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IbpA DR2 subunit immunization protects calves against *Histophilus somni* pneumonia

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ABSTRACT

Histophilus somni is a prevalent cause of pneumonia and septicemia in cattle. Yet evidence for protection against pneumonia by current vaccines is controversial. We have identified a new *H. somni* virulence factor, IbpA. Previous studies implicated three likely protective subunits or domains in IbpA (A3, A5, and DR2), which were expressed as recombinant GST fusion proteins and purified for systemic vaccination of calves. After two subcutaneous immunizations, calves were challenged intrabronchially with virulent *H. somni* strain 2336 and clinical signs were monitored for four days before necropsy. Serum samples were collected throughout. At necropsy, the area of gross pneumonia was estimated, bronchial lavage fluid was collected, lesions were cultured and tissue samples were fixed for histopathology. Results showed that calves immunized with IbpA DR2 had a statistically lower percentage of lung with gross lesions than controls, fewer histologic abnormalities in affected areas and no *H. somni* isolated from residual pneumonic lesions. Calves immunized with the control GST vaccine, IbpA3 or IbpA5 had larger *H. somni* positive pneumonic lesions. ELISA results for serum antibodies showed that calves immunized with the IbpA DR2 antigen had high IgG1 and IgG2 and lowest IgE responses to the immunizing antigen. Specific IgG responses were also high in the bronchial lavage fluid. High specific serum IgE responses were previously shown to be associated with more severe pneumonia, but high IgG specific anti-IbpA DR2 responses seem to be critically related to protection. Since the IbpA DR2 Fic motif has been shown to cause bovine alveolar cells to retract, we tested the neutralizing ability of pooled serum from the IbpA DR2 immunized group. This pooled serum reduced cytotoxicity by 75–80%, suggesting that the protection was due to antibody neutralization of IbpA cytotoxicity, at least in part. Therefore, IbpA DR2 appears to be an important protective antigen of *H. somni*. The study shows, for the first time, that immunization with a purified Fic protein protects against disease in a natural host.

1. Introduction

Histophilus somni (formerly *Haemophilus somnus*) [1] causes pneumonia, septicemia, thrombotic meningoencephalitis, myocarditis, arthritis and reproductive failure in cattle and other ruminants [2–6]. We previously reproduced pneumonia in young calves [7] and showed that convalescent phase serum passively protected calves against *H. somni* pneumonia in this model [8]. The major antigens recognized by the protective convalescent phase serum include 78 kDa and 40 kDa outer membrane proteins [8] (OMPS) and the 270 kDa surface/secreted protein [9]. This 270 kDa antigen is an immunoglobulin binding protein (now called IbpA) which is detected as a series of protein bands spanning 350–76 kDa [10,11]. It was later shown to be a surface fibrillar network which also is shed into the culture supernatant [12]. The gene encoding *H. somni* IbpA (*ibpA*) is homologous with *Bordetella pertussis* filamentous hemagglutinin (*Fha*) in the N terminal half and with *Yersinia* spp. YopT near the C terminus [13]. Deletion of *ibpA* resulted in loss of toxicity for macrophages [14].

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Fig. 1. Diagram of *H. somni* IbpA and the subunits used for vaccination (A3, A5 and DR2). Other motifs include HBD (heparin binding domain), CRD (carbohydrate recognition domain), RGD (integrin binding motif), several approximately 200 bp repeats, the DR1/Fic + DR2/Fic cytotoxic domains and the YopT homology region.

The IbpA deduced amino acid sequence reveals putative adherence domains in the Fha homologous region, a series of approximately 200 bp tandem repeats in the middle and two direct repeats just upstream from the YopT homologous region (Fig. 1) [13]. The two direct repeats (DR1 and DR2) each contain a cytotoxic Fic motif [15]. Three domains (IbpA3, IbpA5 and IbpA DR2) were chosen for expression and testing as recombinant subunit vaccines because of their probable function and because they were recognized by passively protective convalescent phase serum [13,16]. First we showed that IbpA3 and IbpA DR2 provided partial protection in a murine model of *H. somni* septicemia [17]. Now we report studies on immunization of calves with these three recombinant GST fusion proteins followed by intrabronchial challenge with *H. somni*. The experiments showed that IbpA DR2 protected calves against bronchopneumonia and induced high antibody levels specific for IbpA DR2. The pooled post immunization serum from these calves neutralized IbpA DR2/Fic cytotoxicity for bovine alveolar type 2 cells, providing a mechanism for protection.

2. Materials and methods

2.1. Calves

Healthy 5-week-old Holstein bull calves were obtained from the Van Ommering Dairy in San Diego County. All calves received colostrum. Dams were vaccinated against IBR, PI3, BVD, BRSV and Leptospira (Covert 10, Boehringer Ingelheim Vetmedica Inc., St. Joseph, MO). Samples for viral serology were taken at the beginning of the experiment and two weeks after the second IbpA subunit vaccination. Assays for antibodies to BRSV, BVD, PI-3 and IBR were done at the Washington Animal Disease Diagnostic Laboratory, Pullman, WA. All calves had positive titers for all four viruses. Titers either fell or remained stable throughout the experimental period, indicating passive immunity from Covert 10 vaccinated calves, rather than active infection. Calves were housed outside in pens at the UCSD Elliott Research Station. They were fed milk replacer and then calf starter without antibiotics. After a minimum of three days to acclimatize, baseline samples and clinical signs were taken. Calves were randomly assigned to four groups of 5–6 calves each. All experiments were approved by the UCSD Institutional Animal Care and Use Committee.

2.2. Bacteria

H. somni strain 2336 was originally isolated from the lung of a veal calf which died of pneumonia. It was passed once in a calf (#93) in a previous study [18] and then again just before use in this study (calf #5166). Bacteria were stored at −80 °C in 60% glycerol. On removal from the freezer, the isolate was cultured on Columbia Blood Agar (CBA) plates (Difco Laboratories) with 10% bovine blood in Alsever's solution (Colorado Serum Co., Denver, CO) in a candle jar at 37 °C.

2.3. H. somni antigen preparation for in vitro tests

Brain Heart Infusion (Becton, Dickinson and Company, Sparks, MD) broth with 1% Trisma base and 0.01% thiamine monophosphate was inoculated with *H. somni* scraped from an 18 h CBA plate and incubated with shaking for 6 h because preliminary data indicated that little free endotoxin was released by 6 h (unpublished data). The culture was then centrifuged at 8000 × *g* for 10 min, the supernatant removed, filtered through a 0.2 μM filter to remove any residual bacteria and then dialyzed through a 10,000 MW Snake Skin membrane (Pierce, Rockford, IL) overnight against PBS. The bacterial pellet was washed three times with PBS and used as the whole cell antigen in ELISA. The supernatant was used as a source of enriched shed IbpA for Western blots to detect reactivity of pooled antibody from each immunized group with IbpA.

2.4. Recombinant IbpA subunit fusion protein production

Three IbpA domains (A3, A5 and DR2) were cloned and expressed as GST fusion proteins for purification (Fig. 1). Previously described plasmids pHS140 and pHS134 were used for cloning IbpA3 and IbpA5 [13,19] and pHS139 for the DR2 domain [15,17]. The forward and reverse primers were as follows:

for A3:

Forward: 5′-AGCTGGATCCAGTGAACGAATCACTGTAGG-3′,
Reverse: 5′-AGCTGAATTCGAAGCTTTCACAAAACCTGTTTTA-3′;

for A5:

Forward: 5′-AGCTGGATCCGATTTGGATCTTGTAGCGG-3′;
Reverse: 5′-AGCTGAATTCGAGGCATAAATATGATCTGCCG-3′;

for DR2:

Forward: 5′-AGCTCCATGGGAAAATCATCTCCGCAAGAG-3′;
Reverse: 5′-AGCTGGATCCTGATTTTTTTGCCAACTCTTTTAAA-3′.

The PCR-amplified A3 and A5 sequences were ligated into the pET-GSTx vector using the Rapid DNA Ligation Kit (Roche, Nutley, NJ) and the amplified DR2 sequence was ligated into the pET41a vector (Novagen, Darmstadt, Germany). Both vectors carried a glutathione-S-transferase (GST) tag at the N-terminus of the inserted DNA sequence. *Escherichia coli* BL21 DE3 cells were transformed with each of the plasmids. After growing in LB media and inducing with IPTG, the suspension was lysed using a French Press and the recombinant proteins purified with glutathione–agarose beads (Sigma, San Louis, MS). The purified proteins were dialyzed in PBS overnight and protein concentrations determined with the Bio-Rad Protein Assay (BioRad Laboratory Inc., Hercules, CA). Purity was determined by SDS–PAGE and Western blotting (data not shown but previously published [17]).

2.5. Vaccination and challenge of calves

After stabilization for a few days, calves were randomly assigned to vaccine groups. Vaccines were coded so that the experimental team was blinded to the antigen groups. Calves were vaccinated with recombinant subunits as GST fusion proteins (200 μg per dose) or GST alone (negative control at 67 μg which equals the amount of GST in 200 μg of fusion protein). Each of the four treatment groups consisted of 6 calves (except for one group which had 5 calves). Initial 1 ml doses of subunit antigens or the GST control protein were emulsified with 1 ml of Complete Freund's Adjuvant and given subcutaneously. Three weeks later calves were immunized a