EXPLORING BOVINE PNEUMONIC PASTEURELLOSIS

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CHAPTER I

INTRODUCTION AND REVIEW OF RELEVANT LITERATURE

Rivolta first recognized a disease afflicting fowl 122 years ago (1877), which was subsequently named fowl cholera (80). However, it was not until 1884 that Louis Pasteur identified and characterized the bacterial agent from fowl cholera. It is in his honor that the genus is called *Pasteurella* spp. The family *Pasteurellaceae* Pohl was later expanded to encompass other genera containing Gram-negative, fermentative, facultatively aerobic rod bacteria. *Pasteurellaceae* now contains *Haemophilus* spp., *Pasteurella* spp., *Mannheimia* spp. and *Actinobacillus* spp.. Even today the taxonomic structure of the family *Pasteurellaceae* is evolving through advances in molecular techniques, such as DNA – DNA hybridization and 16S rRNA sequence similarities. (2-4).

P. multocida is a heterogenous species with a large spectrum of host preferences, together with variable pathogenic, serological and cultural characteristics. Today, *P. multocida* is classified into 5 serogroups using Carter's classification system for capsular antigens (58, 113). These serogroups are given the letters A, B, D, E, and F (similarities between groups and the lack of capsule led to the inclusion of members from an original group C into other serogroups). A somatic antigen classification scheme leads to further classification of *P. multocida* into 16 serotypes (24, 58). Therefore, *P. multocida* isolates are traditionally expressed as A:3, B:2, etc., to designate both serogroup and serotype. Important *P. multocida* strains include serotype A:3, which are commonly isolated from pneumonic diseases of cattle, sheep and goats; serotypes B:2 and E:2, which are associated with hemorrhagic septicemia in cattle and buffaloes; toxigenic serogroup D and A strains, which are associated with atrophic rhinitis in swine; and serotypes A:1, A:3, A:4, and A:5 which cause fowl cholera.

PATHOGENESIS

Bovine respiratory disease (BRD) is a frequent and significant cause of morbidity and mortality in cattle. According to the National Animal Health Monitoring System, respiratory disease results in the loss of billions of dollars in annual revenue incurred through mortality, reduced production efficiency, disease prevention, and treatment cost. (55, 125, 141, 143, 144)

Confusion has existed by the use of a simple term, BRD, to describe what is a complex and multifactorial group of diseases. This is primarily the result of the failure to fulfill Koch's postulates with any single infectious agent under physiologic conditions, leading many investigators to the use of the term "Disease Complex" to describe BRD. It is postulated that BRD is the result of several synergistic factors, such as bacterial or viral infections and/or stress. Primary viral or bacterial infections are thought to enhance the prevalence of BRD by direct damage of the respiratory mucosa or through immunosuppression. Some viruses incriminated in BRD included Bovine Parainfluenza-3 (PI-3) (14, 68, 129), Bovine Herpesvirus-1 (BHV-1) (62, 76), Bovine Respiratory Syncytial Virus (7, 135) and Bovine Viral Diarrhea Virus (97). Primary bacterial etiologies include *Mycoplasma* spp. (56, 142, 145) and *Chlamydia* sp. (89) infections. In addition, there is a lengthy list of proposed stress factors, which correlated with a higher incidence of BRD, such as weaning, transportation (45, 136), extremes in environmental temperature and condition (62, 126), noxious gases (including ammonia), overcrowding, branding, and irregular feeding and watering.

Primary lung damage leads to severe pneumonia in beef and diary cattle. In the order of prevalence, the most common bacterial isolates from BRD pneumonic lungs are

Mannheimia haemolytica 1 (formerly *Pasteurella haemolytica* A:1), *P. multocida* A:3 and *Haemophilus somnus* (145). In dairy calves *P. multocida* A:3 is by far the most common isolate from pneumonic lungs (14). The remainder of this discussion will focus on *P. multocida*, with special emphasis on *P. multocida* A:3.

Pasteurella multocida A:3 can be isolated from 21% - 41% of beef cattle and >60% of dairy cattle with pneumonic pasteurellosis (14, 145). The peak incidence of pneumonia has traditionally been regarded as afflicting 2 to 5 month old calves. A recent study, however examining transtracheally obtained bacterial cultures from calves with clinical respiratory disease found that infection occurs as early as 2 weeks of age, with the peak incidences observed at 3 to 5 weeks (142). In another study using producer-diagnosed pneumonia, peak incidence was 5 to 6 weeks (143), and in a study using a combination producer, veterinarian and post-mortem findings, peak incidence was 10 weeks of age (125).

The morphologic appearance of the *P. multocida*-associated pneumonia varies depending on the combination of agents involved in the disease. Traditionally, the pneumonia is characterized as a subacute to chronic fibrinopurulent bronchopneumonia with a cranioventral distribution. Grossly, the lesions are characterized as irregular, but well demarcated reddish-black to grayish-brown regions of consolidation and moderately firm lung parenchyma. On the cut surface, one often can express purulent exudate from bronchioles. Severe acute to subacute lesions often have dull pleural surfaces covered with a thin layer of fibrin. Histologically, bronchiolar and alveolar lumina are partially occluded by a markedly suppurative infiltrate admixed with hemorrhage, edema and fibrin. Often, there is exfoliation of the bronchiolar and alveolar epithelium in to their

respective lumina. The infiltrate continues into and expands the alveolar septal and interlobular interstitium. With time, the reparative process begins and the lesion partially or fully resolves. Severely damaged parenchyma frequently develops fibrous adhesions between visceral and parietal pleural surfaces with concurrent marked effacement of the normal lung parenchyma. Respiratory compromise often develops as a sequela (35).

BACTERIAL FACTORS ASSOCIATED WITH VIRULENCE

To aid in the discussion of virulence factors, a brief discussion of the Gram-negative cell envelope morphology is indicated. The envelope is composed of three loosely arranged layers (Fig. 1). The inner layer, or cell membrane, is a phospholipid bilayer that provides structural support and acts as an osmotic barrier. Many of the proteins embedded in this membrane serve specific functions such as transport, energy production, biosynthesis and motility.

The outer membrane is a bilayer membrane with embedded proteins and is intimately associated with the underlying peptidoglycan layer. The outer membrane markedly differs in composition from the inner membrane. Within the outer leaf of the outer membrane is a large quantity of lipopolysaccharide (see Lipopolysaccharide).

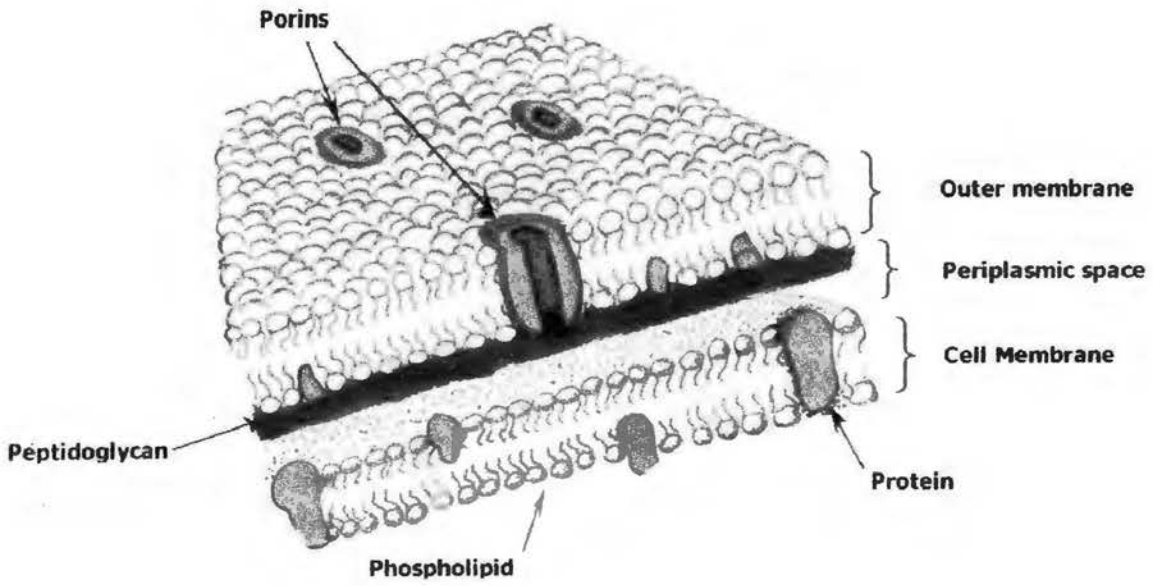

Fig. 1: Gram-negative bacteria cell envelope

Several outer membrane-associated virulence factors have been identified in *P. multocida*. These include a polysaccharide capsule, lipopolysaccharide (LPS), neuraminidase (NA), outer membrane proteins (OMP), iron-regulated outer membrane proteins (IROMP), multocidin (siderophore), porins, fimbriae, and *Pasteurella multocida* toxin (PMT or dermonecrotic factor).

Capsule

Early attempts to classify *P. multocida* on the basis of capsular antigens were often fraught with discordant results. The discrepancies in results were most likely attributed to crude extraction techniques and the intimate association of the capsule and underlying lipopolysaccharide. The system used today was developed, in part, by Carter and has evolved with time to include Groups A, B, D, E and F (12, 15-18, 21).

Capsules are extracellular acidic or neutral polysaccharides that form a distinct surrounding structure. A major capsular polysaccharide of *P. multocida* Carter-type A, but not other serotypes, is hyaluronic acid (HA; Fig. 2) (12, 15-17, 21, 30, 31, 92, 115). HA is a high molecular weight polymer composed of linear and non-branching disaccharide units. The HA unit consists of repeating glucuronic acid (GlcUA) and N-acetyl glucosamine (GlcNAc) linked with the assistance of a membrane-bound enzyme, hyaluronic acid synthetase (HAS) (Fig.3).

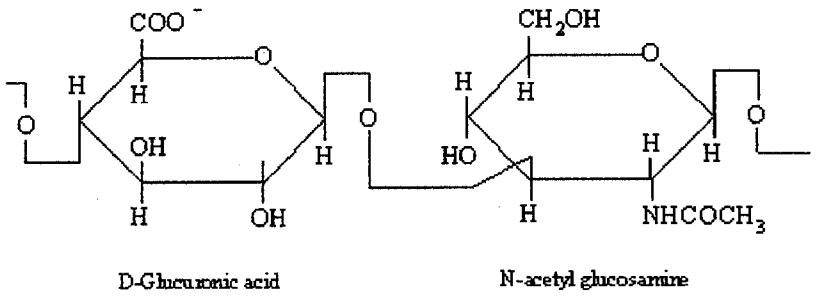

Fig. 2: Hyaluronic acid

The overall reaction for HAS is given in figure 3.

$$\text{UDP-GlcA} + \text{UDP-GlcNAc} + (\text{HA})_n \xrightarrow{\text{HAS}} (\text{HA})_{n+1} + 2\ \text{UDP}$$

Fig. 3: Hyaluronic acid synthesis

De Angelis et al. has cloned and extensively studied HAS from *P. multocida* serogroup A strain P-1059 (PmHAS) (31). When compared to other well-define HAS from *Streptococcus* spp., *Paramecium bursaria Chlorell* virus-1 or higher animals, PmHAS differs in many important respects and may represent a new class of HAS. One differing aspect of PmHAS from HAS from other species is its significantly larger size, 972 amino acid residues versus 417-588 residues, respectively. PmHAS nucleic acid

sequence is markedly variable with dissimilar predicted hydrophobicity plots and the loss of several motifs that are conserved in other HAS. In addition, PmHAS is kinetically optimized by the incorporation of Mn^{+2}, while streptococcal HAS is optimized with the addition of Mg^{+2} (30).

The capsular biosynthetic locus of *P. multocida* A:1 has been defined, and consists of three regions: Regions 1 – 3. Region 1 contains the open reading frames (ORFs) for 4 proteins involved in polysaccharide transport to the surface. Region 2 consists of five ORFs and is believed to encode for proteins involved in assembly of monosaccharides into capsular polysaccharides. Region 3 encodes for 2 proteins involved in phospholipid substitution at the reducing end of the capsular polysaccharide, presumably anchoring the capsule in the lipid of the outer membrane.

Rosner et al. revealed a second major neutral polysaccharide in capsular fractions from serotype A strains. The polysaccharide structure, identified as (1→4)-β-D-xylan, is a common polysaccharide in plants (115). Little is known about the biological function of the newly identified capsular polysaccharide, which is not immunogenic in mice (Rosner, unpublished data).

P. multocida capsule enhances pathogenicity through several mechanisms. Hyaluronic acid is a major component of the extracellular matrices for a variety of mammalian and avian tissues. This is fortuitous for *P. multocida* serogroup A, because under normal conditions the host does not recognize HA as immunogenic, enabling the bacteria to evade the immune response.

Various models and bacterial strains have shown that the capsule of *P. multocida* serogroup A significantly impairs phagocytosis by mouse neutrophils (72), rabbit

neutrophils (1), and avian macrophages (102). In each of these studies, hyaluronidase decapsulation or naturally decapsulated bacterial variants were consistently phagocytosed at higher rates when compared to capsulated homologous or parental strains.

The presence of capsule influences bacterial binding to cell surfaces. Capsulated strains of *P. multocida* serogroup A bound at significantly higher rates to the surface of avian air sac macrophages (105), HeLa cells, and macrophages of guinea pigs, rats and mice (43) when compared to hyaluronidase decapsulated bacteria. Interestingly, in each study, macrophages that were preincubated with hyaluronic acid consistently had decreased surface binding of bacteria. Another unexpected finding was that phagocytosis of cell surface bound capsulated bacteria was inhibited when compared to hyaluronidase decapsulated bacterial (105). Implying that from the above studies was the possibility of a specific and common HA receptor on the surface of macrophages.

Recent advances in the fields of organogenesis and tumorigenesis led to the identification of hyaladherins. Hyaladherins are transmembrane receptors found on many mammalian tissues that play crucial roles in cell behavior, organogenesis and tumorigenesis (64, 65, 124, 152, 153). With this knowledge, Pruimboom et al. explored the role of CD44, a hyaladherin group member, in the adhesion of *P. multocida* A:3 to turkey peripheral blood monocytes (TPBM). Their data revealed a significant decrease in bacterial adhesion when TPBMs were preincubated with monoclonal anti-CD44 antibodies (104).

One interesting study presents a paradox that has not been addressed by additional studies. Jacques et al. found that *P. multocida* capsular types A and D that were grown *in vitro* under iron-restrictive conditions, similar to *in vivo* conditions, or within

intraperitoneal chambers, have thinner capsules (61). The use of polymyxin gold labeling revealed significantly more staining of the lipopolysaccharide in bacteria grown in iron restrictive environments. Intuitively, one would expect that in a hostile environment the bacteria would develop a thicker capsule.

Several other hypothesized mechanisms for capsular virulence factors are shedding of polysaccharide components, hiding of outer membrane epitopes, and activation of T suppressor cells (114). For now, all virulence mechanisms for the *P. multocida* serotype A capsule have not been fully elucidated, but with time should be revealed.

Lipopolysaccharide

Most of the knowledge of lipopolysaccharide (LPS) is from the study of bacteria within the Enterobacteriaceae, especially *Escherichia coli*, *Salmonella* spp., and *Shigella* spp.. It is beyond the scope of this introduction to discuss all attributes of LPS, so the reader is referred to several comprehensive references (86, 103, 148).

LPS emanates from and is a major component of the outer leaf of the outer bacterial membrane. It is responsible for many of the physiologic responses observed in mammals due to Gram-negative sepsis. The general architecture is shown in Figure 3.

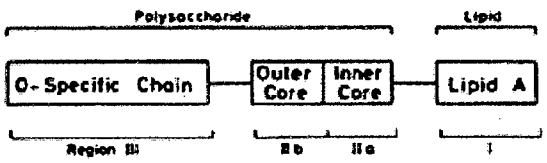

Fig. 3: General lipopolysaccharide structure

LPS consists of three regions: Regions 1 – 3.

Region 1 is Lipid A, which is responsible for endotoxic activity of Gram-negative bacteria. Lipid A is composed of a phosphorylated N-acetylglucosamine (NAG) dimer with 6 – 7 attached fatty acids, giving region 1 hydrophobic properties. This region is highly conserved among Gram-negative bacteria, embedded within the outer cell membrane, and is the membrane anchor for LPS.

Region 2 is the core polysaccharide. The structure of the core oligosaccharide varies slightly among different genera of Gram-negative bacteria, but remains fairly consistent within genera. This region is arbitrarily separated into an inner and an outer core. The inner core contains 2-keto-deoxyoctonic acid (KDO) and heptose, usually L-glycero-D-manno-heptose, which are linked to Lipid A. Mutant bacteria lacking the inner core region have not been identified. It is assumed that the inner core region is involved in growth and survival of the bacterial cell (112). The outer core region in most Gram-negative bacteria is responsible for linking the O-antigen side chain. However, there are a few bacteria, such as *Neisseria* spp., *Bordetella* spp., *Branhamella* spp. and *Campylobacter jejuni* that naturally lack the O-antigen; this low-M_r LPS is often referred to lipooligosaccharide (LOS). LOS is an area of intense research for its role in molecular mimicry to host antigens, such as paragloboside which is the precursor of the ABH glycolipid antigen on human erythrocytes [Preston, 1996 #763; Moran, 1996 #875].

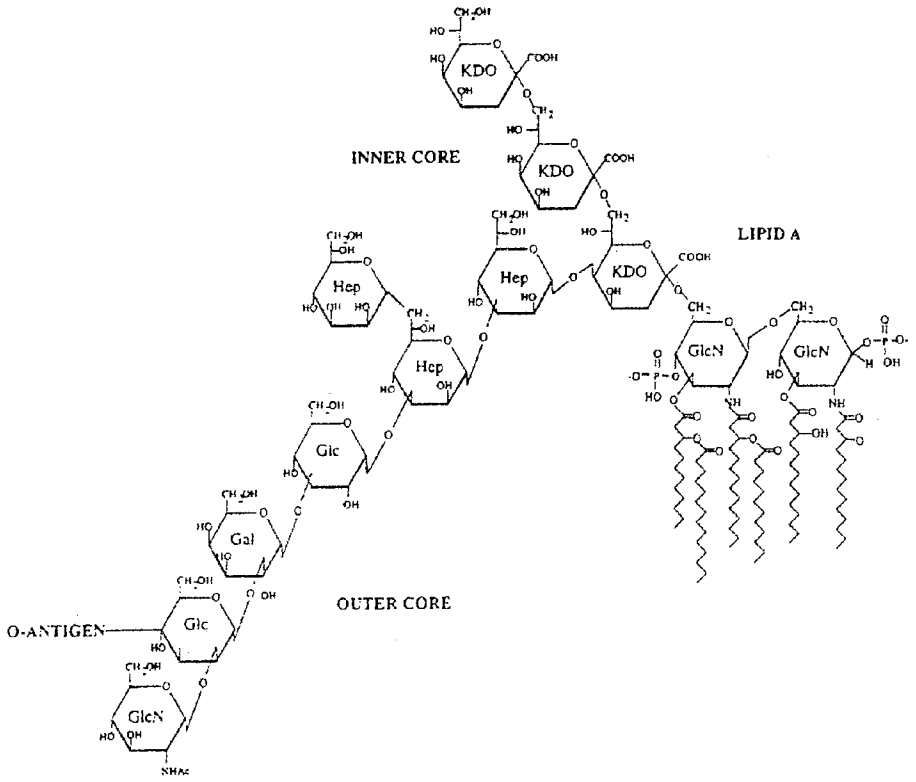

Fig. 4: *Escherichia coli* lipopolysaccharide

Region 3 is the highly immunogenic polysaccharide O antigen. The O antigen is composed of repeating (up to 25) oligosaccharide subunits consisting of 3 to 5 sugars that extend from the bacterial surface. The oligosaccharide composition lends hydrophilic properties to region 3. Limited bacterial serotype classification is accomplished by variations in saccharide composition, linkage and sequence of region 3. Loss of region 3 through mutation or chemical removal causes a change in colony morphology from "smooth" (S form) to "rough" morphology (R form).

During the past 40 years there have been numerous studies that characterize LPS by chemical structure and by isolation techniques. Erler et al. have extensively studied

the LPS polysaccharide content of *Pasteurella multocida* (36-42). In one study, Erler et al. tried to reclassify or sub-classify serotypes from numerous isolates of *P. multocida* representing 5 serogroups and 9 serotypes. Based on variations in the LPS content of glucosamine, galactosamine, L-glycero-D-mannoheptose, D-glycero-D-mannoheptose, glucose and galactose, Erler divided *P. multocida* into 4 groups. However, they obviously were not equivalent to standard Heddleston serotypes (38). Studies by Coy et al. examined strain variation of LPS using lectin affinity. Their data shows some variation in the affinity of LPS from three strains of *P. multocida* A:3 and 4 to bind to *Ricinus communis* agglutinin (RCA), a lectin that binds β-galactose residues. RCA-positive variants were more likely to bind to epithelial cells, to have increased resistance to complement-mediated killing, and to have increased virulence in experimentally infected turkeys (26).

Others characterized LPS on the basis of the extraction techniques used to obtain the molecule and the fractions obtained. A common LPS extraction procedure used is the Westphal technique (146), a technique based on phenol-water fractionation of formalinized whole cell bacteria. The Westphal extracted LPS has good correlation with heat-stable typing antigen, and in gel diffusion-precipitin reactions, in which precipitation only occurs with its homologous antiserum (13, 109).

The lipid A component has the ability to activate the acute inflammatory response, which can have beneficial as well as deleterious effects within the infected animal. LPS-mediated damage can occur indirectly by the release of cytokines such as tumor necrosis factor-α (TNF-α) (9), interleukin-8 (IL-8) and interleukin-1 (IL-1) (27) from activated macrophages and monocytes. *In vitro* studies have shown a dose

dependent increase in TNF-α and IL-1 release from bovine alveolar macrophages exposed to *M. haemolytica* LPS (9, 150). These cytokines are responsible for cell death (123), alterations in endothelial morphology, complement activation, intravascular coagulation (11, 119, 144), and polymorphonuclear cell trafficking and activation (27). *In vitro* studies revealed a time-dependent and dose-dependent cytotoxicity to endothelial cells exposed to *M. haemolytica* LPS (10, 93-95). The cytotoxic changes consisted of extensive cell detachment from the monolayer, cell contraction and cell blebbing.

Several studies in animal models demonstrated the ability of LPS extracts to induce the Shwartzman reaction consisting of generalized or dermal reactions that are histologically characterized by vascular endothelial cell damage with concurrent neutrophil or heterophil infiltrate, microthrombus formation and hemorrhage (75, 107, 111). Typically, this reaction is subclinical with the first LPS exposure, but the second or provacative LPS dose exaggerates the injury. Dermal and generalized Shwartzman reactions have been elicited in rabbits (107) and in turkey poults (75). For unknown reasons, it appears that adult chickens and turkeys are resistant to both dermal and generalized Shwartzman reactions. If dermal reactions do occur, they are significantly attenuated (75, 111).

The role of anti-LPS antibodies has been studied in a variety of *in vivo* challenge models. These studies frequently yield conflicting results, most likely the result of differences in LPS extraction techniques and immune responsiveness of the selected animal model. In a study by Reber et al. (109), the difference in immune response was clearly dependent on the animal species vaccinated. Mice, rabbits and chickens were vaccinated with aqueous components after an aqueous/phenol extraction technique (an

abbreviated Westphal procedure) of *P. multocida* strain X-73. Evaluation of their sera by Ouchterlony gel diffusion assay or by hemagglutination did not detect anti-LPS antibodies in the mice or rabbits, but did in the chickens. Not surprisingly, mice and rabbits were not protected against challenge, whereas chickens were resistant to homologous challenge, again supporting the concept of the variable immune responsiveness of each species (109). Schmerr and Rebers found similar results in mice inoculated with Westphal extracted LPS, then challenged with *P. multocida* strain P-1234 or strain X-73 (122).

Neuraminidase

Neuraminidase (N-acetylneuraminate glycohydrolase, EC 3.2.1.18) is a high molecular weight (160,000 - 500,000 daltons) enzyme that is found in most strains of *P. multocida* (34, 121), several strains of *M. haemolytica* (131), *Corynebacterium diphtheriae* (79), *Diplococcus pneumoniae* (88) and other bacteria and viruses.

Scharmann et al. examined 104 strains of *P. multocida* belonging to the serotypes A, B, C, D or E for neuraminidase production. They found 102 strains had cell-associated neuraminidase activity. Less than 5% of the total neuraminidase activity was found in the cultural supernatant (121). In the same study, it was observed that neuraminidase could be induced by the addition of N-acetyl-D-mannosamine, fetuin, N-acetylneuraminic acid, and sialyl lactose to the culture medium. Drezienk et al. (34) observed induction during the logarithmic phase and in stationary cultures as old as 39 hours. They also found serogroups A and D to have the highest concentration of cell-bound neuraminidase (34).

Neuraminidase cleaves α-ketosidic linkages of N-acetylneuramin lactose, α-1-acid glycoprotein, fetuin, colominic acid, bovine submaxillary salivary gland mucin (130), and bovine serum albumin (147). Additionally, neuraminidase cleaves rabbit, bovine and human transferrin into multiple proteins (120).

The virulence mechanism of neuraminidase has not been elucidated; however, Gottschalk proposed that removal of sialic acid groups (N-acetylneuramininc acid) from mucoproteins decreases viscosity due to a conformational change from an extended protein shape to a more compact shape. He hypothesized that the change in viscosity may inhibit the protective abilities of glycoproteins in salivary, respiratory and urogenital secretions (54).

Several *in vivo* and *in vitro* observations support the importance of neuraminidase as a virulence factor. In goats experimentally infected with *M. haemolytica* and *P. multocida*, there was a strong correlation between severity of disease and bacterial neuraminidase expression and secretion (128, 132). Passive transfer of anti-neuraminidase antibodies protected mice from *P. multocida* challenge at a dose of 30 times the LD50 for unprotected controls (59). *In vitro* studies by Lee et al. showed an increase in bacterial invasion in turkey poult kidney epithelial cell cultures pretreated with neuraminidase (67). Guinea pigs and humans infected with *P. multocida* had altered electrophoretic mobilities of glycoproteins such as, α-glycoprotein, haptoglobin, transferrin, hemopexin and IgA (120).

Multocidin

Iron is an essential component of energy metabolism in bacteria through the cytochrome (heme-protein) oxidase system. At least one micromolar iron is needed for optimal growth; however, under biological conditions, uncomplexed ferric (Fe^{3+}) iron is not greater than 10^{-12} μM (83). To meet the minimal needed iron concentration, bacteria have developed iron scavenging systems.

Two independent strategies are used by *P. multocida* for the aquisition of iron. One effective mechanism is the synthesis and secretion of a siderophore (Gr. *iron carriers*). Siderophores are low molecular weight iron-chelating proteins that have a high affinity for Fe^{3+}. After formation of an iron-siderophore complex, the complex will bind to an iron-regulated outer membrane protein (IROMP) receptor, enabling transport of the iron across the cell wall (83).

Hu et al. were the first to isolate and partially purify a soluble protein "growth-enhancing factor", later called multocidin, from *P. multocida* serogroup A grown in iron-restrictive medium. In a series of salient studies, they characterized multocidin's ability to bind iron in the presence of iron-loaded transferrin and in spent chemically defined medium from *P. multocida* (57). Reissbrodt et al. examined several strains of *P. multocida* of unspecified serogroup/type for the ability to utilize well-characterized siderophores in "cross feeding tests". All strains utilized ferrioxamine B, E and G, and Fe-rhizoferrin for sources of iron (110). Choi-Kim et al. characterized the multocidin receptor of *P. multocida* A:3. They demonstrated antibodies to three IROMPs of molecular masses 76, 84 and 94 kDa significantly decreased binding of ^{59}Fe-multocidin complexes to these proteins.

In another novel adaptation, *P. multocida* A:3 can mimic the mammalian host by having outer membrane transferrin receptors. Transferrin is a host glycoprotein that transports iron from sites of absorption and storage media, like the intestine and liver, to peripheral cells. Ogunnariwo et al. identified an 82kDa OMP from *P. multocida* A:3 that acts as a transferrin receptor. In addition, the receptor only possessed affinity for bovine, but not avian, equine, human or porcine transferrin (87). Similar affinities for transferrin have been observed in serogroups B and E; however, the receptor has not been characterized (140).

Outer membrane proteins

Outer membrane proteins (OMPs) function in transmembrane solute transport and membrane structural integrity. These proteins may be fully imbedded within the outer membrane or partially surface exposed. Several of the surface exposed proteins are major antigenic determinants and often can stimulate protective immunity.

A 28 kDa, major OMP was identified and partially characterized (28, 73). Partial N-terminal sequencing revealed homology between this protein and OmpA, a major outer membrane protein (porin) of *E. coli*, *Salmonella typhimurium*, and *Shigella dysentery*. The *P. multocida* OmpA homologue migrates at 35kDa on SDS-PAGE when heated to 100°C in the presence of 2% SDS. When heated to 37°C, the OmpA homologue migrates at 28kDa, a characteristic of porins. Additional characterization of the *P. multocida* OmpA homologue resulted in conflicting reports as to whether it is surface exposed. Using immunoelectron microscopy and monoclonal antibody (MAb MT4.1), Marandi and Mittal failed to demonstrate surface exposure (73). In addition, mice were not

protected from an intraperitoneal challenge with *P. multocida* when using the backpack hybridoma tumor elaborating MAb MT4.1. Dabo et al. however, demonstrated surface exposure by sensitivity of the 28kDa protein to protease treatment of whole organisms (28).

Recently, studies have identified a major 37.5 kDa OMP porin (OmpH) of *P. multocida.* OmpH up-regulates the secretion of IL-1, IL-6, IL-12, TNF-α and interferon-γ (IFN-γ) from murine splenocytes (60). In additions, neutrophils exposed to OmpH elicit f-actin polymerization, chemotaxis and increased oxidative burst capabilities (47). Monoclonal antibodies to OmpH have been shown to be protective in mice challenged by intraperitoneal inoculation of *P. multocida* (139).

Pasteurella multocida grown in iron-restricted *in vitro* conditions or *in vivo* express novel and highly conserved outer membrane proteins (IROMPS). The M_r of these proteins varies slightly with strain and study. Glisson et al. observed *P. multocida* strains X-73 and P-1059 grown in iron-restrictive environments expressed four additional proteins with molecular weights of 52, 77, 91 and 96kDa, and 63, 77, 91, and 99kDa, respectively (50). Strain 1059 grown under iron-restrictive conditions by Choi-Kim et al (23) produced three major proteins with molecular weights corresponding to 76, 84, and 94kDa. These proteins are known to bind multocidin and to a lesser extent free iron (23). The complete mechanism(s) for iron transport through the periplasmic space and cytoplasmic membrane in *P. multocida* is not fully understood. *Neisseria* sp., which also expresses IROMPS, have been studied more extensively (8, 51-53, 149). There is evidence in this organism for a ferric binding protein (Fbp) used in the transport of iron through the periplasmic space (51). A cytoplasmic membrane permease is postulated for

iron transport through the cytoplasmic membrane. Studies have also revealed IROMPS can elicit serum resistance with occasional cross protection (23, 25, 116).

Fimbria

Adherence to mucosal surfaces is an important criterion for bacterial colonization. For instance, toxigenic strains of *P. multocida* A and D were consistently isolated from the tonsils of experimentally infected SPF pigs, but they were inconsistently re-isolated from the nasal passages (101). The author suggested that toxigenic *P. multocida* reside in the tonsils and cause a toxemia resulting in atrophic rhinitis. Fimbria can be found on most toxigenic *P. multocida* isolates from the nasal passages of swine suffering from atrophic rhinitis. Conversely, most isolates from pneumonic pasteurellosis in swine are non-toxigenic and non-fimbriated (138). Fimbria are thin filamentous structures, measuring approximately 6.0 – 7.0 nm x 25.0 – 50.0 nm, which protrude from the bacterial surface, and may aid in adherence. Fimbriae of *P. multocida* serogroup A (PtfA) have been characterized as type 4 fimbriae, consisting of repeating 18 kDa fimbrial subunits. Partial N-terminal sequencing of PtfA revealed homology to PilA of *Pseudomonas aeruginosa*, FimA of *Dicholobacter nodosus*, βpilin of *Moraxella bovis*, HtfA of *Haemophilus influenza*, and PilE of *Neisseria gonorrhoeae* (117). Through immunostaining with anti-PtfA antibodies, common epitope(s) were found on fimbriae from all *P. multocida* serogroups A, B and D, as well as recombinant βpilin and FimA. .

Pasteurella multocida toxin

Pasteurella multocida toxin (PMT) or dermonecrotic factor is a cell-associated toxin elaborated primarily from certain strains of *P. multocida* serogroups D and A. This toxin is associated with atrophic rhinitis in swine when given parentally or in association with natural mucosal infection. Using experimental conditions and laboratory animals, a variety of lesions such as dermal necrosis, hepatic necrosis, nasal turbinate atrophy, pneumonia and death, may be observed. The mechanism of action is unknown, but may be mediated through the host cell phospholipase C and protein kinase C signaling cascade. Several studies found that lesions associated with PMT may be reduced in severity through parental or intranasal vaccination using either recombinant or native PMT (44, 85, 98, 133, 137).

BIOLOGICALS

The use of biologicals for control of *P. multocida*-induced disease has produced inconsistent results. *P. multocida*-induced bovine pneumonic pasteurellosis is complicated by the paucity of studies to determine the efficacy of vaccination. However, by using vaccination data obtained from studies of hemorrhagic septicemia, fowl cholera, and pneumonic pasteurellosis in rabbits and swine, as well as studies related to bovine pneumonic pasteurellosis, we can better understand the immune response to various *P. multocida* antigenic determinants.

Biologicals: Attenuated live bacterial vaccines

Attenuated live *P. multocida* vaccines have the potential advantage of stimulating a strong humoral response that may cross protect against different serotypes. Often, bacteria produce protective epitopes only under certain growing conditions or *in vivo* (118); therefore, the replication of an attenuated live vaccine *in vivo* should stimulate antibodies to the appropriate epitopes. However, attenuated vaccines carry a greater potential risk such as reversion from an avirulent to a virulent state, inducing a disease in an unintended or immunocompromised host, or anaphylaxis (77, 118).

One hundred and sixteen years ago Louis Pasteur used an attenuated live vaccine for the prevention of the *P. multocida*-induced disease, fowl cholera. Since then, several attenuated strains of *P. multocida* have been somewhat successful at inducing a protective immune response in chickens and turkeys. Even with the advent of efficacious vaccines, fowl cholera remains a threat to poultry producers. Dick et al. quantified fowl cholera antibodies in eighteen broiler-breeder flocks containing unvaccinated 2 to 10 week old chicks. He found chicks in 5 flocks were positive, indicating exposure (unpublished data) (33).

Friedländer et al. examined the efficacy of three live attenuated *P. multocida* vaccines, M-9, Minnesota (Mn) and Clemson University (CU) strains administered via drinking water and subcutaneous inoculation in turkeys and chickens. Their results determined that a combination of the CU vaccine given subcutaneously at 7 weeks and followed by a booster 1 week later with live CU in the drinking water resulted in better protection than did other strains and dosing combinations. In addition, with that dosing combination, there was a significant correlation with the mean antibody titer, as

determined by the microtiter agglutination test, and survivability after an oral challenge with *P. multocida* A:3 (46). In another study, vaccination of chickens and turkeys with CU strain stimulated protective immunity for 25-30 weeks (49). Others have shown that a commercially available attenuated live vaccine can stimulate protection for up to 80 weeks post vaccination in broiler-type chickens (32).

A protective immune response is not only dependent on the *P. multocida* vaccine, but also the competence of the immune system. A positive correlation was observed between immune system maturation and vaccine timing. Chicks were vaccinated once at 1, 2, 3, 4, 5 or 6 weeks of age and again at 11 weeks with live CU strain demonstrated higher mean antibody titers were correlated with increasing age at the time of vaccination. In addition, chicks less than 3 weeks old needed an extended time in order to reach peak antibody production (33).

Vaccination of cattle with live *P. multocida* A:3 by aerosol or parental routes, then subsequently transthoracically challenged with a homologous strain, produced significantly elevated antibody titers and reduced pneumonic lesions. These studies also found aerosolization to yield the best protection (84, 90, 91). Similar results were obtained through parental vaccination with a streptomycin-dependent strain (Catt) and with a commercial live vaccine (Chengappa) of *P. multocida* with subsequent transtracheal challenge with a heterologous strain (19, 20). Catt et al. in a field trial compared immunity elicited by one and two dose regimens after a parental vaccination of preconditioned and non-preconditioned calves with a streptomycin-dependent *P. multocida* vaccine. The twice-vaccinated preconditioned calves had a higher average

daily weight gain, lower clinical scores, and lower death rate than single-dose vaccinates and non-preconditioned calves (63).

In experimental hemorrhagic septicemia a single subcutaneous or intradermal vaccination with *P. multocida* B: 3.4, an avirulent strain for cattle and buffalo, increased resistance to challenge with a virulent, heterologous strain of *P. multocida.* Protection remained 13 months post-vaccination, the longest time point examined (81, 82). De Alwis et al. vaccinated buffalo and cattle by subcutaneous and intramuscular routes using *P. multocida* strain 33/100, a streptomycin-dependent mutant. Significant protection was obtained from a single dose regimen in buffaloes and a double dose regimen in cattle, as determined in a mouse protection assay (29).

Biologicals: Bacterins

Bacterins are often favored over live vaccines because of the ease of production and the advantage of producing autogenous bacterins. Bacteria grown under iron-restrictive conditions often express several high molecular weight outer membrane proteins not observed with bacteria grown in iron-rich medium. Because bacteria grown *in vivo* produce similar outer membrane proteins, the protective IROMPs have been the focus of numerous studies. Glisson et al. immunized chickens and turkeys with two different bacterins produced from *P. multocida* serotype 1, strains X–73 and P-1059 grown in an iron-deficient medium. Protection was observed with homologous challenge, however no protection was observed with heterologous challenge (50). Similarly, Ruffolo et al. vaccinated mice with *P.* multocida strain PBA100 or PBA101 grown in iron-replete or iron-deficient medium. Mice vaccinated with PBA101 grown in

iron-deficient medium were fully protected from homologous and heterologous intraperitoneal challenge. Mice vaccinated with PBA100 grown in iron-deficient medium were fully protected from homologous challenge, but only partially protected from heterologous challenge (116).

Biologicals: Subunit vaccines

Subunit vaccines are vaccines composed of portions of the bacteria that can elicit a protective immune response. This type of vaccine is intriguing, because one can control the level of contaminants and associated toxicity as well as selectively tailor the proper immune response for the desired protection. The desired response would be an increase in plasma cell production with concurrent antigen-specific IgG production or, for a mucosal pathogen, IgA (118).

One approach to subunit vaccines has been to use ribosomal RNA. Mice vaccinated with various concentrations of purified 70S ribosomes from *P. multocida,* strain X-73 were not protected from challenge with the homologous strain (100). In a similar investigation, *P. multocida* strain X-79 ribosomal vaccines were partially effective in preventing experimental fowl cholera when isolated ribosomes were used in combination with *P. multocida* lipopolysaccharide using a 2-dose vaccination regimen at a 3-week interval. It is interesting to note that substitution of the bacterial ribosomes with 70S chicken ribosomes and LPS did not confer protection, suggesting the *P. multocida* ribosomes participated in stimulating the immune response (99). Baba observed similar protection following vaccination of chickens with ribosomes using *P. multocida* strain P-1059 (5).

Several investigators have determined that *P. multocida* LPS is antigenic, but the protective response afforded by an LPS vaccine is variable. Early studies, using crude LPS extracts as experimental vaccines, found some level of protection. Those vaccines, however, were toxic in mice, rabbits and calves (6, 108) due to endotoxin. LPS-protein extracts of *P. multocida* were evaluated as vaccines by several investigators and typically produced a protective immune response in chickens, turkeys, mice and rabbits (66, 107). In addition, vaccination with the soluble fraction of an LPS-protein extract of *P. multocida* strain P1059 elicited a protective immune response in turkeys to a homologous challenge (66). Gaunt et al. found that vaccination of chickens with a potassium thiocyanate (a chaotropic agent) extract of *P. multocida* strain P-1059 produced a protective immune response in chickens to homologous and heterologous challenge (48). A species-specific immune response was observed when *P. multocida* strain X-73 LPS was extracted using the Westphal procedure and used as a vaccine. The extracted LPS did not induce a protective immune response in rabbits and mice, but did so in chickens (109). Ramdani et al. further characterized the immune response of mice to *P. multocida* LPS. Using six monoclonal anti-*P. multocida* LPS antibodies, it was shown that anti-LPS antibodies in vitro were opsonic but not bactericidal. Mixtures of these same monoclonal antibodies, however, were not protective to mice in a passive mouse protection assay from challenge with homologous *P. multocida* strain. In addition, mice vaccinated weekly for 9 weeks with LPS developed anti-LPS antibody titers but were not protected from homologous challenge (106).

P. multocida capsular extracts containing a combination of proteins and polysaccharides have been examined and shown to be protective. Rabbit and bovine

antibodies to cetyl pyridinium chloride-precipitated capsular antigen from *P. multocida* type B were protective, as determined by the passive mouse protection assay (96).

Biologicals: *P. multocida* OMPs

The potential importance of *P. multocida* outer membrane proteins as immunodeterminants that possess the ability to stimulate a protective immune response has been recognized in numerous studies. Several major OMPs of *P. multocida* have been identified and partially characterized (22, 28, 73, 74). A high antibody response to *P. multocida* OMPs correlated directly with protection after challenge in cattle (25), rabbits (71, 134), chickens (151) and in mice (69, 70, 127). Confer et al. showed by ELISA an increase in OMP antibodies following vaccination by subcutaneous or aerosol routes with live *P. multocida* A:3. Lung lesion scores were inversely correlated to OMP antibodies in vaccinated calves following challenge, and were significantly different than unvaccinated controls and whole cell-bacteria vaccinated calves (25).

Biologicals: Toxoid

Pasteurella multocida toxin (PMT) is a virulence factor principally of *P. multocida* type A and D isolates, and is responsible for osteoclastic bone resorption leading to nasal turbinate atrophy in swine. Heat-inactivated PMT alone and with the cholera toxin (PMT-CT) as the adjuvant was used to intranasally inoculate rabbits at 0, 7 and 14 days. Rabbits were challenged on day 21 with PMT, had a decreased severity of pleuritis, pneumonia, turbinate atrophy, and hypospermatogenesis in both PMT and PMT-CT groups compared to nonvaccinated controls. Use of CT as an adjuvant

enhanced protection and increased anti-PMT nasal IgA production compared to the PMT vaccinated group (133). Vaccination of pregnant sows with formaldehyde-inactivated PMT produced colostral antibodies that protected piglets against intranasal *P. multocida* and *Bordetella bronchiseptica* infection. Protected piglets had a decrease in nasal turbinate atrophy, *P. multocida* and *B. bronchiseptica* CFU in nasal swabs, and an increased daily weight gain (44). With a similar experimental design, Nielsen et al found vaccination with a recombinant nontoxic PMT derivative (dO) induced protective colostral antibodies in piglets (85).

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CHAPTER II

SUMMARY AND STATEMENT OF RESEARCH PROBLEM

M. haemolytica serotype 1 and *P. multocida* A:3 are part of the normal upper respiratory bacteria flora of cattle. Through a combination of synergistic factors the bacteria are able to colonize the lower respiratory tract, causing bovine pneumonic pasteurellosis. Two different approaches were taken in this study of bovine pneumonic pasteurellosis.

First, to study immunity to *P. multocida*, we looked at a major OMP that was previously found to be highly immunogenic in cattle and to which high serum antibody responses correlated with resistance to experimental challenge (Confer et al., 1996). We hypothesized that the 28kDa *P. multocida* OMP (Omp28) was a surface exposed porin and vaccination of mice with purified Omp28 would stimulate a protective immune response against homologous challenge. The study of Omp28 was done in three phases, each examining various attributes of the protein. During the first phase Omp28 was purified through a combination of detergent fractionation and chromatographic procedures. The second phase examined the physical characteristics of the protein such as surface exposure, determination of porin activity and N-terminal sequence homology. The third phase examined the immune response to homologous challenge in Omp28-vaccinated mice.

Second, because of the difficulties and expense of using cattle to study pasteurellosis, the prevalence of the organism in cattle, and the lack of a good laboratory model, we undertook the development of a laboratory animal model for bovine pneumonic pasteurellosis. We looked at a potential chimeric mouse model that used mice with severe combined-immunodeficiency and beige genetic mutations (SCID-beige mice) reconstituted with bovine fetal hematolymphoid tissues (SCID-bo) (Denny et al.,

1996). We hypothesized that experimental *M. haemolytica* infection in SCID-bo mice would mimic bovine pneumonic pasteurellosis and could be a model for the disease in cattle. The first phase will characterize and histologically graded the pulmonary inflammation observed after the intratracheal administration of *M. haemolytica* 1 to SCID-bo mice. The lesions were correlated to the bacterial count per gram of lung tissue. The second phase examined the pulmonary inflammatory response after intratracheal administered live and formalin killed *M. haemolytica* serotype 1 in SCID-bg mice.

CHAPTER III

ISOLATION AND CHARACTERIZATION OF THE OMPA-EQUIVALENT OUTER MEMBRANE PROTEIN OF *PASTEURELLA MULTOCIDA* SEROTYPE A:3 (OMP28)

ABSTRACT

A major immunogenic 28 kDa outer membrane protein (Omp28) from *Pasteurella multocida* A:3 (strain 232) was purified, and partially characterized immunologically and structurally. Omp28 was extracted from N-lauroylsarcosine-insoluble protein (OMP) preparations by a combination of detergent fractionation with Zwittergent 3-14 and chromatography. Partial N-terminal amino acid sequence confirmed Omp28's as a member of the OmpA-porin family. However, porin activity for Omp28 could not be demonstrated in a lipid bilayer assay. Omp28 surface exposure was examined by partial protease digestion of intact bacteria and by immunogold labeling with polyclonal anti-Omp28 antibodies. Surface exposure was not demonstrated by either method. Omp28 was immunologically evaluated in a mouse protection assay. CD-1 mice vaccinated with purified Omp28 developed a significant antibody titer ($P<0.05$) compared to the control treatment group, but were not protected from a homologous intraperitoneal bacterial challenge. Treatment groups vaccinated with *P. multocida* outer membrane, formalin-killed *P. multocida* or a commercial vaccine were significantly protected from challenge. *In vitro* complement-mediated killing of *P. multocida* was observed from post-vaccination sera of outer membrane, formalin-killed *P. multocida,* and commercial vaccine-treatment groups, but not with sera from the Omp28-treatment group. This is the first report in which the 28 kDa outer membrane protein of *P. multocida* A:3 was purified and partially characterized immunologically and structurally.

INTRODUCTION

Pasteurella multocida is an important pathogen for several animal species, including humans, and is associated with a variety of diseases. In cattle, *P. multocida* A:3 is one of the etiologic agents of bovine respiratory disease (BRD). Our understanding of BRD is incomplete, and many aspects of its pathogenesis are still debated. Several studies have shown the influence of environmental stressors (16, 28, 59, 64), concurrent viral (4, 7, 28, 31, 44, 55, 61, 63) or bacterial infection (22, 50, 68, 70), and host immune status (15, 56) as having a role in the development of BRD. The lack of full comprehension of the disease pathogenesis and immunity has allowed the disease to prevail with significant economic burden to the cattle industry. In a recent study by Bowland and Shewen, the estimated annual economic loss to the cattle industry in the United States due to BRD is 640 million dollars (6).

Several studies have demonstrated the importance of outer membrane proteins (OMPs) in the development of a protective antibody response against Gram-negative bacteria. Vaccination of cattle (12), mice (60, 67), chickens (74) and rabbits (33) with *P. multocida* OMPs stimulated significant protection against challenge with the live bacteria. Protection has also been obtained by vaccination with selected and purified OMP (37) or by passive monoclonal antibody reconstitution to purified OMPs (1, 32, 67). OMPs have been examined in several *in vitro* assays for the ability to influence phagocytosis (66, 67), complement-mediated bacterial killing (43), activation of neutrophils (17), indirect hemagglutination (53), and cytokine expression (25).

Marandi et al. identified an immunogenic 28 kDa OMP from *P. multocida* serogroup B (strain 656) with some N-terminal amino acid sequence homology to

Escherichia coli OmpA, a porin protein (42). A similar 28 kDa OMP was found in *P. multocida* A:3 (strain 232) by Dabo et al.. That study revealed that the 28 kDa OMP had OmpA homology and partial surface exposure (13). Further characterization of the 28 kDa OMP revealed significant genomic sequence homology to *E. coli* OmpA, as well as, hydrophobicity-predicted surface exposure (Dabo et al.; unpublished data).

Porins represent a group of channel forming proteins that traverse the outer membrane of several genera of Gram-negative bacteria. They have been extensively studied (5, 9, 30, 48, 49, 54, 57, 62) and share a basic structure containing a high proportion of antiparallel β-chains that take on a barrel conformation. Depending on the type of porin, the β-barrel can associate in the outer membrane as a homotrimer or remain as a monomer. The channel formed allows for the passive passage of small solutes based on size, shape, hydrophobicity or charge (26, 27, 48). Only a few substrate-specific porins have been identified (19, 35, 40, 65). In addition, porins have a role in bacteriophage attachment (27, 36) and structural integrity of the cell envelope (36).

In this report, we describe the purification and characterization of the 28 kDa OMP from *P. multocida* A:3 strain 232, and elucidate the role of the protein in stimulating immunity in mice. The 28 kDa OMP will be hereafter designated as Omp28.

MATERIALS AND METHODS

Animals

All mice used in these studies were 5-week-old CD-1 mice housed in a climate-controlled facility at 22°C, ambient humidity and a 12/12 day/night cycle. Animal facilities and care meets the standards set forth by the National Research Council in the Guide For The Care and Use of Laboratory Animals (24).

Bacterial cultures

P. multocida A:3 (strain 232), originally isolated from a case of bovine pneumonia and kindly donated by Dr. John Berg (University of Missouri, Columbia, MO), was plated onto brain-heart infusion (BHI; Becton Dickinson, Sparks, MD) agar containing 5% bovine blood and grown at 37°C in a 5% CO_2 environment for 18 hours (51). An isolated colony was transferred to 3.0 ml of BHI broth in a 15 ml sterile polystyrene tube and grown at 37°C with rotatory shaking at 120 oscillations/min. for 4 hours. A 200 µl culture aliquot was transferred to 2.0 L of BHI broth in a 4.0 L Erlenmeyer flask and grown at 37°C with rotatory shaking at 120 oscillations/min. for 14 hours.

Bacterial outer membranes preparation

Bacterial envelopes were prepared by sonication and centrifugation as previously described (58). Outer membranes were extracted with 0.5% sodium N-lauroylsarcosine (Sarkosyl; Sigma; St. Louis, MO) in 0.01 M Tris buffer and collecting the insoluble

fraction by centrifugation. The Sarkosyl-insoluble fraction, is nearly identical to the outer membrane fraction purified by sucrose gradient centrifugation on SDS-PAGE (13). The Sarkosyl-insoluble fraction is heretofore referred to as the OMP-enriched fraction.

Zwittergent 3-14 detergent fractionation

The OMP-enriched fraction was solubilized at 1% (w/v) Zwittergent® 3-14 (Calbiochem-Novabiochem Corp., La Jolle, CA), and 20 mM 3-[N-morpholino]propanesulfonic acid buffer (MOPS; Sigma Chemical Co., St. Louis, MO), pH 7.5 for sonication for 15 sec on ice with a 50% duty cycle (Bransonic 450, Danbury, CT). Insoluble protein was sedimented by centrifugation at 150,000 x *g* for 60 min at 4°C. The insoluble protein was solubilized for sonication as above in 0.1% Zwittergent 3-14, 50 mM ethylenediaminetetraacetic acid (EDTA; Sigma; St. Louis, MO) in a 20 mM MOPS buffer at pH 7.5. Zwittergent-EDTA insoluble protein was centrifugated and solubilized for sonication as above in 0.1% Zwittergent 3-14, 0.4 M NaCl and 20 mM MOPS buffer at pH 7.5. The supernatant, designated Zwittergent fraction 3 (Zfr3), was retained and stored at -70°C. The fourth Zwittergent fraction was obtained by solubilizing the insoluble pellet in 1.0% Zwittergent 3-14 and 20 mM MOPS at pH 7.5 and centrifuging, as previously described.

In addition to Zwittergent extraction, isolation of Omp28 was attempted using n-octyl polyoxyl ethylene (OPOE; Bachem Bioscience Inc., Philadelphia, PA) as previously described (38).

Electrophoresis and immunoblot analysis

OMP-enriched fractions were equilibrated to 1 mg protein/ml and subjected to discontinuous SDS-PAGE (13). Gels were stained with Coomassie Brilliant Blue. Coomassie-stained gels were further analyzed by densitometry using a video densitometer in transmittance mode (46). Analyses of peaks were performed, and data were expressed as total peak area optical density for each band (Multi-Analyst; Bio-Rad Co., Hercules, CA).

Proteins were transferred to nitrocellulose membranes by the method of Towbin et al.. (1979). Antigens were identified immunologically, using bovine or mouse sera at a 1:25 and 1:250 dilutions, respectively. Immune complexes were detected with alkaline phophatase-conjugated, goat anti-bovine IgG or rabbit anti-mouse IgG (1:25,000) catalyzing precipitate formation by an alkaline phosphatase sensitive substrate (Sigma Fast™ BCIP/NBT; Sigma Co., St. Louis, MO)

Enzyme-linked immunosorbent assay

Antibodies to outer membranes were determined by an enzyme-linked immunosorbent assay (ELISA) as previously described with minor modifications (11). Wells of 96-well microtiter plates (Costar®; Corning Corp., Corning, NY) were coated with 100 ng of *P. multocida* OMP-enriched fraction. Preliminary assays were done to determine appropriate serum dilutions to use in the assay. To accomplish this, 2-fold serial dilutions of sera from vaccinated or non-vaccinated mice were tested against the *P. multocida* antigen in an ELISA. OD_{405} values were determined for each dilution and plotted against the serum dilutions (41). Results indicated that serum dilutions of 1:4,000 were consistently in the titratable range regardless of whether the sera had high or low antibodies to *P. multocida.* Primary antisera were subsequently tested in triplicate

at a 1:4,000 dilution in PBS containing 1% BSA and 0.5% polyoxyethylene sorbitan monolaurate (Tween 20; Sigma Co., St. Louis, MO). The extent of antibody binding to OMP preparations was detected using a 1:400 dilution of alkaline phosphatase-conjugated, affinity-purified goat anti-mouse IgG. Antibody responses were based on a colorimetric response using p-nitrophenyl phosphate substrate (Sigma Fast™; Sigma Chemical Co., St. Louis, MO) and expressed as OD_{405}. All sera were assayed at one time. A control high responder serum and a low responder serum were present on each microtiter plate, and minor plate-to-plate variation adjusted as previously described (69).

Production of polyclonal antibodies

Polyclonal antibodies (pAbs) for OMPs of *P. multocida* strain 232 were produced in CD-1 mice by administering 40 µl subcutaneous inoculations of 10, 25, 50 or 100 µg of OMP-enriched fraction admixed with an equal volume of adjuvant (TiterMax®; CytRx Co., Norcross, GA) on days 0 and 14. On day 28, the mice were deeply anesthetized with methoxyflurane (Metofane®; Schering-Plough Corp., Union, NJ) and exsanguinated. The sera collected and stored at -70°C.

The Hybridoma Laboratory at Oklahoma State University produced Omp28-specific pAbs using a hyperimmunized mouse ascites model (14). Briefly, *P. multocida* OMP-enriched fraction was separated on 10% SDS-PAGE. The Omp28 band was identified with an aqueous Coomassie stain, excised from the gel, and the protein eluted using a buffer composed of 50mM ammonium bicarbonate and 0.1% SDS. Balb/C mice were subcutaneously vaccinated with 50 µg of the eluted Omp28 preparation on days 0, 14, 21, and 37. On day 37 the mice were intraperitoneally injected with 10^6 T-180

sarcoma cells (ATCC TIB-66) to induce antibody-rich ascites. The ascites was collected and stored at -70°C.

Omp28 surface exposure determination

Surface exposure of Omp28 from the outer bacterial membrane was examined by two different methods. The first method examined immunoblotted intact *P. multocida* after protease treatment of accessible epitopes. The immunoblots were then probed with Omp28 mouse ascites pAbs as previously described Pandher et al. (52).

Surface exposure was also examined by immunogold labeling. A 10 µl aliquot of mid-log phase (OD_{650} = 0.60 – 0.65) *P. multocida* grown in BHI broth was sedimented by centrifugation and washed once with 500 µl of PBS. After washing, the bacteria were suspended in either 500 µl of PBS or hyaluronidase 200 IU/ml (Sigma Chemical Co., St. Louis, MO) at 37°C for 1 hour with gentle rocking to maximize surface exposure of OMPs by capsule digestion. The bacteria were then washed in 500 µl of 1.0% bovine serum albumin in PBS (BSA-PBS). The outer surface was probed by suspending the bacteria in 200 µl of Omp28 pAbs diluted 1:40 in BSA-PBS, OMPs pAbs (1:40), or BSA-PBS for 2 hours at room temperature with gentle rocking. Following the incubation, the bacteria were washed 3 times with BSA-PBS and incubated in 200 µl of goat anti-mouse IgG conjugated with gold particles (1:5) (Electron Microscopy Sciences, Ft. Washington, PA). The bacteria were then washed 6 times in BSA-PBS, fixed in 2% glutaraldehyde for 5 minutes and then washed 2 times in ddH_2O. Five microliter aliquots were placed on Formvar (Electron Microscopy Sciences, Ft. Washington, PA) coated

electron microscopy grids and allowed to air dry. The bacteria were then examined using transmission electron microscopy (JEM-100CXII; JOEL Ltd., Akishima, Japan).

Porin activity determination

Membranes were made from 1% lipid (comprising 0.2% diphytanoyl phosphatidyl glycerol and 0.8% diphytanoyl phosphatidyl choline) in n-decane. Bilayers were painted across a 2 mm^2 hole in a Teflon divider separating two compartments containing 5.0 – 6.0 ml each of a bathing solution of 1.0 M KCl. At this time the putative porin protein (1 nmol or less in 1% Triton X-100) was then added to the aqueous phase bathing the lipid membrane. Voltages were applied across this membrane through Calomel electrodes connected by a salt bridge and the resultant current boosted 109 to 1010-fold by a current amplifier, monitored on Tektronix model 7633 oscilloscope and recorded on Rikadenki R-01 strip chart recorder (5).

Complement-mediated killing assay (CMK)

The CMK assay was modified from a previously published protocol by Wijewardana et al. (71, 72). Serum was used from a colostrum-deprived neonatal calf with a low antibody titer against *P. multocida*, as determined by ELISA and Western blot analysis, for a complement source. To assay CMK, sample mouse sera (12 µl), PBS (43 µl), and approximately 550 CFU of logarithmic phase *P. multocida* (5 hour broth culture) were added in duplicate wells in sterile tissue culture microtitre plates (Elkay Products, Inc., Shrewsbury, MA). Forty microliters of the complement source were added to each replicate, except for internal controls, which lacked either a complement or antibody

source. Each plate was sealed with an adhesive plastic film (Seal Plate™; ISC BioExpress, Kaysville, UT) and incubated at 37°C with gentle rocking for 45 minutes. At the end of the time period, using a modified Miles-Misra technique (45), six 10µl aliquots were removed from each well and spotted on BHI agar plates. The average CFU were determined for each sample after 12 hours of growth at 37°C in a 5% CO_2 environment.

Chromatography

All buffers were filter sterilized and degassed prior to use. Detergent-extracted fractions of *P. multocida* OMP were analyzed by Western blot analysis and N-terminal animo acid sequence analysis.

Zfr3 was loaded onto an anion exchange chromatography (High-Q chromatography column (5.0 ml); Bio-Rad laboratories, Richmond, CA) previously equilibrated with buffer A (0.1 % Zwittergent 3-14, 10% methanol, and 20 mM MOPS at a pH 7.3). Proteins were eluted by applying a 0 – 100% gradient of buffer B (0.1 % Zwittergent 3-14, 10% methanol, 20 mM MOPS, and 1.0 M NaCl at a pH 7.3). Fractions containing Omp28 were collected and stored at -70°C.

Pooled anion-exchange fractions were concentrated using centrifugal concentrators (Centricon®; Millipore Corp., Bedford, MA). The concentrate was then loaded onto a gel filtration chromatography column (Superdex 75 HR; Amersham Pharmacia Biotech Co., Piscataway, NJ) previously equilibrated with buffer B (see anion-

exchange chromatography), and the protein eluted at 0.5ml/min. A UV detector at 290nm detected protein-containing fractions; the appropriate fractions were collected and stored at -70°C.

N-terminal amino acid sequencing

Purified Omp28 was subjected to SDS-PAGE. The protein was transferred onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Richmond, CA), Coomassie brilliant blue stained, and the amino-terminal end sequenced as described previously. (13).

Vaccination and Challenge

To determine the lethal dose 50% (LD_{50}) for *P. multocida* challenge, 60 CD-1 female mice were randomly divided among equal groups and each group was intraperitoneally inoculated with 100 μl of a 10-fold dilution of washed *P. multocida* or PBS. The cumulative mouse death was recorded for 5 days after challenge, and the LD_{50} calculated by the method of Reed-Muench (39).

To study the immunogenicity of Omp28, 108 female CD-1 mice were randomly divided among 6 groups of 18 mice each (Table 2). On day 0, four mice from each group were deeply anesthetized with methoxyflurane and exsanguinated. Pre-vaccination sera were collected and stored at -70°C. Subsequently, mice were subcutaneously vaccinated on days 0 and 14 with 40 μl of PBS, PBS with adjuvant (TiterMax®; CytRx Co., Norcross, GA), 75μg of purified Omp28 with adjuvant, 75μg of *P. multocida* strain 232

OMP-enriched fraction, or 75μg of formalin killed *P. multocida* strain 232 whole cells. As a known positive control, a commercial vaccine (Presponse HM®; Fort Dodge Animal Health, Fort Dodge, IA) was administered intraperitoneally at 1/20 the stated cattle dose as recommended by the U.S. Department of Agriculture, Biological Division for *P. multocida* vaccine testing (10). On day 28, four mice from each group were deeply anesthetized with methoxyflurane and exsanguinated. Post-vaccination sera were collected and stored at -70°C. The remaining mice were challenged with a 100 μl intraperitoneal injection of live *P. multocida* (1 x 10^8 CFU/ml) and monitored for death for 96 hours.

Statistical analyses

Mean antibody responses among the various vaccine groups were analyzed by a one-way analysis of variance and Tukey HSD post-hoc multiple comparisons. Antibody responses between days 0 and 28 were compared for each group by paired *t* tests. Survival rates were compared among groups by chi square analysis (2).

RESULTS

Extraction and purification of Omp28

Different combinations of detergents were tested for selective isolation of Omp28 from the OMP-enriched fraction. Attempts to solubilize and extract Omp28 with OPOE detergent were unsuccessful (data not shown). Using the Zwittergent 3-14 procedure, Omp28 was present in each fraction and in the remaining pellet as observed on SDS-

PAGE and confirmed by Western blot analysis (Fig. 1); Omp28 was most prevalent in Zfr3. Heat-modifiability was demonstrated for Omp28 as it migrated from 28 kDa to 35 kDA when incubated at 37°C and 100°C, respectively (Fig. 1).

Even though Zfr3 was Omp28 enriched, further purification was needed to remove several contaminating proteins. The solubilized proteins were fully bound to the anion exchange column at pH 7.3 and could be eluted with a linear gradient of elution buffer (Fig 2). Densitometric analysis of the pooled Omp28–rich fractions separated on SDS-PAGE and then stained with Coomassie brilliant blue revealed 96.6% purity (data not shown). Additional separation of proteins was achieved by gel filtration chromatography, which revealed 3 absorbance peaks. The shaded area under the elution profile contained Omp28 (Fig. 3). Western blot analysis of the pooled Omp28 fractions probed with either hyperimmune sera from a calf vaccinated with *P. multocida* or from Omp28 ascites pAbs, identified two protein bands corresponding to 28 and 35 kDa (Fig. 4).

Omp28 surface exposure

Surface exposure of Omp28 from the outer bacterial membrane was examined by two different methods. Immunoblot analysis of intact *P. multocida* following chymotrypsin treatments resulted in inconsistent results when probed with Omp28 ascites pAbs. Also, Omp28 did not appear to be susceptible to trypsin and *Staphylococcus* V8 protease (data not shown). Immunogold labeling of intact encapsulated and hyaluronidase decapsulated bacteria using Omp28 pAbs also failed to demonstrate Omp28 surface epitopes (Fig. 5).

Omp28 porin activity

The ability of Omp28 to reconstitute channels in membranes was tested by the planar bilayer method, which has been successfully used to determine large numbers of non-specific, specific, and gated porin proteins from Gram-negative bacteria (18, 38). Control proteins (e.g. *P. aeruginosa* OprD and OprM), formed channels, but no porin activity was observed from the Omp28 preparation.

Mouse vaccination and challenge

Vaccination with Omp28, OMP-enriched fraction, and commercial vaccine resulted in a significantly ($P<0.05$) higher mean antibody response between days 0 and 28 when compared to the responses of the other treatment groups. Antibody responses to the Omp28 and OMP-enriched groups were not significantly different ($P>0.05$) from each other (Table 1). Mean antibody responses in formalin-killed whole cells vaccinates was not significantly different from responses for control groups. Vaccination with OMPs, formalin-killed whole cells, and the commercial vaccine resulted in significant protection ($P<0.05$) from challenge. Protection did not occur in control and Omp28 groups; all animals in these groups died within 52 hours following challenge.

Anti-Omp28 antibodies in complement-mediated killing of *P. multocida*

We examined the contribution of anti-Omp28 antibodies in CMK of *P. multocida*. As shown in Fig. 6, significant ($P<0.05$) CMK was achieved from day 28-sera from the OMP-enriched and formalin-killed whole bacteria vaccinates with 0% bacteria

survivability. CMK activity of sera from the Omp28 and commercial vaccine groups resulted in 95.3% and 59.3% bacteria survivability, respectively, and were not significantly different ($P>0.05$) than that for the control group.

DISCUSSION

With bacterial relatedness and structural similarities between Omp28 from *P. multocida* and the PomA from *Mannheimia haemolytica* (formerly, *Pasteurella haemolytica*), we initially attempted to extract Omp28 using OPOE detergent fractionation, as previously described by Mahasreshti et al. for *M. haemolytica* (38). After several unsuccessful attempts, OPOE detergent was abandoned in favor of Zwittergent 3-14, which was successful in extracting Omp28. Purified Omp28 N-terminal amino acid sequence was examined and found to be identical with the sequence previously reported by Dabo et al. (13). Dabo revealed that the partial Omp28 N-terminal amino acid sequence had 80% homology to the OmpA porin protein of *E. coli* and 60% homology to the OmpA-family proteins from *Salmonella typhimurium* and *Shigella dysentery.* Repeated evaluation of Omp28 in a lipid-bilayer assay did not demonstrate porin activity. It is possible that purification conditions were too harsh, resulting in partial denaturation of Omp28 with concurrent loss of porin activity. However, Zwittergent 3-14 is a mild non-denaturing detergent that has been shown to preserve isolated cell-bound receptor conformation (73), native epitopes from isolated P2 protein of *Haemophilus influenza* (47), and allow for reconstitution of native epitopes in outer membrane proteins of

Bacillus subtilis after SDS detergent isolation (23). Porin activity is further in doubt based on the lack of significant, if any, surface exposure.

Studies on surface exposure indicate that Omp28 is not surface exposed. The results contradict the report by Dabo et al. (13) in this laboratory, but are in agreement with a report by Marandi and Mittal (42) in immunoblot analysis of protease-treated intact *P. multocida* probed with hyperimmune calf sera and immunogold labeling with monoclonal antibody, MT4.1, specific for Omp28, respectively. Differences amongst these studies are likely due to methodology and/or sensitivity in assay systems. Monoclonal antibody directed epitopes might be buried in the membrane. In addition, hydrophobicity plots of the recently cloned Omp28 gene predict minimal surface exposure (Dabo et al., unpublished data).

Vaccination of mice with Omp28 stimulated a significantly higher ($P<0.05$) antibody response as compared to formalin-killed whole cells. (Table 1). However, Omp28 was neither protective from an intraperitoneal challenge with *P. multocida* nor was it able to activate complement. The strong, but ineffectual host antibody response to Omp28 was likely generated by epitopes normally buried within the bacterial outer membrane, or from the exposure of hidden epitopes through partial denaturation of Omp28 during the purification process. The inaccessibility of these epitopes in the intact *P. multocida* would negate any protective properties. Vaccination with the OMP-enriched fraction, formalin-killed whole bacteria, and the commercial vaccine were able to stimulate significant ($P<0.05$) protection to challenge, but only OMP-enriched fraction and commercial vaccine vaccinates had significant ($P<0.05$) antibody responses (Table 1).

Several studies have successfully used antibodies against *P. multocida,* in ELISA, as a predictor of protection against challenge. These studies involved cattle (12), buffalo (8), rabbits (29, 34), chickens (3, 20) and mice (53, 71). In the present study, high antibody responses to *P. multocida* proteins, in ELISA, were found in groups of mice that were good predictor of protection against challenge except for Omp28. Confer et al. revealed that high antibody response to Omp28 consistently correlated with resistance to challenge with virulent *P. multocida (12).* However, as observed in this study, a strong antibody response alone is not always a good predictor of protection. In fact, several studies have identified bacterial epitopes that are detrimental to the host defenses (21, 66).

In conclusion, although Omp28 is a major immunogenic OMP from *P. multocida* A:3, antibodies to it do not appear to be protective. This could be due to its lack of adequate surface exposure.

Fig. 1. SDS-PAGE (10% acrylamide gel, silver stain) showing the Zwittergent 3-14 fractions containing soluble outer membrane protein of *P. multocida*. Lanes 1- 4 correspond to Zwittergent detergent fractions. Lane 5 corresponds to the protein pellet remaining after the extraction procedure. Arrows indicate higher molecular weight migration of the 28kDa band to 35kDa when solubilized at 100°C in loading buffer.

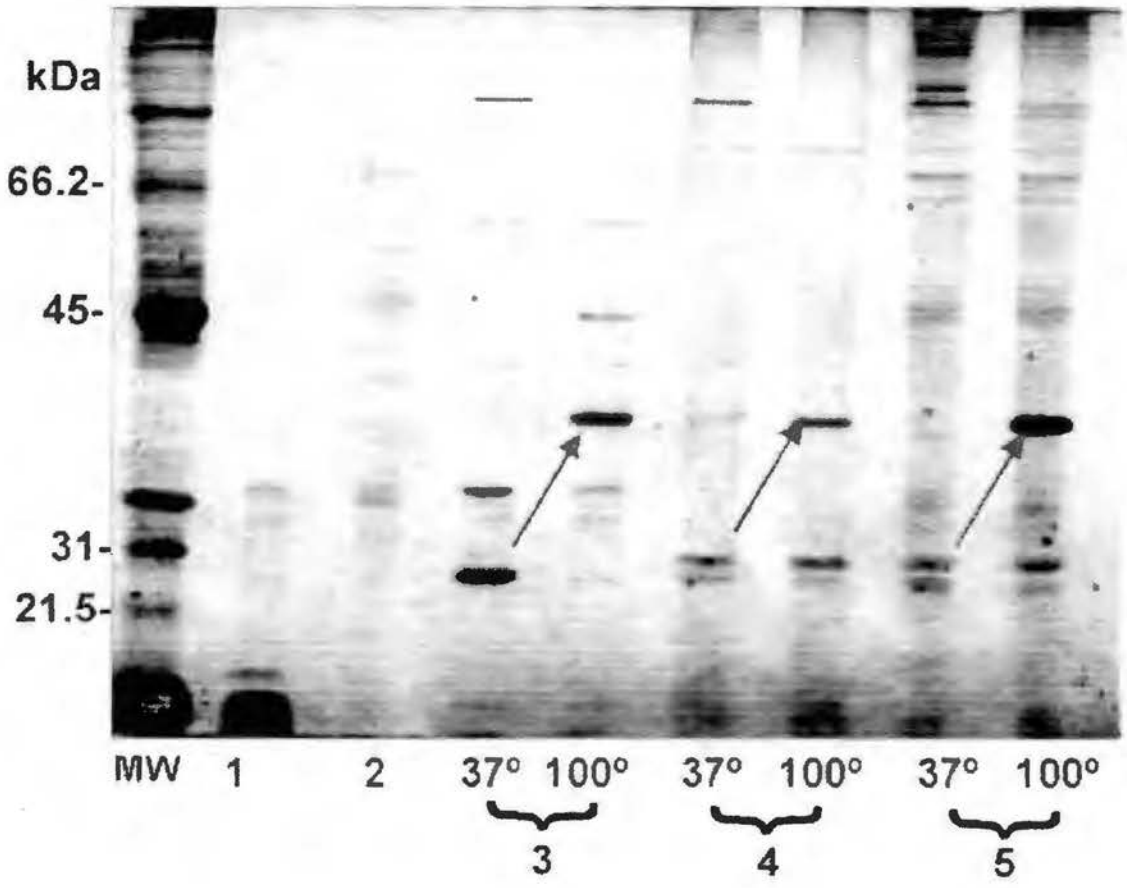

kDa
66.2-
45-
31-
21.5-
MW
1
2
37°
100°
37°
100°
37°
100°
3
4
5

Fig. 2. Anion exchange chromatography elution profile. The shaded area under the profile corresponds to Omp28 containing fractions.

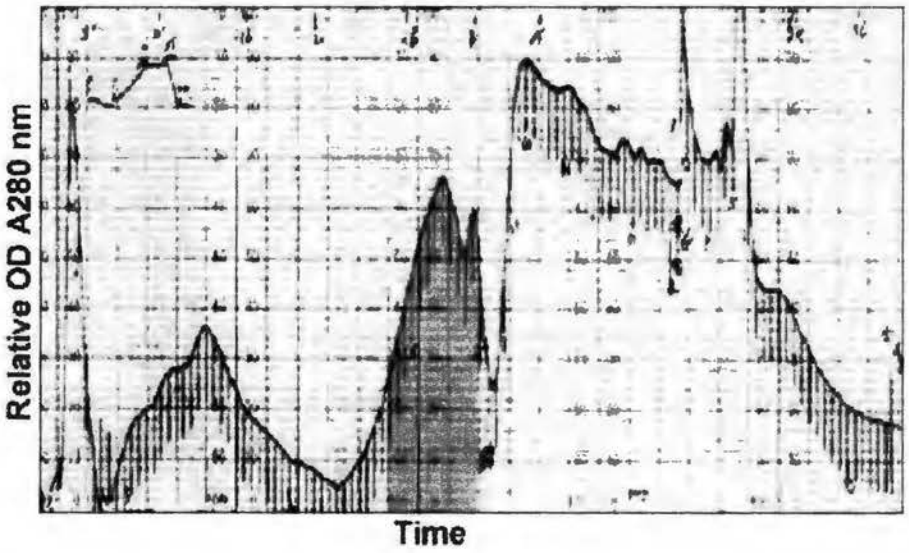

Relative OD A280 nm
Time

Fig. 3. Gel filtration chromatography elution profile. The area shaded under the profile corresponds to fractions containing OMP28. Fractions were pooled, concentrated and analyzed for protein composition, as described in Material and Methods and Fig. 4.

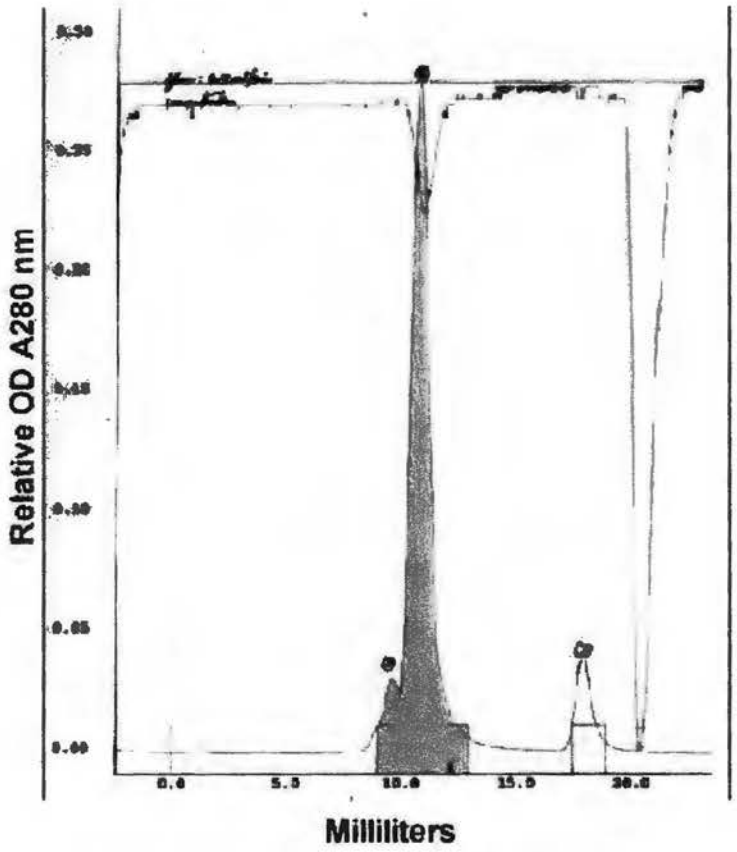

Relative OD A280 nm
0.0
5.0
10.0
15.0
20.0
Milliliters

Fig. 4. Immunoblot analysis of pooled Omp28 containing fractions from gel filtration chromatography. The blot was probed with bovine sera hyperimmunized with *P. multocida* 232.


kDa
35
28

Fig. 5. Immunogold labeling of *P. multocida* 232. (A) Immunogold labeling of hyaluronidase decapsulated bacteria probed with polyclonal mouse sera from mice hyperimmunized with Sarkosyl extracted OMPs from *P. multocida 232.* Note the widespread surface labeling (arrow head). (B) Hyaluronidase decapsulated bacteria probed with polyclonal OMP28 antibodies. Note the lack of surface labeling

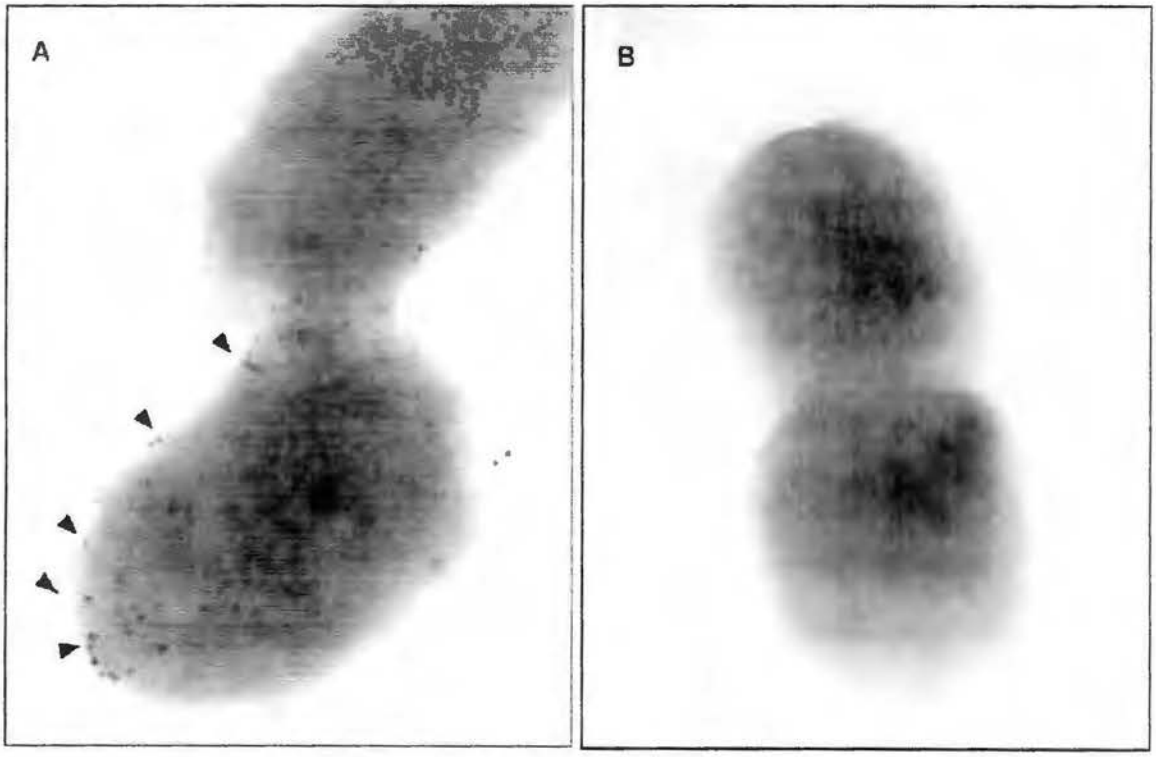
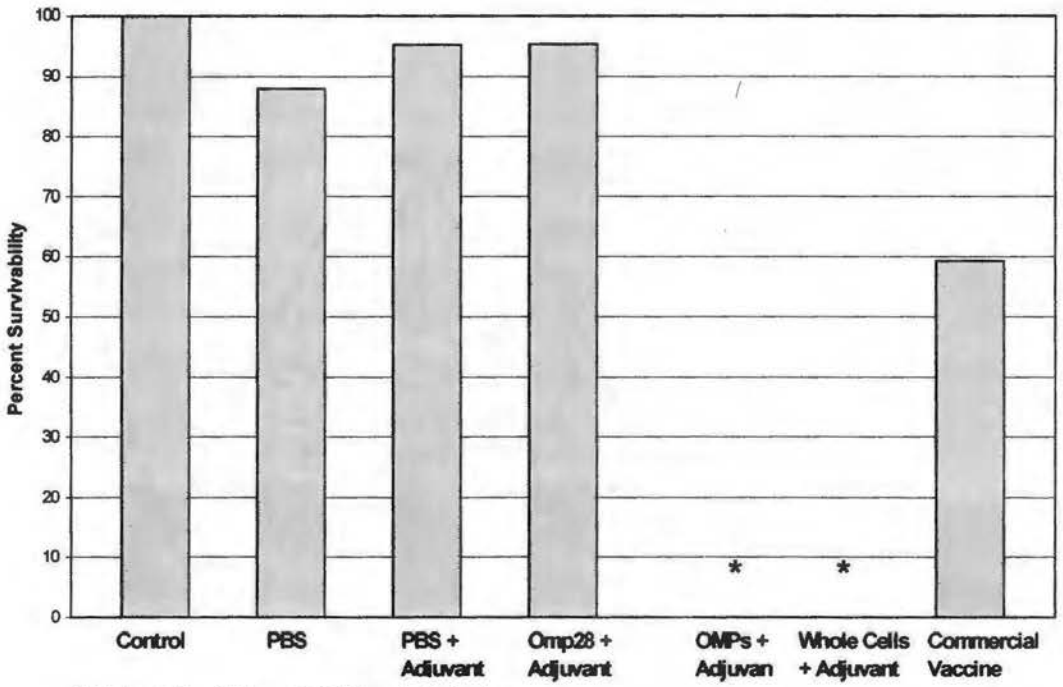

A
B

Fig 6. Complement-mediated killing of *Pasteurella multocida* by post-vaccination sera from CD-1 mice.



* indicates significant difference (P<0.05) from other treatment groups
Percent survivability = (treatment group mean / control group mean) x 100
Adjuvant is TiterMaxÒ; CytRx Co., Norcross, GA
Commercial vaccine is Presponse HMÒ; Fort Dodge Animal Health, Fort Dodge, IA
OMPs are outer membrane proteins of P. multocida 232

Table 1. Evaluation of CD-1 vaccinated mice from *Pasteurella multocida* challenge and corresponding antibody responses to *Pasteurella multocida* outer membrane proteins as measured by ELISA.

Experimental Group	Immunization	Route of immunization	Antibody Response (OD_{650}) to *P. multocida* 232 OMPs		No. of dead/no. challenged
			Day 0	Day 28	
1	PBS	subcutaneous	0.000 ± 0.000^{a}	0.010 ± 0.004^{a}	$10/10^{a}$
2	PBS + adjuvant	subcutaneous	0.008 ± 0.016^{a}	$0.0135 \pm 0.006^{a, b}$	$10/10^{a}$
3	Omp28 + adjuvant	subcutaneous	0.000 ± 0.000^{a}	0.386 ± 0.128^{c}	$10/10^{a}$
4	OMPs + adjuvant	subcutaneous	0.007 ± 0.012^{a}	0.475 ± 0.155^{c}	$1/10^{b}$
5	formalin-killed *P. multocida* 232 + adjuvant	subcutaneous	0.001 ± 0.001^{a}	$0.121 \pm 0.124^{a, b, d}$	$3/10^{b}$
6	Commercial vaccine	intraperitoneal	0.000 ± 0.000^{a}	$0.201 \pm 0.109^{a, b, c, d}$	$2/10^{b}$

Adjuvant is TiterMax®; CytRx Co., Norcross, GA
Commercial vaccine is Presponse HM®; Fort Dodge Animal Health, Fort Dodge, IA
OMPs are outer membrane proteins of *P. multocida* 232
$^{a, b, c, d}$ Differences in superscript letters designates non-relational treatment groups ($P < 0.5$)

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CHAPTER IV

THE PATHOLOGICAL AND BACTERIOLOGICAL CHARACTERIZATION OF SCID-BO AND SCID-BG MICE AFTER INTRATRACHEAL CHALLENGE WITH *MANNHEIMIA HAEMOLYTICA* SEROTYPE 1

ABSTRACT

SCID-beige (SCID-bg) mice engrafted with bovine fetal hematolymphoid tissues (SCID-bo) were evaluated histologically and bacteriologically for use in the study of bovine pneumonic pasteurellosis. In experiment A, SCID-bo and SCID-bg mice were intratracheally challenged with *Mannheimia haemolytica* leukotoxin + (Lkt) wild-type, *M. haemolytica* Lkt–, *M. haemolytica* Oklahoma strain, or phosphate buffered saline solution (PBSS) and sacrificed at variable times post-challenge. In experiment B, SCID-bg mice were intratracheally challenged with live or formalin-killed *M. haemolytica* Lkt+ WT and then sacrificed at 24, 48 and 96 hours. In these experiments, SCID-bo and SCID-bg mice developed pneumonia with similar lung lesion scores between the various treatment groups and experimental studies. In addition, mice in experiments A and B were able to partially or fully eliminate bacteria from the lung. Histologically, lung lesions were dissimilar with respect to those in cattle naturally infected with *M. haemolytica.* These results suggest that the SCID-bo mouse would not be a suitable model for bovine pneumonic pasteurellosis.

INTRODUCTION

Bovine respiratory disease complex (BRD) has a substantial negative impact on the health of cattle, and in the economics of cattle production. A recent study by Bowland and Shewen estimates the financial burden to the cattle industry in the United States to be 600 million dollars per annum.[1] In the order of prevalence, bacterial isolates from BRD infected animals are *Mannheimia haemolytica* serotype 1 (formerly, *Pasteurella haemolytica* A:1), *Pasteurella multocida* A:3, and *Haemophilus somnus.* [2] It is generally accepted that BRD results from the interaction of stressful management practices [3,4] and/or concurrent viral [5-12] or bacterial infection [2,13-15] which through direct damage to the respiratory tract or immunosuppression, allows colonization of the lower respiratory tract by one of the etiologic agents. Infection frequently culminates in a severe fibrinous pleuropneumonia.

Among the several virulence factors identified in *M. haemolytica* is a cytotoxic exotoxin specific for bovine leukocytes [16-20] and platelets. [21] The leukotoxin (Lkt) is a member of the calcium-dependent, RTX (Repeats in ToXin) bacterial protein family produced by several genera of Gram-negative bacteria.[22] Other members of this family of toxins include *Escherichia coli* hemolysin (HlyA) [23,24] and enterohemorrhagic toxin (Ehx) [25], *Actinobacillus pleuropneumoniae* hemolysin (Apx)[26], *Actinobacillus actinomycetemcomitans* leukotoxin (Aalt)[27], *Actinobacillus suis* (AshA)[28], *Moraxella bovis* hemolysin [29] and *Bordetella pertussis* adenylate cyclase/haemolysin (Cya).[30] Lkt, like other RTX members, form transmembrane pores in target cells with subsequent osmotic cell swelling and eventual lysis. As a result of Lkt-leukocyte interation several

eicosanoid mediators, such as leukotriene B_4, prostaglandin E_2 and $F_{1\alpha}$, and thromboxane B_2, are formed through the activation of phospholipase A_2 that results in amplification of the inflammatory response. [31-33] Eventual inflammatory cell lysis causes the release of several lysosomal proteolytic enzymes, reactive oxygen intermediates and fibrinogen, which enhance tissue damage. [34,35] Several studies have revealed a correlation between morbidity and mortality in cattle and Lkt activity or Lkt neutralizing antibodies. [36-38]

Many variations in animal models and experimental designs have been used to characterize the various facets of the host immune response, pathogenesis, and efficacy of immunization to *M. haemolytica*. Each model has certain limitations with respect to its comparison to the natural bovine pneumonic pasteurellosis. Most often cattle models have been favored over goat [39], sheep [40-43], mouse [44-47] and rabbit [43,48,49] models due to Lkt's specificity for bovine leukocytes and platelets. However, cattle models can produce highly variable results due to intrinsic factors such as, a nonuniform genetic background, uncertain health status, the lack of bovine-specific diagnostic reagents, commensal infection or prior naturally acquired disease due to *M. haemolytica,* and high cost and low numbers of animal that can be used in an experiment. To attempt to overcome some of the problems associated with cattle infectious disease research, a xenochimeric mouse model possessing both bovine humoral and cellular immune responses has been developed [50] and marketed (SCID-bovi™; BioNova, Columbia, MO). The model exploits two recessive genetic mutations, the Severe Combined Immune Deficiency (SCID) and beige (bg) mutations. The *scid/scid* mutation causes impaired recombination of the antigen receptor genes with arrest of T and B-cell maturation. [51] Through an undefined mechanism, the *bg/bg* mutation causes a complete functional impairment of the natural

killer (NK) cell lineage [52-54], decreased macrophage inflammatory protein-1β (MIP-1β) and MIP-2 production [55], impaired lysosomal fusion in leukocytes [56], decreased hydroxide formation and myeloperoxidase degranulation [57], and a decrease in neutrophil serine proteases elastase and cathepsin. [58-60] Engraftment of mice having both genetic mutations, *scid/scid:bg/bg* (SCID-bg), with bovine hematolymphoid tissue (SCID-bo) allows for the establishment of a long-term and functional bovine immune system. [50,61]

In these studies, we histologically and microbiologically evaluated the SCID-bo and SCID-bg mouse after intratracheal inoculation with two live wild-type (WT) strains, a formalin-killed strain and a leukotoxin (Lkt)-deficient strain of *M. haemolytica.* [62]

MATERIALS AND METHODS

Animals. A total of 54, 25 day-old, female SCID-beige mice were obtained from Charles River Laboratory (Wilmington, MA). The mice were maintained in sterile microisolator cages with polycarbonate HEPE-filtered tops and manipulations were performed under a laminar hood. The animal room was kept at 22 C, with a 12/12-hour light/dark cycle and ambient humidity. All animals were given autoclaved water and diet (Purina Laboratory Rodent Chow 5010; Ralston Purina Company, St. Louis, MO), and a sulfamethoxazole/trimethoprim suspension (200 mg sulfamethoxazole and 40 mg trimethoprim per 320 ml of water) was added to the drinking water on alternating days prior to surgery. After a two-week acclimation, mice were engrafted with bovine fetal liver, lymph node, and thymus as previously described.[50] Previous studies by studies by

Denny et al.[50] and Smith et al.[61] have extensively characterized SCID-bo reconstitution. Subjective evaluation of reconstitution could be judged by an increase in splenic periarterial lymphoid sheaths (personal communication with DM Estes, Columbia, MO). In our studies, SCID-bo reconstitution was subjectively evaluated by histologically comparing splenic lymphoid architecture to age matched SCID-bg mice. The Institutional Animal Care and Use Committee at the University of Missouri, Columbia, approved the protocol.

Bacterial culture and quantification: *M. haemolytica* Oklahoma Strain (OK WT), *M. haemolytica* 89010807N wild-type (Leukotoxin $^{+}$ ($LktA^{+}$) WT), and *M. haemolytica* 89010807N lkt- (an $LktA^{-}$ isogenic mutant of strain 8901807N) frozen stock cultures were thawed, plated onto brain-heart infusion (BHI; Becton Dickinson, Sparks, MD) agar plates containing nalidixic acid and grown at 37°C in a 5% CO_2 environment for 18 hours. An isolated colony from each was propagated in 10 ml BHI-nalidixic acid broth with rotatory shaking at 120 oscillations/min. for 18 hours at 37°C. For each organism, a 1.0 ml aliquot of the broth culture was used to inoculate 100 ml of BHI-nalidixic acid broth in a 250 ml Erlenmeyer flask and incubated with rotatory shaking at 250 oscillations/min at 37°C to an optical density of A_{600}= 0.65. The bacteria were sedimented by centrifugation at 6000 x *g* for 15 minutes, washed in 125 ml sterile phosphate buffered saline solution (PBSS) and re-centrifuged as above 6000 x *g* for 15 minutes. The bacteria were re-suspended in PBSS and adjusted spectrophotometrically to

a final concentration of 1.0 x 10^9 CFU/ml. Actual CFU/ml were determined on each culture, using a modified Miles-Misra spot-plate counting technique. [63]

Formalin-killed bacteria were prepared by suspending washed, concentrated bacteria in 0.3% formalized PBSS for 48 hours at 4°C. Prior to use, the bacteria were washed 3 times in PBSS as previously described.[64]

Determination of lung, liver and spleen tissue bacteria counts per gram was accomplished by first weighing and homogenizing selected tissues (Kontes Pellet Pestle; Kontes Glass Co., Vineland, NJ) in PBSS. Actual CFU/gm of lung were then determined on the homogenate, using the spot-plate counting technique.

Experimental design. Two experiments were conducted (Table 1). Experiment A consisted of 5 groups of mice that were each administered 25 µl intratracheal challenge and then sacrificed at variable times post-challenge. Group 1 (negative control) consisted of 4 SCID-bo mice that were intratracheally challenged with PBSS. Group 2 was composed of 4 SCID-bg (non-engrafted) mice that were intratracheally challenged with 6.5 x 10^7 CFU of *M. haemolytica* WT. Group 3 was composed of 10 SCID-bo mice that were intratracheally challenged with 6.5 x 10^7 CFU *M. haemolytica* Lkt–. Groups 4 and 5 each consisted of 8 SCID-bo mice that were intratracheally challenged with 6.5 x 10^7 CFU *M. haemolytica* Lkt+ WT, and 1.5 x 10^8 *M. haemolytica* OK, respectively.

Experiment B was composed of 4 groups, each containing 5 SCID-bg mice. The mice were each administered 25 µl intratracheal challenge consisting of 1.0 x 10^8 CFU of live or formalin-killed *M. haemolytica* Lkt+ WT and then sacrificed at 24, 48 and 96 hours.

Challenge was done after a recuperation period of 4-weeks from the engraftment procedure. The mice were anesthetized (Metofane; Pitman-Moore, Mundelein, IL), and the trachea surgically isolated through a ventral midline cervical incision. The bacterial challenge (25 μl) was instilled directly into the tracheal lumen by 25 g needle, and the skin edges apposed with surgical adhesive (Nexaban™; Veterinary Products Laboratory, Phoenix, AZ). The mice were sacrificed by cervical dislocation at times indicated by experimental protocol or when humanely necessary. The thorax was immediately opened using sterile technique. The bronchus of the right cranial lung lobe was ligated with a surgical hemostasis clip (Hemoclip; Edward Weck, Inc., Research Triangle Park, NC), transected distal to the hemostasis clip, weighed and frozen at 0° C for future bacterial culture. The remaining lung lobes were insufflated with 2% glutaraldehyde or modified 2% glutaraldehyde (Karnovsky's fixative) to a pressure equivalent to a column fluid 10 cm in height from the thoracic inlet by an endotracheal cannula. Upon full insufflation, the trachea was ligated, and the lungs removed and placed in 2% glutaraldehyde for 24 hours. After a 24hr period the tissue was transferred to 10% sucrose.

Histologic examination. After complete fixation, a sagittal section of the left lung lobe, transverse section of the right caudal lung lobe and spleen were processed by routine histologic methods, sectioned at 6.0μm, and stained with hematoxylin and eosin. For each histologic section, a digital image was captured using a video microscope (Nikon Microscope, Alphaphot-2 VS2; Japan and Hitachi Color Video Camera; Japan), and the image analyzed with the assistance of computer-aided software (Image-Pro® Plus, Version 1.1; Media Cybernetics, Silver Spring, MD). Each section was graded 0 to 15 by

a blinded investigator (NTG or AWC) based on percentage of lung lobe affected and intensity of the lesion. The two scores were added for a total mouse score with higher scores indicating the more severe lesions (Table 2).

Transmission electron microscopy. After fixation, the lung tissue was washed 3 times in 0.2 M Sodium cacodylate buffer (pH 7.4) supplemented with 0.2 M sucrose and postfixed in 1% osmium tetroxide 0.27 M cacodylate buffer for 1 hr. The tissues were washed twice as previously described, and dehydrated in a graded series of ethanol. [65] The tissues were then transferred to propylene oxide for 30 minutes and embedded in epoxy resin (Polysciences, Inc., Warrington, PA). The embedded tissue was sectioned at 70 to 90 nm, placed on copper grids, and stained with 5% uranyl acetate and lead citrate. After staining, the sections were examined by transmission electron microscopy (TEM) (JEM-100CXII; JOEL Ltd., Akishima, Japan).

Statistical Analysis. Comparison of mean lung lesion scores between groups was done using one-way analysis of variance and Tukey-Kramer multiple comparisons test.[66] All statistical analyses were performed using computer-based statistical software (InStat Software; GraphPad Software, Inc., San Diego, CA).

RESULTS

Hematopoietic reconstitution. The spleens from SCID-bo mice were larger when compared to those of SCID-bg mice. Histologically, SCID-bo mouse spleens were characterized as having a white pulp composed of thick periarterial lymphoid sheaths

containing occasional germinal centers. No lymphoid tissue was observed in the spleens of SCID-bg mice. The red pulp from spleens of SCID-bo and SCID-bg mice contained moderate numbers of hemopoietic cells in variable stages of maturation (Fig. 1).

Clinical evaluation. In experiment A both SCID-bg and SCID-bo mice challenged with each of *M. haemolytica* strains became reluctant to eat or drink, often had a hunched stance, and many became moribund within 24 hours post-challenge. One mouse challenged with Lkt+ WT died within 24 after challenge.

In experiment B, a similar clinical response was seen in SCID-bg mice challenged with live or formalin-killed *M. haemolytica*. Four mice challenged with live bacteria died within 48 hours post-challenge; however, the remaining mice improved clinically as judged by increased activity and resumption of eating and drinking. No mice were lost from the formalin-killed group.

Macroscopic pulmonary lesions. In experiment A, lung lesions were observed in all groups challenged with *M. haemolytica*, but not in the PBSS-challenged group. The macroscopic appearance was typified by multifocal, mild to moderate, red mottling of the visceral pleura with occasional concurrent atelectasis. Macroscopic lesions were similar in *M. haemolytica* Lkt+ and *M. haemolytica* Lkt- challenged mice.

SCID-bg mice in experiment B challenged with both the live and formalin-killed *M. haemolytica* had macroscopic lesions similar to those seen in experiment A. By 96 hours after challenge, however there was a decrease in the intensity of atelectasis and mottling (Fig. 2).

Microscopic pulmonary lesions. In experiment A, inflammatory infiltrates were seen in the lungs of all mice challenged with *M. haemolytica*, but not in those challenged with PBSS. However, lesions scores were significantly different ($P<0.05$) for SCID-bo groups challenged with *M. haemolytica* Lkt+ WT compared to the PBSS group and for *M. haemolytica* Lkt- compared to the PBSS group (Table 1). Lesions were characterized by thickened alveolar septa, and partially occluded bronchiolar and alveolar lumina due to mild to marked inflammatory cell infiltrates (Fig. 3). The infiltrates consisted primarily of neutrophils with lesser numbers of macrophages. Occasionally, lungs with higher overall scores contained moderate expansion of the perivascular stroma with a concurrent lymphangiectasia due to edema and mild hemorrhage. Vasculitis and pleuritis were infrequently seen, but when observed were typically associated with severe lung lesion scorres.

In experiment B, macroscopic and microscopic lung lesions, similar to those observed in experiment A, were observed in all challenged groups, including those challenged with the formalin-killed bacteria. There were no significant differences ($P>0.05$) among lesions scores for the various groups.

Transmission electron microscopy. To attempt to determine whether the pneumonic neutrophil infiltrate was of murine and/or bovine origin, TEM was done. TEM was used because of the lack of commercially available bovine-specific anti-neutrophil antibodies from a source other than the mouse. We were unsuccessful, however identifying the unique large, peroxidase negative-granule within bovine neutrophils [67](data not shown).

Bacterial isolation. In experiment A, *M. haemolytica* was frequently isolated from lung, liver and spleen. Higher concentrations were isolated from mice sacrificed at early time points during the study (20.5 hr), but infrequently isolated and at lower concentrations from mice sacrificed 43.5 hours, the longest interval from challenge (Table 1). Regression analyses of tissue bacteria counts over time were not significant ($r = 0.192 - 0.381$; $P>0.05$).

Similar trends were observed in experiment B, however inference could not be drawn due to small population sizes.

DISCUSSION

As previously mentioned, Lkt is a major virulence factor of *M. haemolytica* that is specific for bovine leukocytes and platelets. We hypothesized that hemopoietic reconstitution of SCID-bg mice with bovine cells (SCID-bo) would allow the development of pneumonic lesions to *M. haemolytica* similar to that seen in bovine pneumonic pasteurellosis. In experiment A, we did demonstrate that pneumonic lesions could be induced in SCID-bo mice after intratracheal challenge with *M. haemolytica.* The lesions were characterized as a mild to marked, neutrophilic bronchiolitis and alveolitis with a concurrent interstitial infiltrate. Occasionally, intense lesions were associated with perivascular edema and hemorrhage. SCID-bo mice challenged with live *M. haemolytica* Lkt+ WT or *M. haemolytica* Lkt- had increasing lung lesion scores up to 43 hours post-challenge that were significantly different ($P<0.05$) from scores for the PBSS-challenged

group. However, the similarity in lesions between the Lkt+ and Lkt- inoculated mice leads us to conclude that Lkt was not a factor in inducing pneumonia in the SCID-bo mice

Because lesions in SCID-bg and SCID-bo mice were similar in experiment A, we undertook further characterization of the pathogenesis of the lesion using SCID-bg mice in experiment B. This decision was due to the increased cost associated with SCID-bo mice compared to SCID-bg mice. In experiment B, SCID-bg mice challenged with live or formalin-killed *M. haemolytica* had similar lung lesion characteristics and scores when compared to each other and to those in experiment A. Some similarities were noted with a recent study by Thorn et al [47] using SCID-bg mice intranasally challenged with 50 µl of 2.8×10^6, 3.3×10^9 or 3.4×10^{11} CFU/ml *M. haemolytica* (American Culture Collection 43720). In that study, mice challenged with 3.4×10^{11} CFU/ml produced increasing lung lesion scores 96 hr after challenge, whereas those challenged at lower concentrations had decreasing lung lesion scores similar to that found in our experiment B. Lesions in the present studies were qualitatively different from those described by Thorn et al. due to the presence of concurrent necrosis and fibrin deposition in their study. Our protocol used insufflation to enhance study of the pulmonary architecture, which may inadvertently prevented detection of small amounts of fibrin exudate. In addition, *M. haemolytica* strains vary in pathogenicity. Therefore, bacterial strain variations could have accounted for variation between studies.

In experiment B, the similarity in lung lesion score and character of the infiltrate in live versus killed *M. haemolytica* suggests that another toxic factor such as lipopolysaccharide (LPS, endotoxin) or lipopolysaccharide-associated proteins are most

likely responsible for the inflammatory changes seen. Several studies have shown LPS to be a potent *in vivo* and *in vitro* inflammatory mediator. Asti et al.[68]observed diffuse neutrophilic infiltrates with concurrent hemorrhage in the interstitium and alveolar lumina of CD-1 mice after intratracheal administration of 10 μg of LPS in a volume of 50 μl. From the examination of bronchoalveolar lavage fluid at various time points, the plateau in the neutrophil intensity was between 12 and 24 hours. Whitely et al. [69] induced pulmonary inflammation in cattle by endotracheal instillation of 96 μg or by 2.5 mg of purified LPS into the distal bronchus. The alveolar luminal and septal inflammatory infiltrate consisted of neutrophils as well as hemorrhage and edema.

The character of the lung lesion in SCID-bo and SCID-bg mice markedly differs between that observed in cattle. *M. haemolytica*-induced pneumonia in cattle is typified as a severe necrosuppurative and fibrinous pleuropneumonia. Histologically, the early lesion is that of a fibrinopurulent alveolitis with sparing of the bronchioles. Leukocytes frequently become elongated and coalesce ("Oat cells"). With time large foci of coagulative necrosis with varying degrees of capillary, venous and arterial thrombosis develop. Necrotic foci are often surrounded by a thick band of degenerate and viable neutrophils and macrophages. Bronchioles and alveoli may contain necrotic epithelium and their lumina partially occluded with neutrophils or may remain intact. Interlobular septa are expanded with a similar inflammatory infiltrate and proteinaceous edema with fibrin. Lymphatic vessels are ectatic and often contain fibrin thrombi. Intense lesions are associated with a thick layer of fibrin deposition on the visceral pleural surface. Unfortunately, many of the above histopathologic features of bovine pneumonic pasteurellosis are lacking in the SCID-bo model with the exception of the neutrophil

infiltrate and edema. Therefore, the SCID-bo mouse, as examined in this study does not appear to be an adequate model for bovine pneumonic pasteurellosis.

Table 1. Evaluation of pneumonic lesions and bacteria quantification from SCID-bo and SCID-bg mice intratracheally challenged with *Mannheimia haemolytica.*

Experiment Group	Challenge[1]	No. of mice	Engrafted[2]	Sacrifice[3] (hr)	Mean Lesion Grade (Score ± SD)	CFU/ gram of Tissue (Mean ± SD) Lung	Liver	Spleen
A	PBSS	4	yes	43	0.00 ± 0^{A}	0.00 ± 0	0.00 ± 0	0.00 ± 0
	Lkt+ WT	4	no	23-28	9.50 ± 7.42	$2.43 \times 10^{4} \pm 3.35 \times 10^{4}$	$1.22 \times 10^{2} \pm 2.11 \times 10^{2}$	0.00 ± 0
	Lkt+ WT	10*	yes	24-44	13.67 ± 5.07^{A}	$1.65 \times 10^{8} \pm 4.23 \times 10^{8}$	$3.11 \times 10^{4} \pm 7.00 \times 10^{4}$	$1.43 \times 10^{3} \pm 3.67 \times 10^{3}$
	OK WT	8	yes	20-43	8.50 ± 5.97	$8.80 \times 10^{7} \pm 1.37 \times 10^{8}$	$5.47 \times 10^{6} \pm 1.3 \times 10^{7}$	$3.02 \times 10^{7} \pm 7.93 \times 10^{7}$
	Lkt-	8	yes	24-44	13.00 ± 5.97^{A}	$4.43 \times 10^{6} \pm 1.10 \times 10^{7}$	$5.84 \times 10^{4} \pm 1.32 \times 10^{5}$	$7.51 \times 10^{2} \pm 1.99 \times 10^{3}$
B	Lkt+ WT	5**	no	24	6.00 ± 10.39	$5.43 \times 10^{8} \pm 9.16 \times 10^{8}$	N.D.	N.D.
	Lkt+ WT	5*	no	48	7.75 ± 10.39	$1.40 \times 10^{4} \pm 1.62 \times 10^{4}$	N.D.	N.D.
	Lkt+ WT	5*	no	96	4.75 ± 5.85	$1.53 \times 10^{4} \pm 3.06 \times 10^{4}$	N.D.	N.D.
	Formalin-killed Lkt+ WT	5	no	48	6.40 ± 5.97	0.00 ± 0	N.D.	N.D.

[1] Challenge dose is 25 µl containing 6.5×10^{7} CFU of *M. haemolytica* WT or *M. haemolytica* Lkt-, or 1.5×10^{8} CFU of *M. haemolytica* Oklahoma Strain
[2] Engrafted with 2nd trimester bovine fetal hematolymphoid tissues
[3] Time from challenge to sacrifice in hours
[A] Differences in superscript letters designates non-relational treatment groups ($P < 0.05$)
*Mouse died following challenge
N.D. = not done

Table 2. Histological grading criteria for pneumonic lung lesions in SCID-bo and SCID-bg mice.

Points Available	Grading Criteria
6	**Percentage of lung involvement.** **0**- 0-5% **2**- 6-20% **4**- 21-60% **6**- 61-100%
6	**Inflammatory infiltrate.** **0**- none to a few random alveoli with luminal macrophages +/- rare neutrophils **2**- mild, alveolar and septal neutrophil and macrophage infiltrate (up to 2 cell thick) with few random alveoli with mild luminal inflammatory cell infiltrate. **4**- moderate, multifocal alveolar and septal neutrophil and macrophage infiltrate (2-3 cells thick) with numerous alveoli containing a moderate (occasional small clusters and/or slightly separated inflammatory cells)inflammatory cell infiltrate. **6**- marked, alveolar and septal neutrophil and macrophage infiltrate (septa frequently 3 neutrophils thick) with numerous alveoli containing small clusters inflammatory cells.
2	**Vasculitis** Expansion of the tunica media with concurrent neutrophilic infiltrate within the tunica media and concurrent endothelial swelling. **0**- no vasculitis **1**- thin walled vessels with vasculitis **2**- muscular arteries with vasculitis
1	**Pleuritis** Mesothelial swelling with concurrent neutrophil infiltrate or a subpleural infiltrate. The mesothelial lining at acute angle is not to be used in scoring. **0**- no pleuritis **1**- pleuritis

Fig. 1. Histologic comparison of SCID-bg and SCID-bo splenic architecture. A) Spleen from a SCID-bg mouse. The spleen is small and lacks peri-arterial lymphoid sheaths. B) Spleen from a SCID-bo mouse. The spleen is large and has well defined peri-arterial lymphoid sheaths.

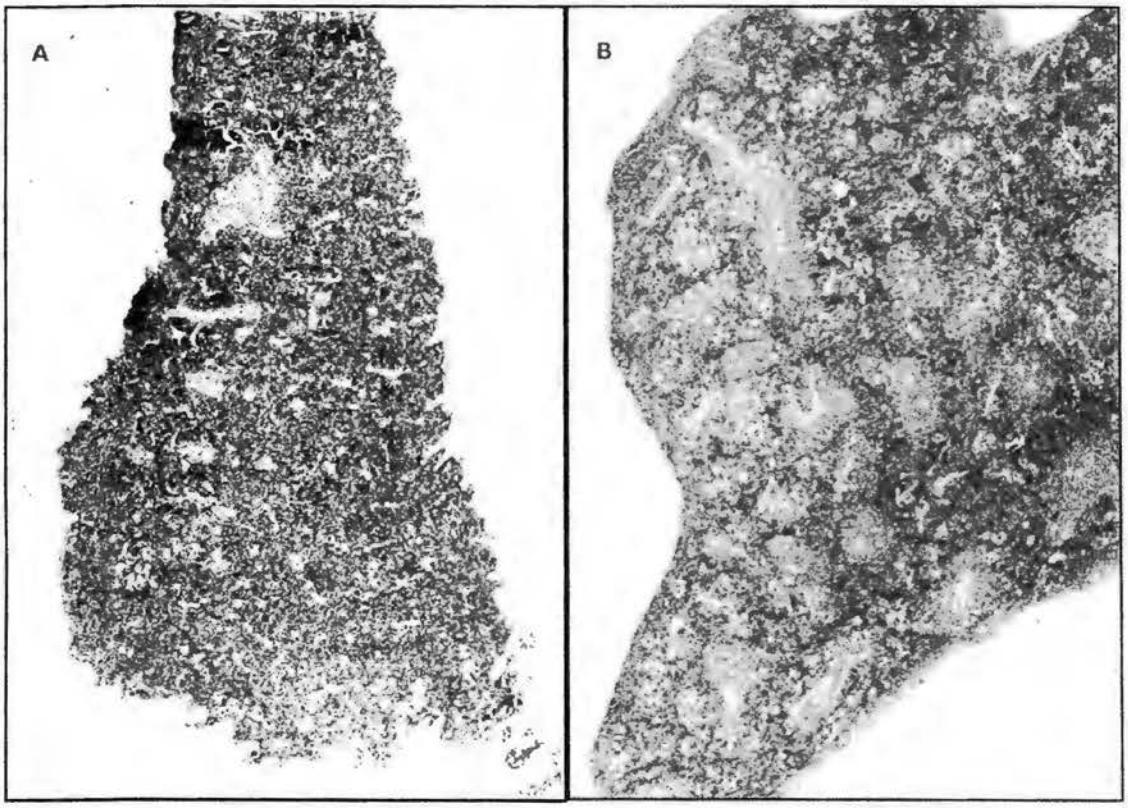
A
B

Fig. 2. Macroscopic lung lesions in SCID-bg mice after intratracheal challenge with *M. haemolytica. A)* SCID-bg lung 48 hr post-challenge. There is atelectasis of a major portion of the lung with concurrent mottling of the lung surface. B) SCID-bg lung 96 hr post-challenge. There is extensive mottling of the lung surface, but minimal atelectasis.

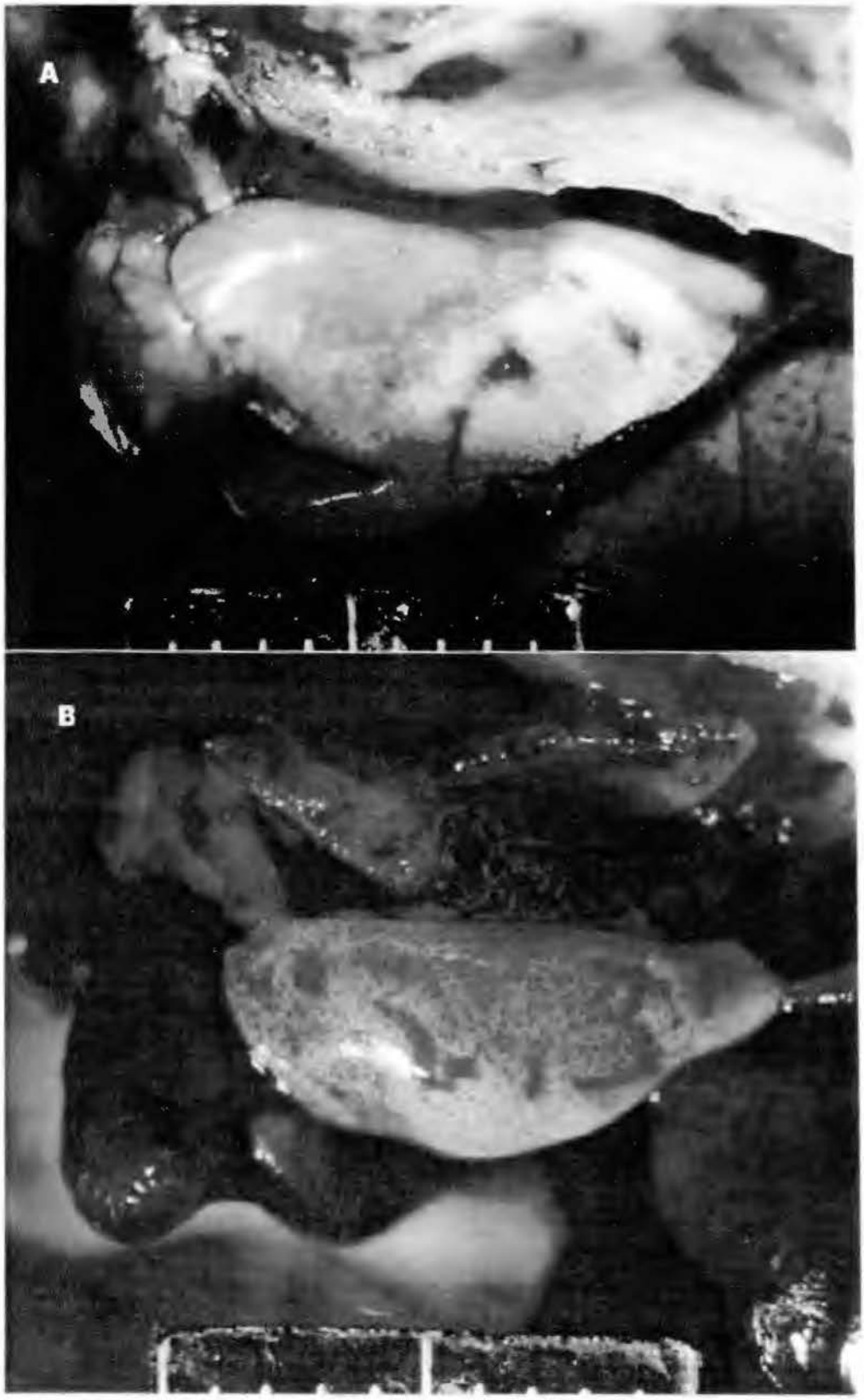
A
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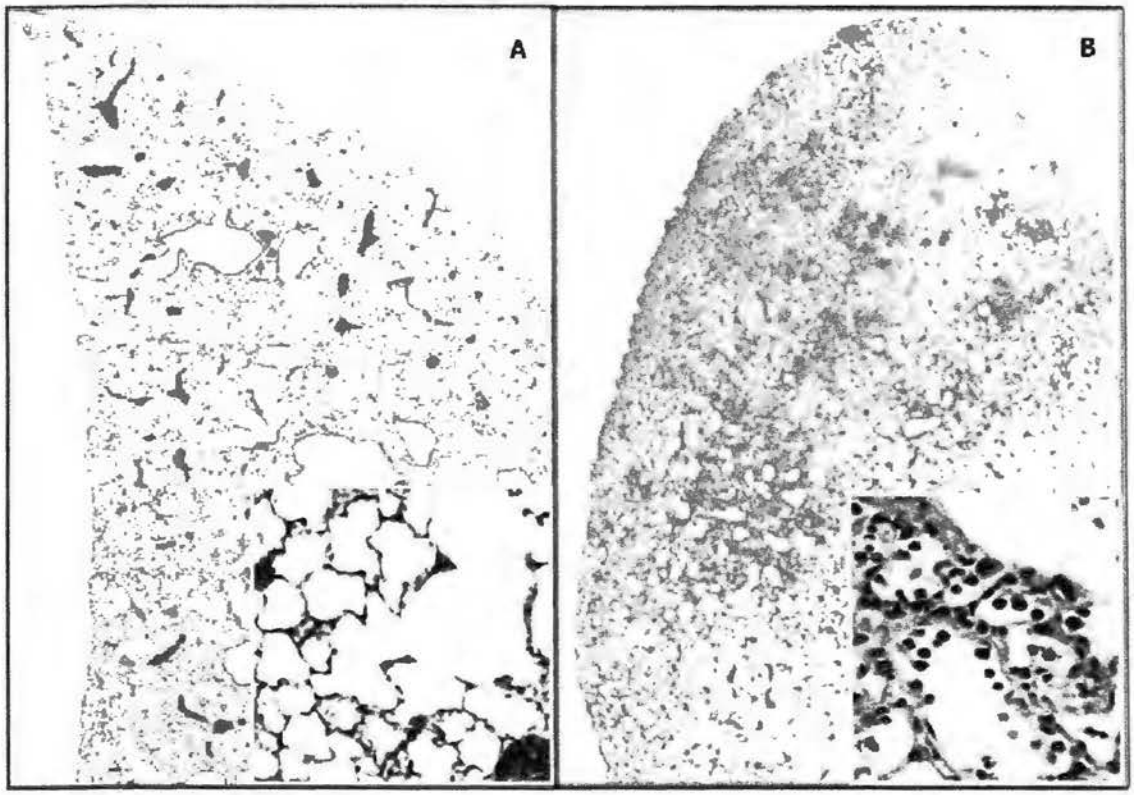
Fig. 3. Histologic lesions observed in SCID-bo mice after intratracheal challenge with *M. haemolytica.* A) SCID-bo lung 43 hr post-challenged with PBSS. Note the lack of inflammatory cells. B) SCID-bo lung 43 hr post-challenge with *M. haemolytica* Lkt+ WT. There is a marked neutrophilic infiltrate within the interstitium, and bronchiolar and alveolar lumina.


A
B

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CONCLUSION

Bovine respiratory disease complex (BRD), or as it is commonly called "Shipping Fever", is a multifactorial disease causing significant morbidity and mortality in cattle. In the order of prevalence, bacteria isolated from the lungs of affected cattle are *Mannheimia haemolytica* 1 (formerly, *Pasteurella haemolytica* A:1), *Pasteurella multocida* A:3 or *Haemophilus somnus.* Induction of BRD is thought to involve a combination of stressful management practices and/or concurrent viral or bacterial infections, which by direct damage to the respiratory tract or immunosuppression culminate in the colonization of the lower respiratory tract by one on the etiologic agents. Pneumonic infection is typified as a fibrinous pleuropneumonia

Several studies have revealed the importance of the host humoral immune response to surface epitopes of *M. haemolytica* and *P. multocida* resistance *in vitro* and in various animal models such as, cattle, rabbit and mouse. Protection from experimental challenge with *P. multocida* and *M. haemolytica* has been established through vaccinating with outer membrane proteins (OMP), lipopolysaccharide, and exotoxins. To develop an efficacious and safe vaccine, the contribution of each immunogen contained in the vaccine should be individually examined.

In these studies, we examined an immunogenic, 28 kDa OMP (Omp28) from *P. multocida* A:3 (strain 232). Omp28 was selected for study because of structural similarities to PomA of *M. haemolytica,* to the OmpA porin protein of *Escherichia coli,* and the OmpA-family proteins from *Salmonella typhimurium* and *Shigella dysentery.* Omp28 was purified through a combination of detergent fractionation and chromatography. Porin activity was not demonstrated on purified Omp28 in a lipid

bilayer assay. Immunogold labeling of intact encapsulated and hyaluronidase decapsulated *P. multocida* with polyclonal anti-Omp28 antibodies failed to demonstrate Omp28 surface exposure. The lack of significant surface exposure was supported by the inability of anti-Omp28 antibodies to activate complement *in vitro.* The efficacy of Omp28 as a vaccine candidate was assessed by subcutaneously vaccinating mice with purified Omp28. Vaccinated mice developed a significant antibody response, however the antibody response was not protective from a homologous bacterial intraperitoneal challenge.

To aid in the study of BRD, SCID-bg^{j} mice reconstituted with bovine immune cells (SCID-bo) were examined. The pneumonic lesions in SCID-bo and SCID-bg^{j} mice after intratracheal challenge with either one of two live strains of *M. haemolytica,* a formalin-killed strain, or a *M. haemolytica* leukotoxin- mutant were characterized as a neutrophilic alveolitis-bronchiolitis with concurrent perivascular edema and hemorrhage. Lung lesion scores were significantly different in groups challenge with *M. haemolytica* Lkt+ WT and *M. haemolytica* Lkt- when compared to the control group, however no significant difference was observed between live or formalin-killed bacteria challenged groups. Mice examined at later time points post-challenge had decreasing lung lesion scores and decreasing lung bacteria isolation.

Pneumonic lesions due to *M. haemolytica* in SCID-bo mice lack many of the unique characteristics observed in cattle. Therefore, the SCID-bo mouse is not an acceptable model for the study of bovine pneumonic pasteurellosis.

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