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Histophilus somni host–parasite relationships

Lynette B. Corbeil[1,2]*

[1]*Division of Infectious Diseases, Department of Pathology, University of California, San Diego, CA, USA, and* [2]*Department of Population Health and Reproduction, University of California, Davis, CA, USA*

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Abstract

Histophilus somni (*Haemophilus somnus*) is one of the key bacterial pathogens involved in the multifactorial etiology of the Bovine Respiratory Disease Complex. This Gram negative pleomorphic rod also causes bovine septicemia, thrombotic meningencephalitis, myocarditis, arthritis, abortion and infertility, as well as disease in sheep, bison and bighorn sheep. Virulence factors include lipooligosaccharide, immunoglobulin binding proteins (as a surface fibrillar network), a major outer membrane protein (MOMP), other outer membrane proteins (OMPs) and exopolysaccharide. Histamine production, biofilm formation and quorum sensing may also contribute to pathogenesis. Antibodies are very important in protection as shown in passive protection studies. The lack of long-term survival of the organism in macrophages, unlike facultative intracellular bacteria, also suggests that antibodies should be critical in protection. Of the immunoglobulin classes, IgG2 antibodies are most implicated in protection and IgE antibodies in immunopathogenesis. The immunodominant antigen recognized by IgE is the MOMP and by IgG2 is a 40 kDa OMP. Pathogenetic synergy of bovine respiratory syncytial virus (BRSV) and *H. somni* in calves can be attributed, in part at least, to the higher IgE anti-MOMP antibody responses in dually infected calves. Other antigens are probably involved in stimulating host defense or immunopathology as well.

Keywords: virulence factors, antibodies, protection, pathogenesis, *Histophilus somni*, Bovine Respiratory Disease, synery, immunoglobulins

Disease

Bovine respiratory disease (BRD) is a polymicrobial infection involving several viruses and bacteria as well as the predisposing factor of stress. The bacterial pathogens most often include *Mannheimia haemolytica*, *Pasteurella multocida* and *Histophilus somni* (Angen *et al*., 2003), also called *Haemophilus somnus*. In addition to BRD, *H. somni* induces respiratory disease in sheep, bison and big horn sheep as well as infertility, septicemia, abortion, myocarditis, arthritis and meningoencephalitis in cattle (Griner *et al*., 1956; Biberstein 1981; Stephens *et al*., 1981; Humphrey and Stephens, 1983; Miller *et al*., 1983; Widders *et al*., 1986; Corbeil *et al*., 1987, 1995; Gogolewski *et al*., 1987a, b, 1989; Corbeil, 1990; Haritani *et al*., 1990; Kwiecien and Little, 1991; Lees *et al*., 1994; Ward *et al*., 1995, 1999, 2006; Dyer, 2001). Not only is *H. somni* the etiologic agent in all of these syndromes but also it commonly exists in an asymptomatic carrier state on the reproductive and respiratory mucosa (Humphrey and Stephens, 1983; Humphrey *et al*., 1985). Several reviews of the naturally occurring disease are available (Humphrey and Stephens, 1983; Miller *et al*., 1983; Harris and Janzen, 1989; Kwiecien and Little, 1991). BRD due to *H. somni* is sometimes seen in young calves (enzootic pneumonia) but most often in feedlot cattle (Humphrey and Stephens, 1983; Harris and Janzen, 1989). A study of hemophilosis in feedlot calves showed that *H. somni* was a significant cause of death even though calves were immunized with a commercial *H. somni* vaccine on arrival (Van Donkersgoed *et al*., 1990). In that study, the median onset of fatal pneumonia was day 12 after arrival in the feedlot and death due to pneumonia was during the first five weeks. Although metaphylaxis (with antibiotics given before clinical signs are evident) has become popular for the prevention of BRD (Vogel *et al*., 1998; Step *et al*., 2007), the long-term results in selecting for

*Corresponding author. E-mail: lcorbeil@ucsd.edu

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antibiotic resistant bacteria is not known. Characteristics of the pathology of naturally occurring *H. somni* pneumonia have been defined in several studies (Andrews *et al.*, 1985; Bryson *et al.*, 1990; Tegtmeier *et al.*, 1999b). Much information on the definition of *H. somnus* disease also has been gleaned from experimental reproduction of the spectrum of syndromes caused by this organism. Several groups have reproduced thrombotic meningoencephalitis (Stephens *et al.*, 1981), reproductive failure (Miller *et al.*, 1983) and pneumonia (Andrews *et al.*, 1985; Jackson *et al.*, 1987; Groom *et al.*, 1988; Potgieter *et al.*, 1988; Tegtmeier *et al.*, 1999a; Tagawa *et al.*, 2005). Interestingly, *H. somni* also is found commonly in an asymptomatic carrier state (Humphrey and Stephens, 1983). The pathogenesis of *H. somni* infection was studied by our group by reproducing both abortion and pneumonia in cattle. To simulate hematogenous infection, 19 pregnant cows were inoculated intravenously or intrabronchially (Widders *et al.*, 1986; Corbeil *et al.*, 1987). Reproductive failure occurred in six cows. Cultures showed massive infection of the uterus and placenta (Widders *et al.*, 1986; Corbeil *et al.*, 1987). Placentitis was characterized by thrombosis and vasculitis (Widders *et al.*, 1986), as in thrombotic meningoencephalitis (Stephens *et al.*, 1981) and pneumonia (Gogolewski *et al.*, 1987a, b, 1988).

In other studies, pneumonia was reproduced in 6–12-week old calves (Gogolewski *et al.*, 1987a, b, 1988), since our earlier studies indicated that calves are most susceptible to pneumonia at this time (Corbeil *et al.*, 1984). At 24 h after intrabronchial inoculation of 10^7 *H. somni*, pneumonia was characterized by neutrophilic to fibrinoid vasculitis, degeneration of alveolar macrophages, necrotizing bronchiolitis, suppurative bronchiolitis, lobular necrosis and dilation and thrombosis of lymphatics (Gogolewski *et al.*, 1987b). Infection lasted for 6–10 weeks in studies of chronic infection (Gogolewski *et al.*, 1989). Interestingly, *H. somni* was isolated more often from the bronchioalveolar lavage fluid than from nasal swabs in weekly samples during these studies on chronic disease. This suggested that *H. somni* may preferentially colonize the lower respiratory tract rather than the nasal mucosa. Severe clinical signs in these chronically infected calves were only obvious for a few days. After that only an occasional cough was detected, indicating that chronic *H. somni* infection of the lower respiratory tract can be almost subclinical for weeks.

Immunodiagnostic assays have proven to be useful for diagnosis of *H. somni* infection. Culture of bacteria has usually been the gold standard for diagnosis of bacterial infection. This is also true for *H. somni* when large numbers are obtained in pure culture from lesions. However, a common genital asymptomatic carrier state and a less common respiratory carrier state complicate diagnosis by culture. In experimental disease, Gogolewski *et al.* (1987b) showed that organisms could be detected in pneumonic lesions by immunohistochemistry. This assay has been developed for diagnosis and used very successfully by Haines and Clark (1991). Microagglutination assays have been used widely for diagnosis but many cattle have high titers, even with no history of *H. somni* disease and no current clinical signs (Widders *et al.*, 1986). This can be explained by several antigens of *H. somni* which are cross-reactive with other members of the family *Pasteurellaceae* (Kania *et al.*, 1990; Corbeil *et al.*, 1991). In addition, the microagglutination test preferentially detects IgM antibodies and natural cross-reactive antibodies are often of this Ig class. Thus the high microagglutination titers in normal cattle with no history of *H. somni* infection are probably due to antibodies induced by other *Pasteurellaceae*. Alternatively, some of these antibodies could be due to induction by *H. somni* in the asymptomatic carrier state, especially in the genital tract. Our laboratory showed that ELISA tests using second antibody conjugates for bovine IgG2 were more specific than secondary antibodies to other immunoglobulin classes (Widders *et al.*, 1986) and IgG2 antibody assays against the 270 kDa FcR (now called IbpA or IgBPs) were most specific compared with other antigens in immunodiagnostic assays (Yarnall and Corbeil, 1989).

Virulence factors

We and others have identified and characterized several *H. somni* antigens or virulence factors that are likely to be important in host–parasite relationships. The 40 kDa outer membrane protein (OMP; p40) is surface exposed and is surely important in protection since convalescent phase serum recognizing p40 and monospecific bovine antibodies against p40 are passively protective against experimental bovine pneumonia (Gogolewski *et al.*, 1987a, 1988; Corbeil *et al.*, 1991). Others have cloned and sequenced a 40 kDa lipoprotein OMP of *H. somni* (Theisen *et al.*, 1992) which may be the p40 protective antigen. The 40 kDa OMP recognized by protective antibodies is different from the 41 kDa major outer membrane protein (MOMP) (Gogolewski *et al.*, 1987a; Yarnall *et al.*, 1988a, b). We showed that p40 was immunodominant (Corbeil *et al.*, 1991) but that the MOMP was not recognized by bovine convalescent phase serum at dilutions of 1 : 500–1 : 1000 or more; the dilutions which we usually use in Western blots (Gogolewski *et al.*, 1987a, b; Ward *et al.*, 1995). Tagawa and colleagues purified and characterized the MOMP (Tagawa *et al.*, 1993b, d; Khan *et al.*, 2005), showing it to be similar to porins of other Gram-negative bacteria. Tagawa also studied immunologic aspects of the MOMP in Corbeil's laboratory (Tagawa *et al.*, 2000). Low dilutions (1 : 50 or 1 : 100) of bovine convalescent serum did recognize the MOMP but higher dilutions did not. Also, the MOMP was shown to be antigenically variable (Tagawa *et al.*, 2000) and it is the predominant antigen recognized by IgE (Corbeil *et al.*, 2006), which was associated with

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enhanced respiratory disease. Thus the MOMP is not likely to be a very good vaccine candidate.

Tagawa *et al.* (1993a) also characterized a 37 kDa heat modifiable OMP with N-terminal sequence homology and immunologic cross-reactivity with OmpA of other Gram-negative bacteria. Studies of surface exposure gave contradictory results. This OMP was recognized by convalescent phase serum at 1 : 800 in Western blots. It is not clear whether it is an important virulence factor. Similarly, a 17.5 kDa OMP has been characterized but its role in virulence is unknown (Tagawa *et al.*, 1993c). Lastly, iron regulated OMPs may be important in host–parasite relationships. Schryvers' group demonstrated transferrin binding proteins (Tbps; Ogunnariwo *et al.*, 1990) which bind bovine transferrin, but not other ruminant transferrins (Yu *et al.*, 1992). Later, Ekins and co-workers (Ekins and Niven, 2001; Ekins *et al.*, 2004) showed that some strains of *H. somni* have two genes for Tbps. Whether antigenic differences among strains result from differences in Tbps is unclear. These Tbps are probably involved in our studies of the role of bovine serum proteins in *H. somni* virulence in a mouse septicemia model (Geertsema *et al.*, 2007). That study demonstrated enhanced virulence for mice by preincubation of *H. somni* in Fetal Calf Serum (FCS) for 5 min before inoculation. To identify the virulence enhancing factor, *H. somni* was preincubated in each of the main components of FCS. Both fibrinogen and transferrin bound to *H. somni* but only bovine transferrin enhanced virulence. Mouse transferrin did not enhance virulence but bovine lactoferrin did enhance virulence for mice. Iron chelation experiments indicated that iron uptake from transferrin (but not lactoferrin) was critical for increased virulence in the mouse septicemia model.

In addition to protein virulence factors, endotoxin or lipooligosaccharide (LOS) is important in pathogenesis (Inzana *et al.*, 1988). Collaborative studies with Inzana (Inzana *et al.*, 1992), showed that *H. somni* LOS undergoes phase and antigenic variation. Since then, Inzana's group has contributed much to the understanding of *H. somni* LOS, including LOS structural analysis of several strains (Cox *et al.*, 1998, 2003; Michael *et al.*, 2004, 2005, 2006), sequences involved in biosynthesis of LOS (McQuiston *et al.*, 2000; Wu *et al.*, 2000), the mechanism of phase variation (Howard *et al.*, 2000; McQuiston *et al.*, 2000) and the role of LOS sialylation in resistance to complement mediated killing (Inzana *et al.*, 1992, 2002). This work was reviewed recently (Siddaramppa and Inzana, 2004), so will not be further discussed here. The antigenic variation *in vivo* suggests that LOS may not be a good vaccine candidate but this may make it a more effective virulence factor. Czuprynski's group (Sylte *et al.*, 2001, 2005a, b, 2006; Kuckleburg *et al.*, 2005) showed that LOS mediated apoptosis of bovine endothelial cells. Later, the same group found that sulfated glycans are involved in adherence to bovine endothelial cells (Behling-Kelly *et al.*, 2006), similar to our observations on the role of heparin binding motifs in studies of Immunoglobulin Binding Proteins (IgBPs) (Tagawa *et al.*, 2005). Whether similar mechanisms of apoptosis are involved in phagocyte dysfunction caused by *H. somni* is not clear but Howard *et al.* (2004) demonstrated that LOS did not contribute to *H. somni* inhibition of superoxide production by phagocytes.

Much of the work from Corbeil's laboratory in the last several years has focused on the surface IgBPs of *H. somni*. Initially, we showed that a high molecular weight (HMW) antigen, comprised of several bands in SDS–PAGE (~120–350 kDa; called p120) as well as a 76 kDa antigen (p76), bound bovine IgG2 by the Fc domain (Yarnall *et al.*, 1988a, b; Widders *et al.*, 1988, 1989; Corbeil *et al.*, 1997a, b; Sanders *et al.*, 2003), meeting the definition of IgBPs. These surface proteins consisted of a fibrillar network on the surface of *H. somni* which was Sarkosyl soluble, unlike integral membrane proteins (Corbeil *et al.*, 1985) and is easily shed from the surface (Yarnall *et al.*, 1988a, b). The role of IgBPs in virulence of *H. somni* is not yet well explained, but it is clear that they are associated with serum resistance (Corbeil *et al.*, 1985; Widders *et al.*, 1989). All disease isolates of *H. somni* had both HMW and p76 IgBPs (Yarnall *et al.*, 1988b; Cole *et al.*, 1992) and five serum sensitive preputial strains from asymptomatic carriers lacked Ig Fc binding activity as well as both groups of IgBPs (Widders *et al.*, 1989). It should be noted that not all isolates from asymptomatic carriers were serum sensitive (Corbeil *et al.*, 1985) and not all serum sensitive isolates lacked IgBPs (Widders *et al.*, 1989). Furthermore, surface proteins p76 and p120 are absent in four preputial serum sensitive strains of *H. somni* (Cole *et al.*, 1992), which lacked the HMW IgBPs and had a truncated MOMP (Widders *et al.*, 1989). Southern hybridization showed that a 13.4 kb segment (including the entire sequence encoding p76 and p120) was absent in these four serum sensitive strains including strains 129Pt and 1P (Cole *et al.*, 1992). This further supports the hypothesis that IgBPs contribute to serum resistance.

To confirm the role of *H. somnus* IgBPs in serum resistance, our goal was to knock out the genes for IgBPs, requiring development of genetic exchange systems for *H. somnus*. This had been a difficult problem for many of the *Pasteurellaceae* (Frey and MacInnes, 1995), except for *Haemophilus influenzae*. Jerry Sanders, a former postdoctoral fellow in our laboratory, with previous experience on *H. influenzae* genetics (Sanders *et al.*, 1993, 1994) succeeded in transfer of DNA from *H. somni* to *Escherichia coli* and back into *H. somni* (Sanders *et al.*, 1997, 2003) using the broad host range vector, pLS88, methylated in *H. influenzae*. The p76 gene was electroporated into *H. somni* serum sensitive strain 129Pt, which lacks the gene for p76 (Cole *et al.*, 1992). Expression of p76 was detected by Western blotting (Cole *et al.*, 1992; Sanders *et al.*, 1997). Later, we found that the HMW IgBPs but not p76 could be partially purified from *H. somni*

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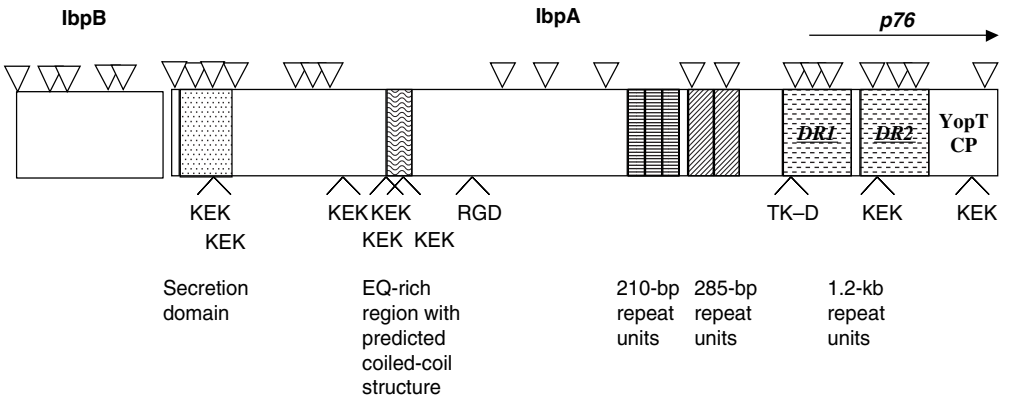

Fig. 1. Sequence diagram for *H. somni* IbpB and IbpA. Multiple ATG translational start sites are shown by small triangles, the p76 gene with a horizontal arrow (labeled p76) and RGD, TKXXD and KEK motifs by open arrows. The EQ-rich region may have Ig binding activity. Sets of repeats are shaded similarly. The 1.2 kb repeat units (DR1 and DR2) are upstream from the cysteine proteinase catalytic domain (YopT) near the C terminus.

culture supernatants by gel filtration (Corbeil *et al.*, 1997a). Extraction of cells and supernatants with Sarkosyl showed that p76 is in the Sarkosyl soluble fraction of the cell pellet, suggesting that it is a peripheral OMP rather than an integral OMP (Corbeil *et al.*, 1997a, b). HMW IgBPs predominated in the Sarkosyl-soluble fraction of the culture supernatant, providing a method for purification (Corbeil *et al.*, 1997a).

Ultrastructural studies showed that virulent IgBP+ strains (2336 and 649) were covered with a fibrillar network but IgBP negative strains (129Pt and 1P) were not (Corbeil *et al.*, 1997a, b). Fibrils on the surface bound gold-labeled bovine IgG2 anti-dinitrophenol (DNP), indicating that fibrils are IgBPs (Corbeil *et al.*, 1997a). Since anti-DNP did not react with *H. somni* in competitive inhibition assays with DNP-albumin or DNP (Widders *et al.*, 1988; Corbeil *et al.*, 1997a), this binding was not antigen specific, so was due to Fc binding. Furthermore, Fc fragments bound much more efficiently than Fab fragments, confirming that IgG2 bound to IgBPs by the Fc portion (Widders *et al.*, 1988). Binding of the IgG2 allotypes to IgBPs was investigated in a separate study. Cattle have two IgG2 allotypes, $IgG2^a$ and $IgG2^b$ (formerly IgG2A1 and IgG2A2), encoded by codominant alleles. In our studies, $IgG2^b$ bound to IgBPs and $IgG2^a$ did not (Bastida-Corcuera *et al.*, 1999b). Also, since binding of IgG2 to IgBPs and complement activation (or lack thereof) may both be related to serum resistance, it is noteworthy that $IgG2^b$ activates C better than $IgG2^a$ (Bastida-Corcuera *et al.*, 1999a). These findings may partially explain differential susceptibility of cattle to *H. somni*.

In order to clone genes for the IgBPs, we made a cosmid library of *H. somni* DNA. Four clones expressed IgBPs and one clone expressed a 60 kDa surface antigen of *H. somni* (Corbeil *et al.*, 1988). We addressed the IgBPs by subcloning linked genes encoding HMW and p76 IgBPs in an approximately 12 kb PvuII fragment excised from the original cosmid clone. First Cole *et al.* (1993) sequenced and defined motifs in p76, including tandem 1.2 kb direct repeats (DR1 and DR2) with insertion sequence structure. Then Tagawa *et al.* (2005) sequenced the DNA encoding HMW IgBPs ranging from above 200 kDa to 120 kDa (p120). The series of bands in SDS-PAGE and Western blotting is characteristic of not only *H. somni* HMW IgBPs (Yarnall, 1988a, b), but also Staphylococcal Protein A (Bjorck and Kronvall, 1984) and Streptococcal Protein G (Fahnestock, 1986; Fahnestock *et al.*, 1987; Akerstrom *et al.*, 1987). The sequence of the *H. somni* ORF encoding HMW IgBPs indicated that it was contiguous with the ORF encoding p76 (Tagawa *et al.*, 2005). So, one ORF of >12 kb encoded both p76 and the HMW IgBPs (Fig. 1). This very large gene (*ibpA*, for IgBP A) contained 18 translational start sites (ATG codons), many of which had potential Shine–Delgarno sequences and putative promoters (−35 and −10 consensus sequences). Deletion analysis of the p76 sequence had shown that several start sites were used to express p76 and its truncated peptides (Cole *et al.*, 1993), perhaps explaining the many bands in SDS–PAGE.

A protein sequence homology search of this very large IbpA sequence (both HMW IgBPs and p76) demonstrated similarity to *Bordetella pertussis* filamentous hemagglutinin (FHA) (which is a surface fibrillar network (Blom *et al.*, 1983) similar to IgBPs), and other large exoproteins (see below). Tagawa *et al.* (2005) also sequenced an additional upstream ORF of 1758 bp, designated *ibpB*. The sequence suggested that IbpB is an integral OMP which is likely involved in secretion of IbpA (see below). At the 3′ end of *ibpA* is another open reading frame, ORF7, with a predicted homology of 64% identity to *P. multocida* thiamin binding protein A (May *et al.*, 2001; Tagawa *et al.*, 2005). The sequences of the predicted IbpA and IbpB proteins demonstrated homology to large exoproteins and the transporter proteins of Gram-negative bacteria that belong to the two-partner secretion pathway family (Jacob-Dubuisson *et al.*, 2001). The large predicted or confirmed exoproteins related to IbpA include

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P. multocida PfhB1 (2615 aa) and PfhB2 (3919 aa) (May *et al.*, 2001), *Haemophilus ducreyi* LspA1 (4152 aa) and LspA2 (4919 aa) (Ward *et al.*, 1998), *B.* FhaB (3591 aa) (Domenighini *et al.*, 1990; Relman *et al.*, 1990) and others. The greatest identity and similarity was observed with PfhB1 and PfhB2 (53.6 and 61.4%, respectively). The N-terminal half of the whole IbpA sequence overlapped in part with FhaB hemagglutination domains (Blom *et al.*, 1983).

Low-level homology regions were detected for several known functional domains such as a heparin binding domain at aa 525–975 of IbpA (aa 442–863 of FhaB) (Hannah *et al.*, 1994) and a carbohydrate recognition domain at aa 1307–1471 of IbpA (aa 1141–1279 of FhaB) (Prasad *et al.*, 1993) at 40.8 and 39.3% similarity, respectively (Tagawa *et al.*, 2005). The integrin binding motif, RGD, was unique in IbpA and FhaB but the TK–D sequence, with a possible role in integrin recognition, was found in all related large exoproteins listed above. Another common motif found in all except FhaB was a putative ATP/GTP binding consensus sequence. Interestingly, multiple KEK motifs were found in IbpA as well as in PfhB1, PfhB2 and LspA2. The KEK motifs in malaria antigens are thought to be involved in binding to erythrocyte membranes (Calvo *et al.*, 1991). A cysteine proteinase catalytic domain with homology to *Yersinia* YopT as well as PfhB1 and PfhB2 has also been demonstrated (Shao *et al.*, 2002). Just upstream from the invariant C/H/D residues of the cysteine proteinase catalytic domain of p76 are the previously described direct repeats, DR1 and DR2 (Cole *et al.*, 1993).

Several virulence factors have been described above, including LOS, the MOMP, the 40 kDa OMP, Tbps and IgBPs. Other surface proteins such as a 37 kDa OMP, a 31 kDa protein (Won and Griffith, 1993), a 78 kDa OMP (Kania *et al.*, 1990), a 60 kDa protein (Corbeil *et al.*, 1988) and a 17 kDa OMP (Tagawa *et al.*, 1993c) are potential virulence factors. In addition to LOS and surface proteins, *H. somni* has been shown to produce histamine (Ruby *et al.*, 2002) and exopolysaccharide (Siddaramappa and Inzana, 2004). The exopolysaccharide is shed from the surface of *H. somni*, so may be more like a slime layer than like a capsule. Passive immunization of mice with antibodies to the purified exopolysaccharide was not protective (Siddaramappa and Inzana, 2004), consistent with it not being a capsule. Lastly, *H. somni* has been shown to form biofilms *in vitro* (Sandal *et al.*, 2007) and may be capable of quorum sensing (Siddaramappa and Inzana, 2004). Since virulence of bacterial pathogens is usually multifactorial, many of the above factors may be involved in *H. somni* pathogenesis. Probably more virulence factors will be identified by comparative genomics now that the compete genome sequence of serum sensitive strain 129Pt, from an asymptomatic carrier (Corbeil *et al.*, 1985) is published (Challacombe *et al.*, 2007) and the sequence of serum resistant strain 2336, demonstrated to be pathogenic (Gogolewski *et al.*, 1987a, b, 1988, 1989), is expected to be published soon.

Protective immune responses

Antibodies are likely to be important in protection for two reasons. Firstly, convalescent phase serum was passively protective (Gogolewski *et al.*, 1987a). Secondly, although *H. somni* survives phagocytosis by bovine macrophages (Lederer *et al.*, 1987; Gomis *et al.*, 1997a, b), it destroys the macrophage within hours *in vivo* (Gogolewski *et al.*, 1987b). Therefore, it is more like an extracellular parasite than a facultative intracellular parasite which multiplies over an extended time inside normal macrophages. Classically, antibodies are most important in protection against extracellular pathogens. Other studies showed that IgG2 antibodies were more associated with protection against *H. somni* (Corbeil *et al.*, 1997a, b). In chronic *H. somni* BRD study, infection was maintained for 6–10 weeks with clearance occurring as IgG2 titers to *H. somni* increased in bronchioalveolar lavage fluids (Gogolewski *et al.*, 1989). Immune responses of infected calves protected rechallenged convalescent calves against re-infection (Gogolewski *et al.*, 1989). Convalescent phase serum from these calves passively protected a second group of calves against experimental pneumonia (Gogolewski *et al.*, 1987a). The protective serum recognized several antigens in Western blots, including 37, 40, 60, 78 kDa proteins (Gogolewski *et al.*, 1987a; Kania *et al.*, 1990; Corbeil *et al.*, 1995; Tagawa *et al.*, 2000) and HMW or p76 IgBPs (Corbeil *et al.*, 1987; Yarnall and Corbeil, 1989) as well as LOS (Gogolewski *et al.*, 1987a; Inzana *et al.*, 1992). Passive protection studies with monospecific bovine antibodies to 78 and 40 kDa antigens showed that antibodies to p40 protected but antibodies purified from monospecific bovine antiserum to p78 resulted in enhanced pneumonia (Gogolewski *et al.*, 1988). Interestingly, the antibodies to p78 consisted of only IgG1 whereas the antibodies to p40 were both IgG1 and IgG2 (Gogolewski *et al.*, 1988). Therefore, passive protection experiments were done with purified IgG1 or IgG2 monospecific bovine antibodies to p40. This experiment showed a trend toward better passive protection with IgG2 anti p40 than IgG1 anti p40 (Corbeil *et al.*, 1997b).

The above studies of passive protection by monospecific antibodies against immunodominant antigens of *H. somni* did not include antibodies to IbpA. At the time the passive transfer experiments were done, we used OMP preparations in SDS–PAGE and Western Blots to define immundominant antigens because we thought only OMPs and LOS were surface exposed. However, Yarnall and Corbeil (1989) found that cattle with experimental or natural *H. somni* infection had strong IgG2 antibody responses to IgBPs/IbpA (then called FcRs). Later, we showed the IgBPs consisted of surface exposed fibrils (Corbeil *et al.*, 1997a). The surface

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exposure and strong IgG2 responses to IbpA suggest that it may be a protective antigen also. Since IgG2 antibodies appeared to be important in protection, we then investigated the role of IgG2 allotypes. We found that IgG2 allotype responses develop at different ages, with $IgG2^a$ (formerly IgG2A1) being expressed early but $IgG2^b$ (IgG2A2) not being expressed until 3 or 4 months of age (Corbeil *et al.*, 1997b). Functional studies indicated that $IgG2^b$ but not $IgG2^a$ bound to HMW IgBPs of *H. somni* (Bastida-Corcuera *et al.*, 1999b), perhaps accounting for one mechanism of genetic susceptibility to *H. somni* infection.

IgE responses also appear to be important in *H. somni* induced BRD. Ellis and Jong (1997) reported systemic adverse reactions similar to anaphylaxis after vaccination with products containing *H. somni* whole cells. Others demonstrated IgE antibodies to *H. somni* in calves immunized with commercially available *H. somni* bacterins (Ruby *et al.*, 2000). In studies with Laurel Gershwin, we found that calves given bovine respiratory syncytial virus (BRSV) six days before *H. somni* had greater clinical scores, longer duration of respiratory disease and higher IgE anti-*H. somni* levels than calves given either pathogen alone (Gershwin *et al.*, 2005). The antigenic specificity of IgE and IgG antibodies in the serum of these calves was studied by Western blotting (Corbeil *et al.*, 2006). Although both isotypes recognized some antigens to a similar extent, the predominant antigen recognized by IgE was the 41 kDa MOMP. The predominant antigen recognized by IgG antibodies was the 40 kDa protective OMP, consistent with our earlier studies (Gogolewski *et al.*, 1987a). Since the MOMP is antigenically variable (Tagawa *et al.*, 2000) and IgE antibodies are associated with worse pneumonia of extended duration (Gershwin *et al.*, 2005), a vaccine without the MOMP epitopes recognized by IgE might be indicated. With this in mind, we determined the reactivity of these IgE antibodies with *H. somni* strain 129Pt, which has a truncated MOMP of 33 kDa (Widders *et al.*, 1989). The IgE antibodies, which reacted strongly with the 41 kDa MOMP of virulent strains, did not react with the truncated MOMP of strain 129Pt (Corbeil *et al.*, 2006). Since this strain does have the protective 40 kDa immunodominant OMP, it may be a good vaccine candidate. These studies of *H. somni* and BRSV infection alone or together were then extended to investigate the effect of vaccination on disease and cytokine profiles (Berghaus *et al.*, 2006). These aspects of the dual infection studies will be covered in the paper on BRSV by Laurel Gershwin in this volume.

Our studies and those of others have shown that antibody specificity as well as isotype and allotype all contribute to protection versus immune evasion or immunopathogenesis. The Fc binding of IgG2 to HMW and p76 IgBPs could be considered to be a mechanism of immune evasion, but specific antibody binding of IgG2 is at least 16 times more avid. Therefore, the demonstrated specific antibody responses to IbpA/IgBPs are likely to be protective rather than primarily immuno-evasive. A vaccination challenge study of IbpA subunits is underway in our laboratory to test this hypothesis. Our previous studies have made it clear that IgG2 antibodies to p40 are protective in a BRD *H. somni* challenge model (Gogolewski *et al.*, 1988; Corbeil *et al.*, 1997b). Other observations confirmed the importance of IgG2 in protection since cattle with low IgG2 anti-*H. somni* titers were more susceptible to experimental *H. somni* disease and IgG2 antibodies increased the most after infection (Widders *et al.*, 1986; Gogolewski *et al.*, 1988, 1989; Corbeil *et al.*, 1997b). Thus adjuvants and antigens which stimulate IgG2 responses are critical. The bovine IgG2 antibody response and cell-mediated immunity are both Th1 responses (Estes and Brown, 2002), so T cell effectors may also play a role. IgG1 and IgE responses are usually considered to be Th2 responses. Our studies suggest that IgG1 antibodies are less critical than IgG2 antibodies and that IgE antibodies are associated with poor outcomes in BRD. Therefore we have concluded that Th1 responses are to be desired in protection against *H. somni* BRD. The role of IgA is relatively unexplored. Both the 40 kDa OMP and the IgBPs are among critical antigens in eliciting protective host responses. Clearly both the arm of the immune response and the specificity are important in protection.

Conclusions

Virulence factors and immune responses involved in the host–parasite relationships during *H. somni*-induced bovine pneumonia have been described. Several of these interactions are likely to account for the pathology after intrabronchial inoculation of *H. somni* into 6–12-week old calves. Histamine production by *H. somni* (and later histamine release by IgE cross-linking of receptors on mast cells) may account for early lesions of edema, increased mucus secretion, bronchoconstriction and vascular constriction. Complement activation by LOS and perhaps Fc binding of IgG2 to the surface fibrillar network (IgBPs or IbpA) would result in chemotaxis of inflammatory cells characteristic of *H. somni* pneumonia. LOS has been shown to result in apoptosis of endothelial cells although it does not account for all the apoptosis detected. LOS also activates platelets. Surely both functions are important factors in the etiology of the vasculitis and thrombosis so characteristic of *H. somni* infection. Vasculitis and histamine release by *H. somni* would both contribute to vascular leakage and edema. Since fibrinogen attaches to *H. somni* it would be interesting to know if fibrinogen binding contributes to pathogenesis of fibrinopurulent bronchopneumonia. The mechanism of the damage to macrophages detected in histopathology at 24 h after inoculation of *H. somni*, is not clear. However, it could also be related to IbpA, similar to the role of the YopT homologous protein in *Yersinia* infection. Of

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course the large influx of inflammatory cells, due to the above mechanisms, contributes to the necrotic lesions seen in severe cases.

In early natural infection, exopolysaccharide release and biofilm production are thought to contribute to colonization. Iron acquisition after binding bovine transferrin to Tbps enhances growth *in vitro* and perhaps *in vivo*. Histamine release with resulting vascular permeability would increase the plasma transferrin concentration in secretions. Although we did not show that lactoferrin provided iron to *H. somni*, it did increase virulence for mice. Since lactoferrin is present in secretions and is released by neutrophils, it may contribute to survival of *H. somnus in vivo*. Unlike several other Gram negative pathogens, the MOMP of *H. somni* does not induce much of an immune response in cattle and it also undergoes antigenic variation, so it is probably a factor in evasion of host responses, contributing to survival. *H. somnus* LOS also undergoes antigenic variation, contributing to evasion of immune responses.

Protective immune responses are complex. The critical antigenic proteins identified so far include the 40 kDa OMP and IgBPs. Other antigens may also induce protective responses. Since *H. somnus* kills macrophages, rather than surviving and multiplying in them for long periods, it is more like an extracellular pathogen than a facultative intracellular pathogen. For this reason and because antibody is passively protective, the antibody response is thought to be most important. Several studies have shown that IgG2 antibodies are most protective. IgE responses, conversely, are associated with more severe disease of longer duration. This accounts for at least part of the synergy of BRSV and *H. somni* in the etiology of BRD. The dynamic balance between immunoprotection and immunopathogenesis is critical in determining the outcome of infection.

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