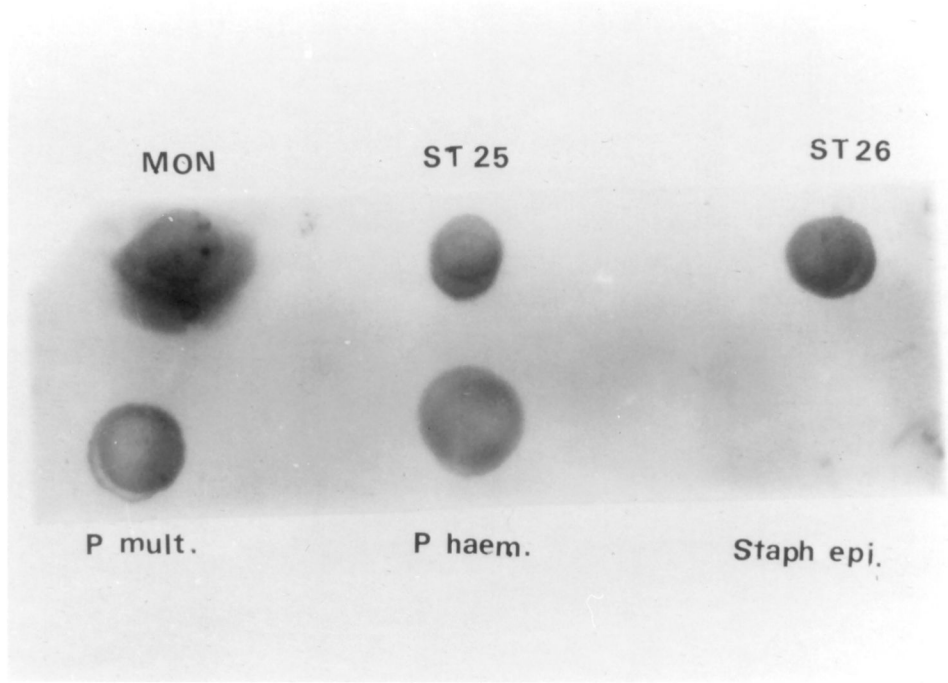

1
6
2
A
5
3
4
7
12
8
A'
11
9
10

FIGURE 2. Dot blot. Bacterial PHE extracts are dot blotted and incubated with M467. Peroxidase conjugated anti-IgA detects antigen-antibody complex. First row; A) MON, B) ST 25, C) ST 26; Second row; *P. multocida*, *P. haemolytica*, and *S. epidermitis*.


MON
ST 25
ST 26
P mult.
P haem.
Staph epi.

Electron Microscopy of Bacterial Cells

Whole bacterial cells were observed by electron microscopy to detect the physical presence or absence of flagella on the surface of the organism in question. Since M467 had been shown to bind flagellar proteins, it was important to eliminate the possibility that these bacteria possessed flagella or flagella-like structures.

Electron micrographs were taken of ST 25, ST 26 and Pasteurella multocida. In Fig. 3, the micrograph of ST 25 clearly demonstrates the presence of numerous flagella, whereas the ST 26 bacterium in Fig. 4 appears to lack flagella, both of which correlates with earlier observations by Carsiotis et al., (1984). P. multocida, pictured in Fig. 5, was also found to be non-flagellated.

It is concluded on the basis of this evidence that ST 25 possess flagella which are the sources of epitopes bound by M467 but no such structure exists in ST 26 or P. multocida. Therefore, it can be assumed that epitopes bound by M467 are located within the structure of the outer membrane as well as a structural component of flagella.

Chromatographic Separation of PSP by HPLC

The phenol soluble proteins of ST 26 and P. multocida were fractionated by HPLC in an initial attempt to isolate the active component recognized by M467. The profiles of the separations are shown in Fig. 6. Individual fractions from the HPLC separation of PSP extracts from ST 26 and P. multocida were collected and tested for activity against M467 by immuno-slot blotting. Activity was present in all fractions tested. Fractions from each peak were run on SDS-PAGE and Western immunoblotted to detect proteins bound by M467. It

FIGURE 3. Electron micrograph of ST 25 at 16,000 X magnification

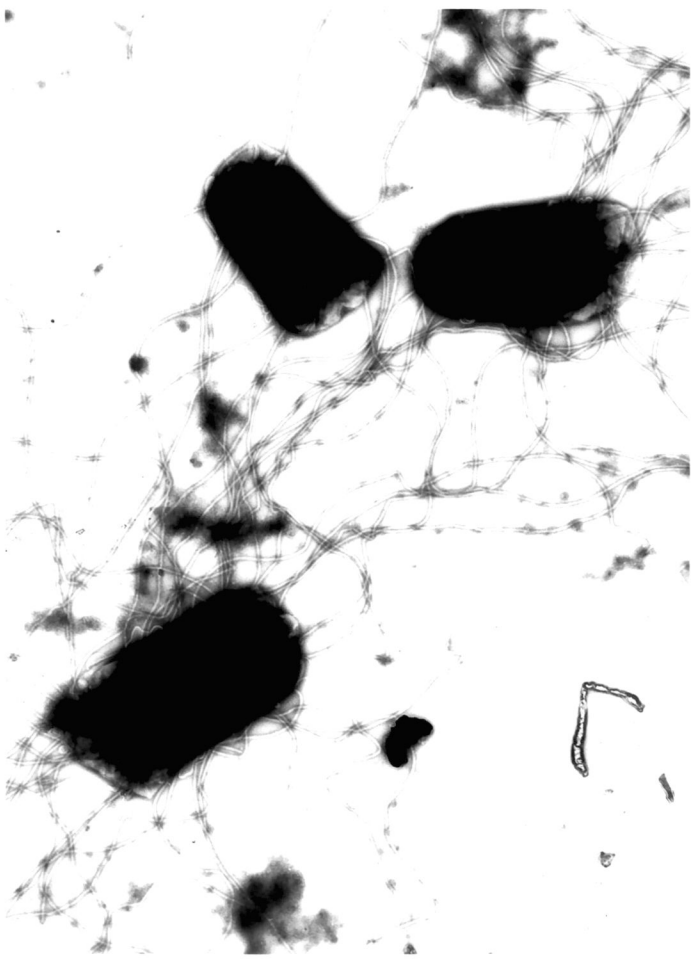


FIGURE 4. Electron micrograph of ST 26 at 15,000x magnification

FIGURE 5. Electron micrograph of P. multocida at 20,000 X magnification

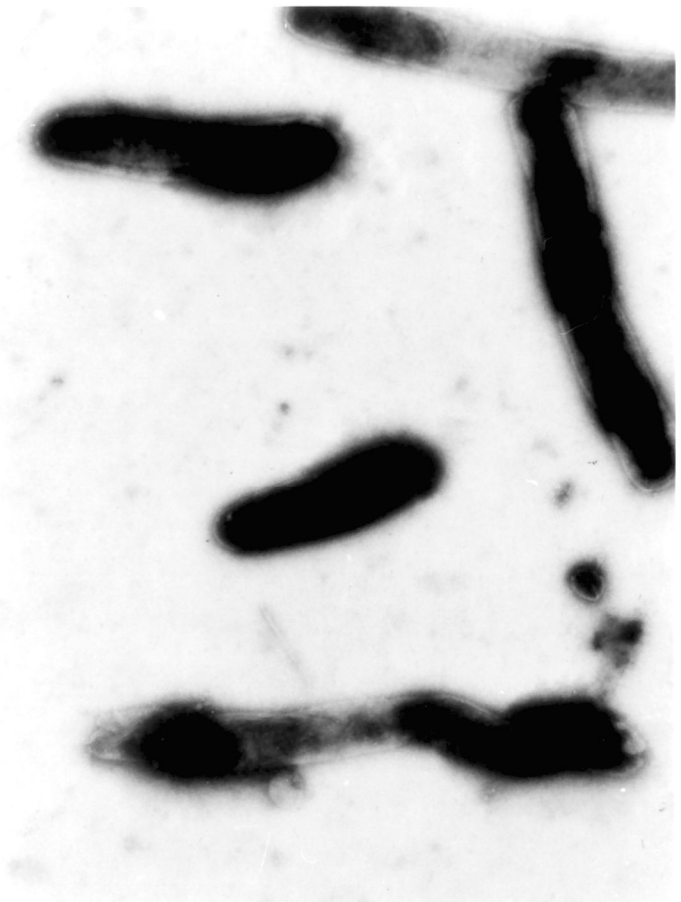
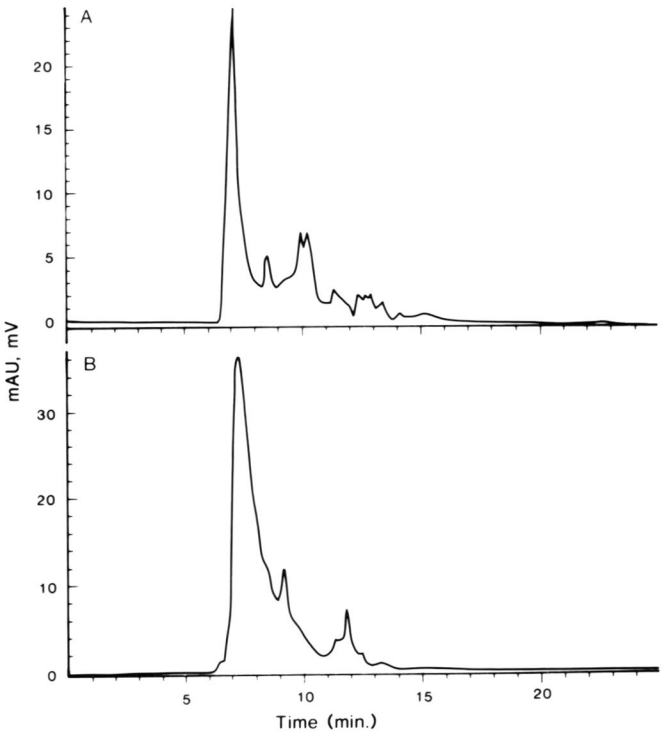


FIGURE 6. High Pressure Liquid Chromatograph. A) OD at 280 nm read during ST 26 PSP elution. b) OD at 280 nm read during P. multocida PSP elution.


A
B
mAU, mV
Time (min.)
0
5
10
15
20
30

was determined that M467 bound epitopes in each fraction, but, other proteins not bound by M467 were also present.

The HPLC data indicate that all fractions contained proteins of various molecular weights with epitopes recognized by M467. Separation by HPLC was unsuccessful for the purpose of isolating pure antigenic components.

Isoelectric Focusing of Phenol Soluble Proteins

The PSP extracts from ST 25, ST 26 and P. haemolytica were focused on an isoelectric focusing agarose gel to determine and compare the pI's of the major proteins found in the extracts. It was determined from the IEF data that the protein bands, stained with Coomassie, had pI's in the range of 3.8 to 4.5 and the number of bands present were variable for each strain. It was concluded that the proteins that possess the epitope recognized by M467 have pI's that range between pH 3.8 and 4.5.

SDS-PAGE Electrophoresis

Experiments were done to compare bacterial antigen extracts to determine the best method for purifying proteins for analysis and immunizations.

Comparisons were made between ST 25 PHE and ST 25 PSP, and ST 26 PHE and ST 26 PSP on 14% SDS-PAGE gels at approximately identical concentrations 50ug/well. It was determined from the SDS-PAGE gel in Fig. 7 that more proteins were extracted in the PSP preparation than in the PHE. On the basis of this gel, it was decided to use PSP extracts for further analysis and immunizations for all bacteria in this study.

FIGURE 7. 14% SDS-PAGE Gel stained with Commassie blue. a) ST 26 PHE; b) ST 26 PSP; c) ST 25 PHE; d) ST 25 PSP.


a
b
c
d

An SDS-PAGE gel comparing the PSP extracts of ST 25, ST 26, Pasteurella multocida, P. haemolytica and P. gallinarium is shown in Fig. 8.

The Coomassie stains of ST 25 and ST 26 PSP extracts display almost identical band patterns. Major bands are located at approximately 15,000 MW, several minor bands between 16,000 and 25,000, and a complex of several major protein bands at approximately 34,000, 35,000 and 36,000 MW are present. A group of proteins was described earlier as components of a porin complex located in the outer membrane of S. typhimurium. There are several protein bands above this complex that are less pronounced. At approximately 48,000 MW, a large band is present in the ST 25 extract that is missing in the ST 26 PSP. It was determined that this band was flagellin, the monomeric protein of flagella, by comparison with pure flagellin on SDS PAGE (lane a and b, Fig. 8).

The profile of Comassie stained bands were not as similar among the Pasteurella species. P. multocida and P. haemolytia showed more similarities in binding patterns than does P. gallinariun. P. multocida had major bands located at approximately 17,000 and 20,000 MW. Minor bands were seen up to approximately 24,000 MW. At approximately 34,000 to 36,000 MW, heavy protein bands were present that strongly resemble the Presumptive porin complex proteins seen in the ST 25 and ST 26 lanes. Additional minor bands can be seen closer to the origin of the gel with one major band located about 46,000 MW. P. haemolytica also had a major band present at about 17,000 MW but lacked the 20,000 MW protein that was prevalent in P. multocida.

Several major bands were located at approximately 25,000 to 28,000 MW. The assumed porin complex was also present in P. haemolytica but was of a slightly larger MW than the complex seen in ST 25, ST 26 or P. multocida. The complex appeared to be approximately in the 40,000 to 42,000 MW range. Several minor bands that stained weakly can be seen above the Presumptive porin complex.

P. gallinarium PSP did not display similarities to the other Pasteurella or to ST 25 and ST 26. Banding patterns were very light in contrast to the other PSP extracts. The bands present were found at 16,000, 32,000 and 40,000 MW. The 40,000 MW band showed a similar migration pattern as the largest protein in P. haemolytica.

In summary, there appear to be multiple protein bands present between the Salmonella strains and Pasteurella spp. used in this study. The range of MW's of the proteins detected in SDS-PAGE was 6,000 to greater than 54,000.

Outer Membrane Proteins of ST 26

Although it can be seen in SDS-PAGE that more proteins were present in the PSP extract, it cannot be determined if these proteins exist in the outer membrane or not. The major proteins of the outer membrane are of interest to this study because of the role they play in a normal immune response. By isolating outer membrane proteins we were able to compare them with the proteins present in the phenol extract to determine the presence of outer membrane proteins in the PSP extract. A comparison of an outer membrane extraction of ST 26 and ST 26 PSP was made by SDS-PAGE gel analysis in Fig. 9.

FIGURE 8. 14% SDS-PAGE Gel stained with Commassie blue. Markers; a) ST25 PSP; b) Salmonella milwaukee flagellin (MON); c) ST26 PSP; d) Pasteurella multocida PSP; e) P. haemolytica PSP; f) P. gallinarium PSP.

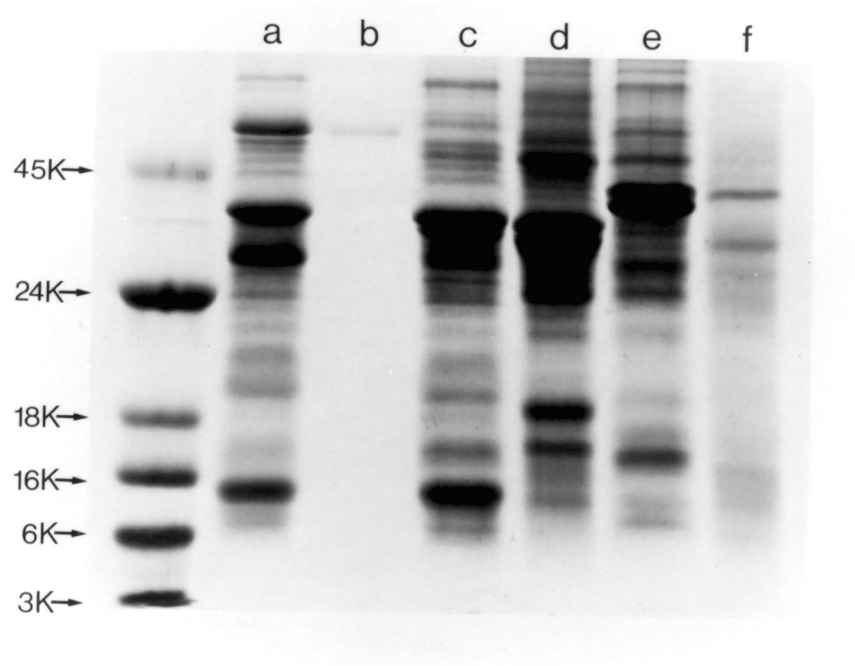

a
b
c
d
e
f
45K
24K
18K
16K
6K
3K

FIGURE 9. 14% SDS-PAGE Gel stained with Commassie blue. Markers; a) ST 26 outer membrane extract; b) ST 26 PSP.

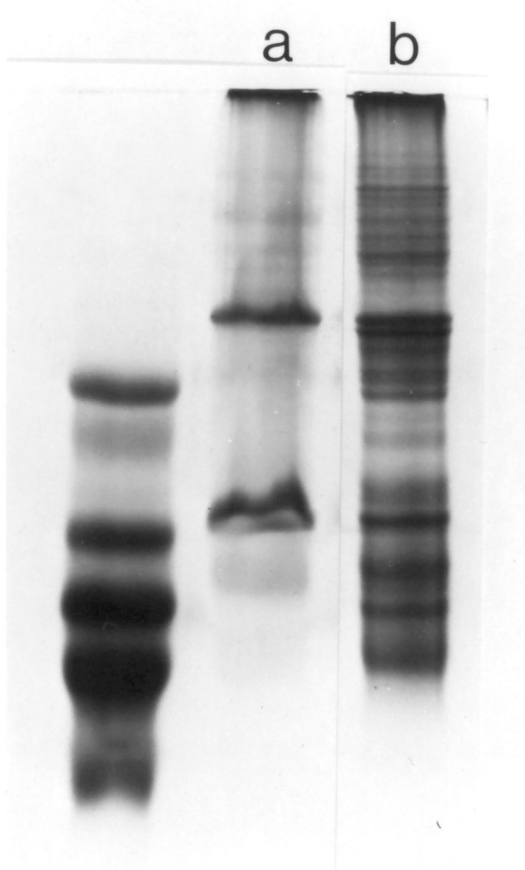
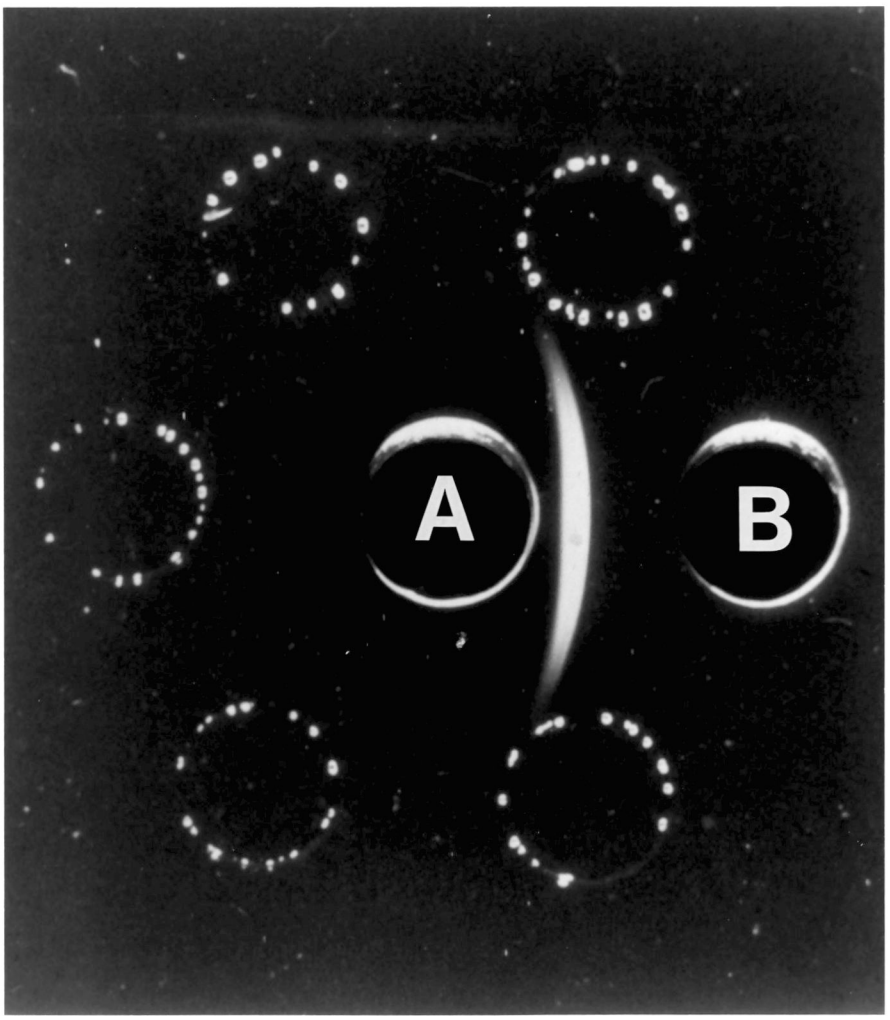

a
b

FIGURE 10. Immunoelectrophoresis. Well number A, M467 ascites fluid; Well number B, affinity column purified M467. Trough 1 contains rabbit anti-whole mouse serum and trough 2 contains rabbit anti-M467.


A
1
B
2

FIGURE 11. Ouchterlony gel demonstrates the binding activity of affinity column purified M467 (well A) for MON (well B).


A
B

Two major bands are present in the outer membrane extract of ST 26. In lane b of Fig. 9, the corresponding bands are seen present in the ST 26 PSP extract. The two outer membrane proteins appear to be 20,000 and 36,000 MW.

Western Immunoblotting with M467

A. Purification of M467

Fig. 10 shows an immunoelectrophoresis gel with the comparison of M467 ascites fluid with purified M467 after affinity chromatography purification.

It can be clearly seen from the lines of precipitation that the anti-whole mouse serum at the first trough reacts with a number of proteins present in the ascites fluid indicating multiple proteins other than M467. However, the affinity column purified M467 is relatively clean of any contaminating proteins.

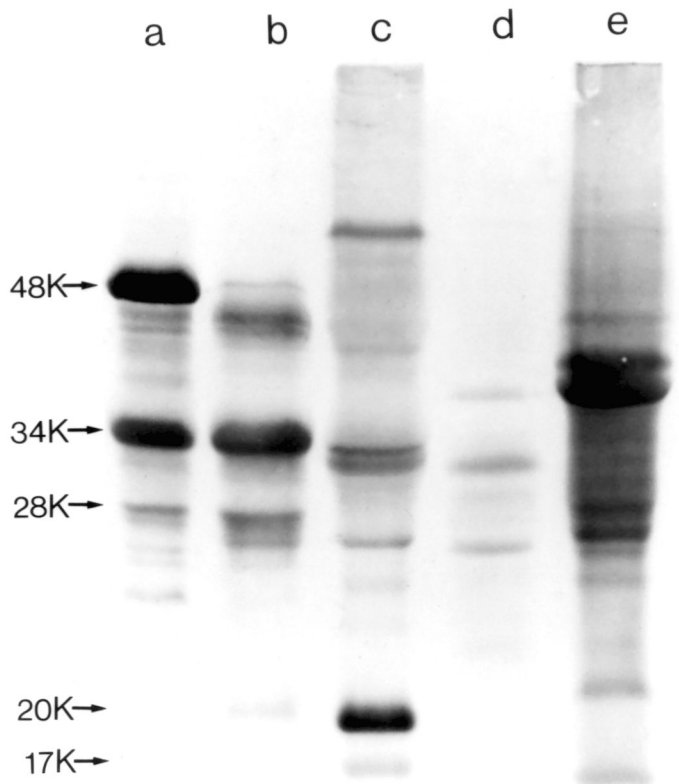
Rabbit Anti-M467 in the second trough provides evidence that the purified IgA is indeed M467.

In Fig. 11, an Ouchterlony gel shows that the purified M467 retains its ability to bind monomeric flagellin protein (MON) from Salmonella milwaukee. Thus far it has been demonstrated that M467 has been purified by affinity chromatography based on immunoelectrophoresis, and that it retained its ability to bind flagellin after purification.

B. Western Immunoblots

The multiple banding patterns seen in the SDS-PAGE of ST 25, ST 26, Pasteurella multocida, P. haemolytica and P. gallinarium phenol extracts were examined for reactivity with M467 by Western

FIGURE 12. Immunoblot of PSP antigens developed with M467. a) ST 25 PSP; b) ST 26 PSP; c) P. multocida PSP; d) P. gallinarium PSP; e) P. haemolytica PSP.


a
b
c
d
e
48K
34K
28K
20K
17K

immunoblots. A comparison of each bacteria's reactivity with M467 is shown in Fig. 12. ST 25 and ST 26 show similar banding patterns, the most noted difference being the presence of a band in the ST 25 PSP (lane a) which appeared at about 48,000 MW. This corresponds to the molecular weight of flagellin and is absent in the ST 26 PSP.

At approximately 34,000 MW, one of the proteins thought to be part of the porin complex reacted very strongly with M467. To a lesser degree the 28,000 MW protein of both ST 25 and ST 26 also reacted with M467. Several minor bands are seen below the 48,000 and 28,000 MW bands. In ST 26, an isolated band at 20,000 MW is visible as a very lightly reacting protein.

P. multocida (lane c) had several bands that reacted with M467. Proteins at molecular weights 17,000, 20,000, 26,000, 32,000 and 33,000 (porin complex) all appear on the blot. One band above the 48,000 MW marker is also present.

There was very little reactivity between M467 and proteins from P. gallinarium (lane d). Three weak bands were present at approximate MW's of 38,000, 32,000 and a band at 26,000 MW. P. haemolytica (lane e) had most of the immunoblot activity in the region of the two porin proteins. Heavy immuno-staining is seen with some minor activity in the bands just under the porin complex. Two low molecular weight bands appear at approximately 15,000 and 22,000 MW.

To determine if these antigenic sites recognized by M467 were proteinaceous, a Pronase protein digestion was performed on ST 26 PSP and the digested material was immunoblotted to determine if the epitopes were destroyed. It is apparent from lane a in Fig. 14 that

the antigen binding sites were destroyed by the protease.

In the same immunoblot the ST 26 outer membrane preparation was run beside a lane that contained ST 26 PSP extract. A comparison of the two extracts indicates that the ST 26 PSP does contain proteins that also appear in the outer membrane preparation with approximately molecular weights of 20,000 and 36,000 and are recognized by M467 (Fig. 13).

Lipopolysaccharide-Protein Complex from Salmonella spp.

Trichloracetic acid (TCA) extracted lipopolysaccharide-protein complex from three different species of Salmonella were immunoblotted with M467 to detect antigenic activity present in the extract. S. typhimurium, S. minnesota and S. abortus equi TCA-extracted endotoxin were used. The immunoblot of the TCA extracts after incubation with M467 appears in Fig. 14. Major bands immunostained by M467 were found between 7,000 and 34,000 MW in all three lanes.

Preparative Technique for Purifying Isolated Bands for Use as Immunogens

Two isolated bands from P. multocida PSP extract were selected that reacted with M467 on the immunoblot. The two bands selected were approximately 17,000 and 20,000 MW and appeared to be isolated from other protein bands. These two proteins were selected for purification for this study because one of the bands appeared to be present in the ST 26 PSP immunoblot (Fig. 12, lane b). It was intended to use these two protein bands, which possess the epitope recognized by M467, as immunogens in order to generate antisera to

compare its reactivity with ST 26 and P. multocida PSP extracts in an EIA assay (Table I) and by immunoblotting (Fig. 19).

It was necessary to perform an immunoblot on the electroeluted bands from P. multocida for two reasons. The immunoblot could provide evidence that the two bands electroeluted from the gel were present in the extracted fraction and free of any contaminating material. It also demonstrated that the electroelution process did not alter the epitope that M467 recognizes. Therefore, the preparation could be used to immunize mice. The immunoblot of the two protein bands is shown in Fig. 15.

EIA for Reactive Antibody Detection

Antisera from groups of BALB/c mice immunized with ST 26 PSP, P. multocida PSP and P. multocida LWP (Fig. 15) were collected and tested by EIA for the presence of antibodies that reacted with the immunogen proteins. Cross-reactivity of antisera was also measured. EIA results are found in Table 1.

As shown in Table 1, the highest titers were measured against homologous antigens. Also, significant levels of cross-reactivity were detected between the ST 26 and P. multocida PSP antigens. Antisera from mice immunized with the electrophoretically purified peptide (17,000 and 20,000 MW) from P. multocida reacted equally well with the homologous antigen, the ST 26 PSP and the P. multocida PSP antigens.

These results indicate that in a normal response to these antigens, antibodies are produced that will recognize common epitopes in Salmonella spp. and Pasteurella spp.

FIGURE 13. Immunoblot developed with M467. a) ST 26 PSP with protease digestion; b) ST 26 PSP extract; c) ST 26 PSP outer membrane extract.

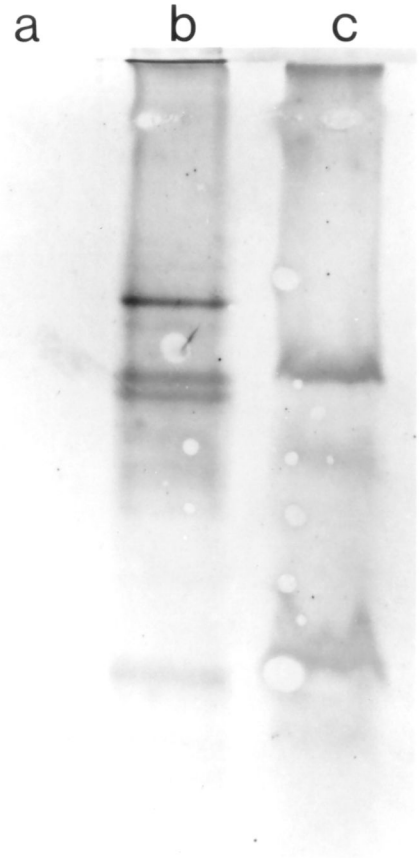

a
b
c

FIGURE 14. A Western Blot TCA extracts of LPS-Protein Complex developed by M467. A) S. minnesota; B) S. abortus equi; C) S. typhimurium.

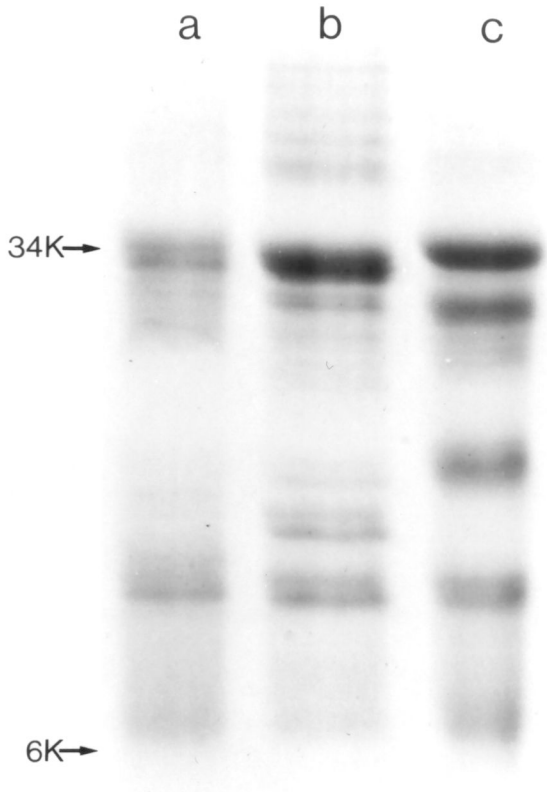
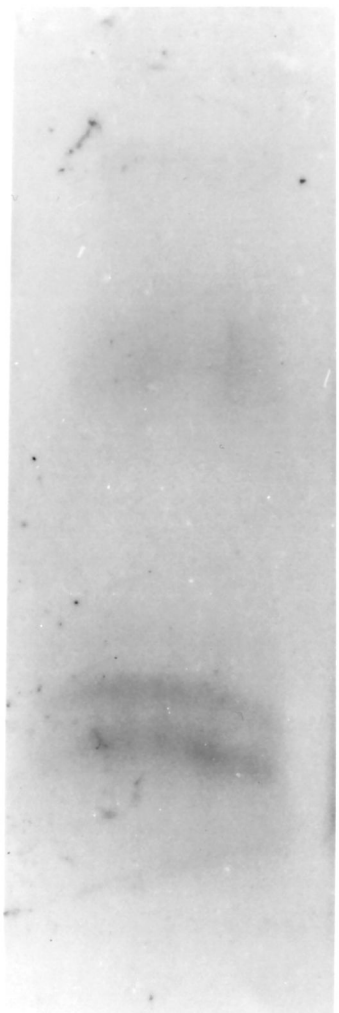

a
b
c
34K
6K

FIGURE 15. Immunoblot of P. multocida PSP electroeluted low molecular weight proteins (LWP) developed with M467.



Western Immunoblotting with Immune Serum

Antisera from mice immunized with ST 26 PSP, P. multocida PSP and P. multocida LWP were used as the primary antibody in the immunoblots with ST 26 and P. multocida PSP. Profiles of the immunoblots indicate which proteins in the phenol extracts were antigenic and which proteins cross-react with the antisera.

In Fig. 16, ST 26 PSP and P. multocida PSP antigens were blotted with ST 26 PSP antisera. Multiple bands were recognized in the ST 26 lane as would be expected using antisera to homologous antigens. However, the ST 26 PSP antisera cross-reacted with two bands from the P. multocida PSP extract. The two bands are located at approximately 20,000 and 34,000 MW, and appear to have identical molecular weights and binding (antigenic) properties as two equivalent bands present in the ST 26 PSP.

A reciprocal immunoblot using P. multocida PSP antisera was performed and is shown in Fig. 17. The antisera generated by P. multocida PSP immunization recognized many of the bands present in the P. multocida PSP extract as would be expected. One band was located at approximately 20,000 MW and several more are located at about 30-35,000 MW. Anti-P. multocida PSP recognized two distinct bands when reacted against ST 26 PSP. A major band was located at approximately 34,000 MW. There appeared to be an equivalent antigen with the same molecular weight present in the P. multocida PSP. A minor band of approximately 48-50,000 MW is also present in the ST 26 PSP with no apparent counterpart located in the P. multocida PSP.

In review of the immunoblots using antisera generated by phenol extracts from ST 26 and P. multocida, it was found that many of the proteins used in an antigen preparation were indeed immunogenic. It also appears that proteins are present that cross-react between the two gram negative bacteria as indicated by the presence of the bands with identical molecular weight and antigenic properties in the immunoblot.

P. multocida LWP antisera was also used as the primary antibody in an immunoblot. The results of the blot are shown in Fig. 18. Several bands are present in both the P. multocida PSP and ST 26 PSP extracts. These results indicate that the two bands of the P. multocida LWP used to immunize these mice are antigenic and possess epitopes shared by other proteins with different molecular weights present in the phenol extracts of P. multocida and ST 26.

Protein bands are seen at 17,000, 22,000, 34,000 and 35,000 MW in the ST 26 PSP lane. In P. multocida PSP, bands are not as clearly resolved as in the ST 26 and the activity appears weaker. Light bands are seen at essentially the same molecular weights that appear in the ST 26 PSP; 17,000, 22,000 and 34,000 MW.

Table 1

Antiserum	Antibody titer against antigen		
	ST 26 PSP	*P. multocida* PSP	*P. multocida* LWP
Anti-ST 26 PSP	1:75000	1:3000	ND
Anti-*P. multocida* PSP	1:2000	1:75000	ND
Anti-*P. multocida* LWP	1:2000	1:2000	1:1000

ND (not done)

Table 1. Antibody serum titers as determined by EIA of mouse anti-ST 26 PSP, anti-*P. multocida* PSP, and anti-*P. multocida* LWP (electrophoretically purified). Antigens were coated on to microtiter plates at a concentration of 100 ng/well. Serial two-fold dilutions of antisera were added to test wells in a volume of 50 microliters/well. Biotin conjugated anti-mouse IgG, IgA, and IgM were diluted 1:1000 and added in a volume of 100 microliters/well. Negative controls of normal mouse serum diluted 1:100 were used. The last dilution of antiserum producing O.D. 405 values greater than 2 times the O.D. value produced by non-immune serum was taken as the titer of that serum.

FIGURE 16. Immunoblot of phenol extracts with ST 26 PSP antisera. a) ST 26 PSP; b) P. multocida PSP.

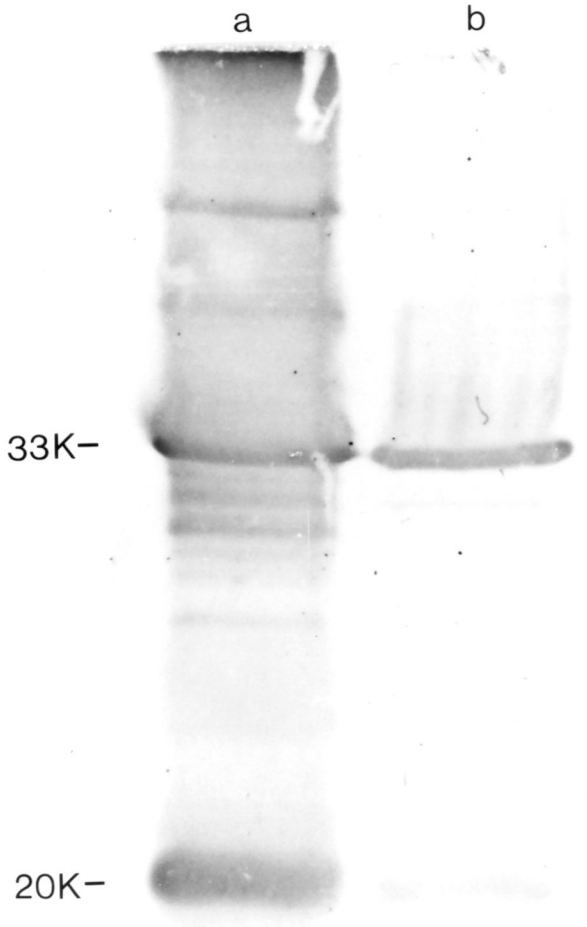

a
b
33K–
20K–

FIGURE 17. Immunoblot of phenol extracts with P. multocida PSP antisera. a) ST 26 PSP; b) P. multocida PSP.

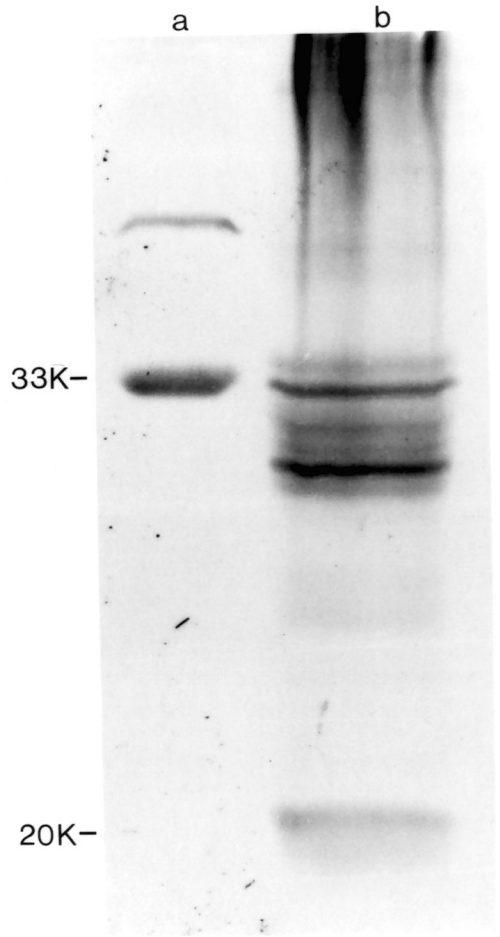
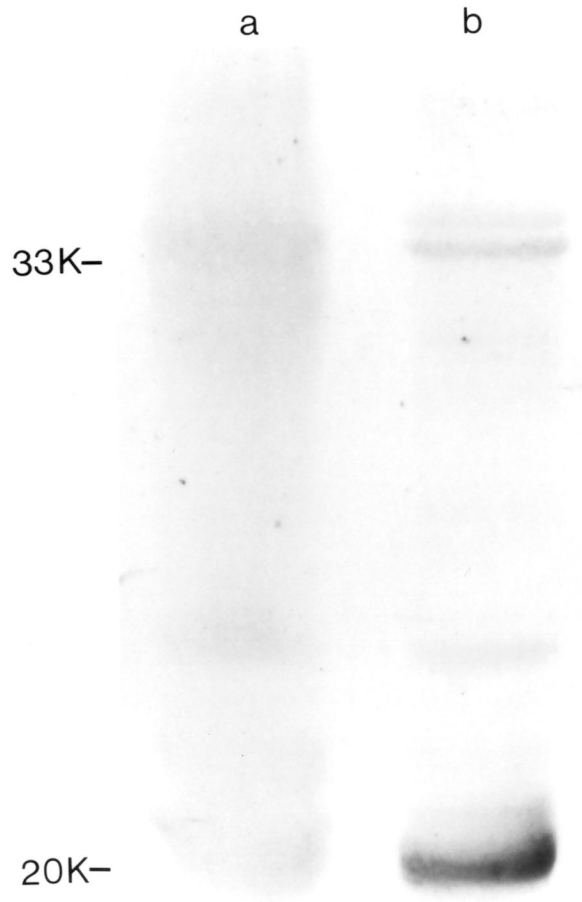

a
b
33K–
20K–

FIGURE 18. Immunoblot of phenol extracts developed with P. multocida LWP antisera. a) P. multocida PSP; b) ST26 PSP.


a
b
33K–
20K–

Discussion

It was initially reported that M467, a protein product of a mouse plasmacytoma, would bind epitopes present in the Salmonella serogroups, S. adelaide, S. thomasville, and S. senftenberg and a member of the Pasteurella group, P. pneumotropica (Potter, 1970). In further studies, it was found that M467 would precipitate antigens present in heat extracts of many Salmonella species, Pasteurella pneumotropica and Herellea vaginicola. Four Salmonella members (S. paratyphi A,B,C, and S. typhosa), were not precipitated by M467 nor were any of the E. coli or Proteus species tested (Potter, 1971). Potter demontrated by trypsin digestion experiments that the antigen bound by M467 was trypsin-sensitive and, therefore, he concluded that the antigen was protein in structure. This finding was further substantiated in my investigation using Pronase digestion to ablate antigenic activity in a Western immunoblot.

Attempts to purify or isolate the antigen present in heat extracts of Salmonella by gel filtration suggested that the antigen was present at various molecular weights. It was thought that the antigen activity could account for the heterogeneity observed if the antigen in question was bacterial flagella of various sizes, fragmented in the collection from the surface of bacterial cells (Smith and Potter, 1976). Results from experiments conducted by Smith showed that purified flagella from S. milwaukee contained the epitopes recognized by M467. The presence of flagella and its soluble component, flagellin, accounts for the ability of M467 to precipitate

the heat extract antigen from S. milwaukee. Pasteurella spp. are non-motile and are described as non-flagellated, therefore, the source of antigens bound by M467 are not from flagella and must reside within or on the outer membrane of these gram negative bacteria.

The preliminary experiments in this investigation were designed to compare the reactivity of M467 with antigens associated with Salmonella spp. and Pasteurella spp. The results of the double diffusion in agar gel experiments indicated that M467 precipitates antigens from ST 25 and ST 26 and the purified flagellin (MON), but does not precipitate any antigens of the Pasteurella species. This would suggest that the flagella or flagellin and the antigenic proteins present in the heat extract of Salmonella must possess multiple epitope sites. The ability of the IgA MOPC 467 myeloma protein to precipitate another protein would require that the antigenic protein possess a characteristic, repetitive determinant or that an antigenic molecular unit have many similar subunits (Potter, 1971). This would account for M467's ability to precipitate the flagella proteins and suggests that the antigens bound from the outer membrane of Pasteurella species do not possess this characteristic repetitive epitope which enables antibodies to cross-link antigens.

In order to determine whether M467 could bind antigens that were not detected by precipitation, immunoblots were used since they are based on binding alone and not cross-linking of epitopes. The results from these experiments demonstrated that M467 bound antigens present in the heat extracts of ST 25, ST 26, all members of the Pasteurella

species in this investigation. No activity was seen with the gram positive organism Staphylococcus epidermidis.

In the process of our investigation, it was determined by SDS-PAGE gel analysis that we could release more available proteins by a hot phenol extraction, a method previously reported (Westphal and Jann, 1965; Wober and Aloupovie, 1971 and Morrison and Ryan , 1976) to extract proteins from LPS. We have used the phenol extraction method for analysis and immunogen preparations in our investigations. Western immunoblotting further substantiated this finding, as more protein bands were present that were reactive with M467 in phenol extracts than in the heat extracts.

We have shown by Western immunoblotting that M467 will bind to membrane proteins of various molecular weights that range between 17,000 and 50,000 from Salmonella and Pasteurella. This finding is supported by data from the HPLC separation of antigens of the phenol extracts of ST 26 and P. multocida. Antigens were present in all fractions of the HPLC separation as detected by M467 using an immunoblot technique.

The molecular weight heterogeneity of the proteins found to react with M467 led us to believe that the epitope may reside on several types of protein associated with the outer membranes of Salmonella spp. and Pasteurella spp.. Lipoproteins are by far the most abundant protein found in the outer membrane of gram negative bacteria and exist in two forms, a bound (attached to the peptidoglycan layer) and a free form with a molecular weight of 7,200 (Braun and Rehn, 1969).

Although this lipoprotein has been described in E. coli, S. typhimurium and Serratia marcescens as being immunologically related (Halegoua et al., 1974), we have not detected any proteins at this molecular weight that appear to be reactive with M467. A second family of lipoproteins, described by Mizuno (1981), has a molecular weight of 21,000 and is immunolgically related in several gram negative bacteria such as E. coli, S. typhimurium , Klebsiella aerogenes and S. marcescens. This protein may be a likely candidate as one of the proteins recognized by M467 since it is in the size range of proteins bound by the antibody.

Another class of proteins thought to be present in all gram negative bacteria are the proteins that exist in porin complexes. Most of the reported work on porin proteins has been done with E. coli and S. typhimurium (Nakae, Ishii and Torunaga, 1979; Tokunga et al., 1979; Osborn et al., 1980). Similarities between the porin proteins of S. typhimurium with molecular weight estimates of 33-36,000 are noted in the Western immunoblot where Salmonella proteins bound by M467 are coincidentally found in approximately the same molecular weight range. The existence of porin complex proteins has also been reported in P. multocida by Lugtenberg (1986) and has been shown to possess many of the physical characteristics of the protein that constitute the porin complexes of E. coli and S. typhimurium. Again, immunoblot data suggest that proteins which correspond to reported porin protein molecular weight ranges are recognized by M467. Therefore, it is very likely that proteins which are associated in the

gram negative bacterial outer membrane porin complex possess the antigenic determinants recognized by M467.

It has been shown in this study that proteins extracted along with LPS from Salmonella by trichloroacetic acid are bound by M467. It is highly probable that some of the protein was associated with the LPS molecule itself as previously described by Wu and Heath (1973). It may well be that the protein moiety functions as an anchor for the LPS into the peptidoglycan of the cell wall. Therefore, such a structural function for a polypeptide may require a highly conserved structure in gram negative organisms.

It is also well documented that proteins associated with the peptidoglycan of Enterobacteriaceae are very common in nature. Three such proteins have been detected in S. typhimurium (Ames, Spudich and Nikato, 1974; Tokunaga et al., 1979) and a large survey has shown that they are present in all Enterobacteriaceae tested (Lugtenberg et al., 1977; Mizuno and Kageyama, 1979; Nixdorff et al., 1977) and in P. aeruginosa (Hancock et al., 1981) and that all of these proteins are cross-reactive with E. coli proteins (Overbeeke et al., 1980; Hofstra and Dankert, 1979; Hofstra, VanTol and Kankert, 1980).

The presence of common protein peptides among the gram negative bacteria has been investigated for several years (Schnaitman et al., 1970; Lugtenberg et al., 1977; Hofstra and Dankert, 1979). The implications of the existence of common proteins or peptides are important in taxonomic classification as well for possible prophylactic uses in vaccines. Investigations by Hofstra and Dankert

(1979) demonstrated that there exists a high degree of outer membrane regularity in PAGE profiles among twenty-three Enterbacteriaceae strains of Shigella flexneri, Edwardsiella turda, S. typhimurium, Citrobacter freundii, Klebsiella pneumonia, Serratia marcescens, Proteus mirabilis and Provencia stuartii and that they were antigenically cross-reactive with outer membrane proteins of E. coli. Proteins found to cross-react were in the 33-38,000 MW range. Their experimental results indicate that antigenic cross-reactivity of the major outer membrane proteins is a general phenomenon in the Enterobacteriaceae independent of variation in the molecular weight of the corresponding proteins in different bacterial strains.

M467 has enabled us to locate and identify proteins that possess common peptides that may be used as potential vaccines against gram negative bacterial outer membranes. Vaccines made with Salmonella typhimurium porin proteins proved to be good immunogens in mice and rabbits (Kuusi et al., 1979). A highly significant protection capacity was noted in mice when challenged by i.p. Salmonella infection. Saline extracted proteins from P. multocida with molecular weights of 44,000, 31,000 and 25,000 were used to immunize and provide protection in turkeys (Syuto and Matsumoto, 1982). Porin proteins and proteins exposed at the surface of bacteria seem to be logical candidates as immunogens since antibodies to these structures would either impair the bacteria or enhance opsonization and clearance by macrophages. Also membrane proteins do not have the toxic properties associated with them that the LPS surface antigens have.

From the studies performed in our laboratory, we are able to report the detection of cross-reacting antigens located in the phenol extracts from non-flagellated S. typhimurium (ST 26) and P. multocida. Detection of cross-reactivity was observed by the highly sensitive EIA and Western immunoblotting techniques. Reciprocal assays of antigens and antisera (ST 26 PSP and anti-P. multocida PSP; P. multocida PSP and anti-ST 26 PSP) showed that antibodies were produced against the two immunogenic extracts that cross-react, which suggests common epitopes within the two bacteria. Data from Western blots suggest, by the presence of a common band of approximately 34,000 MW, that cross-reactivity may be due in part to a protein that appears to be part of a porin complex. A second band common to the Pasteurella and Salmonella observed at approximately 20,000 MW, is close to the size estimate of the peptidoglycan associated lipoprotein described by Mizuno (1981) to be immunologically related among several gram negative enteric bacteria.

We were also able to isolate and purify two protein bands (17,000 and 20,000 MW) from P. multocida that were reactive with M467 in the Western immunoblot. This proved to be a very effective preparative method by which peptides and proteins of interest could be collected in pure form for vaccine purposes or protein analysis. The two bands that were collected show no sign of contamination or loss of epitope activity as determined by immunoblots.

The antisera produced against these two P. multocida proteins were tested in an EIA and Western immunobot. It was determined by EIA

that the antisera reacted equally well against the ST 26 and P. multocida PSP extracts. In the Western blots, the antisera produced against the two proteins also bound proteins at higher molecular weights. This finding suggests that epitopes present in the lower molecular weight proteins are also present in the larger molecular weight proteins.

In this study, we have determined that M467, an IgA myeloma protein, is capable of binding flagellar proteins from Salmonella species and that additional epitopes recognized by M467 are located on proteins within the outer membrane as well. Cross-reactivity of M467 with several Pasteurella species was also observed with some similar reactive protein bands. In both Salmonella and Pasteurella, M467 reacted with proteins of multiple molecular weights. As mentioned earlier, cross-reactivity is a general phenomenon among the gram negative family Enterobacteriaceae (Hofstra and Dankert, 1979) and the data presented in this study suggests that the presence of common proteins or peptides may be extended to a non-Enterobacteriaceae group, the genus Pasteurella. We believe that these two gram negative bacteria share a common peptide sequence expressed in membrane proteins that has been well conserved throughout their evolution from a common ancestral organism. We suggest that this peptide has somehow favored the survival of these organisms and therefore resisted the omission or deletion of the genes that encode for this peptide.

It is further suggested that the precursor cell from which the MOPC 467 tumor cell line arose was a normal plasma cell that had been

programmed to produce antibodies to epitopes associated with gut associated organisms. The transition from a plasma cell to a tumor cell line has provided an amplification of the production of an antibody to a naturally occurring antigen present in several potentially pathogenic gram negative organisms.

The next logical step in the investigation of the common membrane antigens described in this study, is to assess the ability of the protein extract to provide protection in mice challenged with the virulent form of the organism. An investigation is underway at the present time which will evaluate the vaccine potential that ST 26 PSP provides for mice challenged with a wild type of S. typhimurium. Further vaccine application of the phenol soluble extracts would be extended to determine whether cross-reactivity seen in the EIA and Western immunoblots between Pasteurella and Salmonella would provide protection in mice in reciprocal immunizations and challenge experiments.

If future experiments in this area were to show that protection could be induced by a common polypeptide present in the outer membrane proteins then the isolation and characterization of the peptide sequence containing the common epitope would play an important role in the development of vaccines capable of preventing infections caused by a large number of gram negative bacteria.

Bibliography

Ames G.F. E.N. Spudich and H. Nikado. 1974. Protein Composition of the Outer Membrane of Salmonella typhimurium: Effect of Lipopolysaccharide Mutations. J. Bacteriol. 117:406-416.

Bosch V. 1974. The Murien-Lipoprotein of Escherichia coli - Studies on its' Structure, Biosynthesis and Antigenic Determinants. Thesis - Freieuniverstat Berlin.

Bradford, M.M. 1976. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the principle of Protein-Dye Binding. Anal. Biochem. 72:248-282.

Braude, A., E. Ziegler and J. McCutchan. 1978. Antiserum Treatment of Gram Negative Bacteria. Schweiz. Med. Worchenschr. 108:1872-1876.

Braun, V. and K. Rehn. 1969. Chemical Characterization, Special Distribution and Function of a Lipoprotein (murein-lipoprotein) of the E. Coli Cell Wall. The Specific Effect of Trypsin on the Membrane Structure. Eur. J. Biochem. 10:426-438.

Braun, V. 1975. Covalent Lipoprotein from Outer Membrane of Escherichia coli. Biochem. Biophys. Acta 415:335-377.

Carsiotis, M., D.L. Weinstein, H. Karch, I.A. Holder and A.D. O'Brian. 1984. Flagella of Salmonella typhimurium are a Virulence Factor in Infected C57BL/6J Mice. Infect. Immun. 46:814-818.

Elin, R.J. and S.M. Wolff. 1976. Biology of Endotoxin. Annu. Rev. Med. 27:127-138.

Ganfield, D.J., P.A. Rebers and K.L. Heddleston. 1976. Immunogenic and Toxic Properties of a Purified, Lipopolysaccharide-Protein Complex from Pasteurella multocida. Infect. Immun. 14:990-999.

Halegoua, S., A. Hirashima and M. Inouye. 1974. Existance of a Free Form of a Specific Membrane Lipoprotein in Gram Negative Bacteria. J. Bacteriol. 120:1204-1208.

Hancock, R., R. Irwin, W. Costerton and A. Carey. 1981. Psuedomonas aeruginosa Outer Membrane: Peptidoglycan Associated Proteins. J. Bacteriol. 145:628-631.

Hofstra, H. and J. Dankert. 1979. Antigenic Cross-Reactivity of Major Outer Membrane Proteins in Enterobacteriaceae Species. J. Gen. Microbiol. 111:293-302.

Hofstra, H. and J. Dankert. 1980. Major Outer Membrane Proteins: Common Antigens in Enterobacteriaceae Species. J. Gen. Microbiol. 119:123-131.

Hofstra, H., M. VanTol and J. Dankert. 1980. Cross-Reacting Major Outer Membrane Proteins of Enterobacteriaceae, Studied by Crossed Immuno-electrophoresis. J. Bacteriol. 143:328-337.

Kendall, C., I. Ionescu and G.R. Dreesman. 1983. Utilization of the Biotin-Avidin System to Amplify the Sensitivity of the Enzyme Linked Immunosorbent Assay (ELISA). J. Immunol. Methods. 56:329-339.

Klaassen, J.M., B.L. Bernard and R.F. DiGiacomo. 1985. Enzyme-Linked Immunosorbent Assay for Immunoglobulin G Antibody to Pasteurella multocida in Rabbits. J. Clin. Microbiol. 21:159-172.

Kuusi, N., M. Nurmimen, H. Saxen and M. Voltman, P.H. Makela. 1979. Immunization with Major Outer Proteins in Experimental Salmonellosis of Mice. Infect. Immun. 25:857-863.

Laemmli, V.K. 1979. Cleavage of Structural Proteins During the Assembly of the Head of Bacteriophage T4. Nature. 227:680-685.

Lam, J., L. Mutharia and P. Hancock. 1985. Application of Monoclonal Antibodies to the Study of the Surface Antigens in Psuedomonas aeruginosa, pp. 143-157. Monoclonal Antibodies Against Bacteria. Academic Press, Inc.

Lugtenberg, B., H. Bronstein, N. van Selm and R. Peters. 1977. Peptidoglycan-Associated Outer Membrane Proteins in Gram-Negative Bacteria. Biochem. Biophys. Acta 465:571-578.

Lugtenberg, B. and L. van Alphea. 1983. Molecular Architecture and Functioning of the Outer Membrane of Escherichia coli and Other Gram-Negative Bacteria. Biochem. Biophys. Acta 737:51-115.

Lugtenberg, B., R. von Boxtel and M. Jong. 1984. Atrophic Rhinitis in Swine: Correlation of Pasteurella multocida Pathogenicity with Membrane Proteins and Lipopolysaccharide Patterns. Infect. Immun. 46(1):48-54.

Lugtenberg, B., R. Boxtel, D. Evenberg, M. Jong, P. Storm and J. Frik. 1986. Biochemical and Immunological Characterization of Cell Surface Proteins of Pasteurella multocida Strains Causing Atrophic Rhinitis in Swine. Infect. Immun. 52(4):175-182.

Mizuno, T. and M. Kageyama. 1979. Isolation and Characterization of Major Outer Membrane Proteins of Psuedomonas aeruginosa Strains (PAO) with Special Reference of Peptidoglycan-Associated Protein. J. Biochem. 86:979-989.

Mizuno, T. 1981. A Novel Peptidoglycan-Associated Lipoprotein (PAL) Found in the Outer Membrane of Proteins mirabilis and Other Gram Negative Bacteria. J. Biochem. 89:1039-1049.

Morrison, D.C. and J.W. Ryan. 1979. Bacterial Endotoxin and Host Immune Responses. Adv. Immunol. 28:293-301.

Mutharia, L., J. Lam and R. Hancock. 1985. Use of Monoclonal Antibodies in the Study of Common Antigens of Gram-Negative Bacteria. pp. 131-142. Monoclonal Antibodies Against Bacteria. Academic Press, Inc.

Nakae, T., J. Ishii and M. Torunaga. 1979. Subunit Structure of Functional Porin Oligomers that Form Permeability Channels in the Outer Membrane of Escherichia coli. J. Biol. Che. 254:1457-1461.

Nakamura, K., R. Pirtee and M. Inouye. 1979. Homology of the Gene Coding for Outer Membrane Lipoprotein Within Various Gram Negative Bacteria. J. Bacteriol. 137:595-604.

Nixdorff, K., H. Fitzer, J. G. Meimer and H. Martin. 1977. Reconstitution of Model Membranes from Phospholipid and Outer Membrane Proteins of Proteus mirabilis. Eur. J. Biochem. 81:63-69.

Osborn, M.J. and H.P. Wu. 1980. Proteins of the Outer Membrane of Gram Negative Bacteria. Annu. Review. Microbiol. 34:369-422.

Overbeeke, N. and B. Lugtenberg. 1980. Major Outer Membrane Proteins of Escherichia coli strains of Human Origin. J. Gen. Microbiol. 121:373-380.

Plant, J., B. Wilson and A. Glynn. 1982. The Protein-Lipopolysaccharide Complex Extracted with Trichloraietic Acid from Salmonella typhimurium Effective in Protection of Mice Against S. typhimurium Infection Parasite Immunology 4:259-271.

Poolman, S.T., C.P. Hopman and H.C. Zanen. 1978. Immunochemical characterization of Outer Membrane Complexes from Neisseria meningetidis by the SDS-Polyacrylamide-Gel-Electrophoresis Immunoperoxidase Technique (SGIP) IEMS. Microbiol. Lett. 4:245-248.

Potter, M. 1970. Mouse IgA Myeloma Proteins that Bind Polysaccharide Antigens of Enterobacterial Origin. Fed. Proc. 29(1):85-91.

Potter, M. 1971. Antigen Binding Myeloma Proteins in Mice. Ann. New York Acad. Sci. 190:306-321.

Potter, M. 1972. Immunoglobulin-Producing Tumors and Myeloma Proteins of Mice. Physiol. Rev. 52(3):631-710.

Sato, T. and T. Yura. 1979. Chromosomal Location and Expression of the Structural Gene of Major Outer Membrane Protein Ia of Escherichia coli K-12 and of the Homologous Gene of Salmonella typhimurium. J. Bacteriol. 139:468-477.

Schnaitman, C.A. 1970. Comparison of the Envelope Protein Compositions of Several Gram Negative Bacteria. J. Bacteriol. 104:1404-1405.

Smith, A.M. and M. Potter. 1976. A BALB/c Mouse IgA Myeloma Protein that Binds Salmonella Flagellar Proteins. J. Immunol. 114:1847-1850.

Smith, A.M., J. Slack and M. Potter. 1977. Restriction in the Immune Response to Flagellar Proteins in Inbred Mice. Eur. J. Immunol. 7(7):497-500.

Sonnatag, I., H. Schwartz, Y. Hirota and U. Henning. 1978. Cell Envelope and Shape of Escherichia coli. Multiple Mutants Missing Outer Membrane Lipoprotein and Other Major Outer Membrane Proteins. J. Bacteriol. 136:280-285.

Sultzer, B. and G. Goodman. 1976. Endotoxin Protein: A B-Cell Mitogen and Polyclonal Activator of C3H/HeJ Lymphocytes. J. Exp. Med. 144:821-827.

Syuto, B. and M. Matsumoto. 1982. Purification of a Protective Antigen from a Saline Extract of Pasteurella multocida. Infect. Immun. 37(3): 1218-1226.

Tokunaga, M., H. Tokunaga and T. Nakae. 1979. Characterization of Porins from the Outer Membrane of Salmonella typhimurium. Eur. J. Biochem. 95:441-448.

Westphal, O. and K. Jann. 1965. In Methods in Carbohydrate Chemistry, ed. Whistler, R.L., Academic Press, New York. 5:83-91.

Wheat, R.W. (1980) in Zinsser Microbiology, ed. Joklik, W., Willett, H. and Amos, D.B., Appleton-Century-Crofts, New York, pp. 107-134.

Wober, W. and P. Alaupovic. 1971. Studies on the Protein Moiety of Endotoxin from Gram-Negative Bacteria. Eur. J. Biochem. 19:340-356.

Wu, M. and E.C. Heath. 1973. Isolation and characterization of Lipopolysaccaride Protein from Escherichia coli. Proc. Nat. Acad. Sci. 70(9):2572-2576.