Protective Ability and Specificity of Convalescent Serum from Calves with *Haemophilus somnus* Pneumonia

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The ability of convalescent serum to passively protect calves against *Haemophilus somnus*-induced pneumonia was studied. Preimmune and convalescent serum were obtained from calves before or after recovery from experimental chronic *H. somnus* pneumonia. Passive protection was assessed in another group of calves by intrabronchial inoculation of *H. somnus* that had been inoculated with preimmune or convalescent serum. Each calf was inoculated with each treatment in alternating caudal lung lobes. Twenty-four hours after inoculation almost no pneumonia was present in lungs inoculated with bacteria incubated with convalescent serum, whereas severe pneumonia was present in lungs inoculated with bacteria incubated with preimmune serum. Quantitation of calf pneumonia in both treatment groups indicated a significantly different protective capacity between convalescent serum and preimmune serum (*P < 0.0005*). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Western blotting of purified *H. somnus* lipopolysaccharide resulted in intense reactivity with convalescent serum, but no reactivity was detected with preimmune serum. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis of *H. somnus* outer membrane-enriched fractions, Western blots with convalescent serum gave intense reactions against *H. somnus* outer membrane antigens with apparent molecular masses of 78 and 40 kilodaltons and weaker reactions with 60-, 34-, 31-, 29-, 18-, and 15-kilodalton outer membrane antigens. No reactivity was detected with preimmune serum. Antibodies eluted from *H. somnus* after adsorption of convalescent serum reacted almost identically to unadsorbed convalescent serum in Western blots against bacterial outer membrane-enriched fractions. Thus, most of the antigens recognized by convalescent serum are likely to be on the bacterial surface and accessible to antibody. Surface antigens recognized by protective convalescent serum are candidate antigens for a subunit vaccine against *H. somnus* pneumonia.

Pneumonia is one of the most important health problems facing the American cattle industry, with annual losses from respiratory disease estimated to be in excess of $500 million (26). Many agents contribute to this loss, but the role of *Haemophilus somnus* is only beginning to be recognized (40). In addition to causing bovine respiratory disease (2, 17), *H. somnus* is associated with a wide variety of other diseases in cattle, including thromboembolic meningoccephalitis (17, 33) and reproductive failure (7, 17). Clinically normal cattle may also harbor *H. somnus* in the nasal cavity (12, 17) or genital tract (11, 17) or both; however, the relationship between these carrier states and disease has not been established.

Killed *H. somnus* vaccines are commercially available, but there is minimal evidence for the efficacy of such vaccines in the prevention of pneumonia (15, 25). Therefore, development of a more efficacious vaccine is an important research priority. The low level of protection induced by many killed bacterial vaccines is thought to be due to antigenic competition, often resulting in minimal immunity to the most protective antigens (4). Therefore, it is important to know which antigens stimulate protective immunity (27) and to immunize with purified protective immunogens. If humoral immunity is protective, then antigens recognized during the course of infection are potentially protective immunogens. It is probable that humoral immunity is likely to be protective in *H. somnus*-induced pneumonia for several reasons: (i) serum exudation is marked in acute *H. somnus* pneumonia (14a), and thus serum antibody would be present in infected tissues; (ii) immunoglobulin concentrations in nasal secretions and serum are correlated with the susceptibility of young calves to pneumonia (10); and, (iii) antibody is protective in other pneumonias, such as *Pseudomonas aeruginosa* pneumonia in guinea pigs (28). Therefore, we conducted experiments to determine whether preincubation of bacteria with convalescent serum would passively protect calves against *H. somnus* pneumonia and to define the antigenic specificity of protective serum.

**MATERIALS AND METHODS**

**Bacteria.** *H. somnus* 2336 passaged once in a calf was used throughout the study. This strain was isolated at the Washington Animal Disease Diagnostic Laboratory from the lungs of a vealer calf during an outbreak of *H. somnus* pneumonia. Isolates from the primary culture were stored at −70°C in 40% phosphate-buffered saline–60% glycerol.

**Animals.** Holstein bull calves were purchased from dairies in which *H. somnus* disease had not been documented. The calves were housed indoors on bedding of wood shavings or straw.

**Convalescent serum.** Chronic experimental *H. somnus* pneumonia was induced in two 12-week-old male Holstein calves to prepare convalescent serum. The calves were inoculated with 10⁷ CFU of *H. somnus* 2336 prepared from the second 18-h subculture on 10% bovine blood–Columbia blood agar plates (Difco Laboratories, Detroit, Mich.). Bacteria were scraped from the plates, and a standard suspen-

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sion (10^7 CFU) was prepared in 1 ml of sterile saline
and added to 4 ml of sterile RPMI 1640 tissue culture me-
dium (M. A. Bioproducts, Walkersville, Md.). The bacteria were inoculated into a caudal lung lobe with a polyethylene
nasotracheal tube (diameter, 6.5 mm; Bev-a-line tubing; Cole Parmer Instrument Co., Chicago, Ill.). Bronchovascular lavage was performed before inoculation and at weekly intervals after inoculation. Lavage fluid was re-
trieved immediately after instillation of 60 ml of sterile, lacted Ringer solution (Travenol Laboratories, Inc., Deerfield, Ill.). After the fluid was centrifuged at 10,000 x g for 20 min, the pellet was resuspended in 2 ml of superna-
tant, and bacterial plate counts were done in duplicate by the drop method (9). Serum was collected before inoculation and at weekly intervals after inoculation and was stored in small aliquote at -20°C. Calves were necropsied 6 weeks postin-
oculation.

**Enzyme-linked immunosorbent assay.** Antibody titers were determined as described previously (39). Briefly, antigen consisted of Formalin-treated *H. somnus* from a calf-passaged isolate of strain 2336, and antibodies were detected with bovine isotype-specific (immunoglobulin G1 [IgG1], IgG2, and IgM) monoclonal antibodies (provided by A. Guidry, U.S. Department of Agriculture, Beltsville, Md., and W. Davis, Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, Wash.) and peroxidase-conjugated, affinity-purified, goat anti-
mouse IgG (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.). Reactions were developed with hydro-
gen peroxide in 5-aminosalicylic acid, and plates were read in the dual-wavelength mode of an enzyme-linked immuno-
sorbent assay reader (Dynatech Laboratories, Inc., Alex-
andria, Va.). Titers for each serum sample were determined by fitting regression lines of optical density against serum dilution, and from these lines, the reciprocal of the serum dilution at an optical density of 0.2 was determined. Varia-
tion between plates was controlled by including a high-
titered positive control serum on each plate and correcting the sample endpoint as follows: corrected sample endpoint = (sample endpoint for plate X x mean high-tiered serum endpoint of all samples)/high-tiered serum endpoint for plate X.

**Chronic protection experiment.** Pooled convalescent serum samples (collected 6 weeks after infection) or preimmune serum was heated for 30 min at 56°C. Bacteria were prepared as described previously except that 10% fetal calf serum (HyClone Laboratories, Logan, Utah) was substituted for bovine blood in Columbia blood agar plates. Equal volumes of a 1:2 dilution of serum in sterile normal saline and an *H. somnus* suspension (10^7 CFU/ml) were incubated at room temperature for 5 min before intrabronchial inoculation of 2 ml of each reaction mixture (10^6 CFU of *H. somnus*; 1:4 dilution of serum). Bacterial counts were done before and after incubation to determine whether agglutination had occurred. Serum *H. somnus* enzyme-linked immunosorbent assay titers for recipient calves before inoculation were less than 100 for IgG2 and IgM, and mean IgG1 titers were 234 ± 151. Four male Holstein calves (6, 8, 9, and 9 weeks old) were sedated with xylazine (Rompun; Haver-Lockhart, Shawnee, Kans.) and were inoculated by using a flexible fiber-optic bronchoscope (diameter, 6 mm; Machida, Norwood, N.J.). Bacteria incubated with convalescent se-
rum were inoculated into one caudal lung, and bacteria incubated with preimmune serum were inoculated into the contralateral lung. The sides were reversed in each succes-
sive calf. A vehicle control of 2 ml of a 1:4 dilution of normal bovine serum in sterile saline was inoculated into the ante-
rior bronchus of each calf.

Twenty-four hours postinoculation, the calves were hep-
arinized (1,000 IU/kg; intravenous heparin sodium [Ilkins-
Sinn, Inc., Cherry Hill, N.J.J injection) and then killed with an intravenous overdose of sodium pentobarbitone (Veteri-
nary Laboratories, Inc., Lenexa, Kans.). Bronchovascular lavage of left and right caudal lung lobes was done before fixation of the lungs, as described above for preparation of convalescent serum except that only 20 ml of lacted Ringer solution was used. The lungs were perfused with saline before swabs of caudal lungs were taken for bacteriologic and virologic culture and before being fixed by vascular perfusion with 4% formaldehyde–1% glutaraldehyde, as de-
scribed previously (14a). The fixed lungs were sectioned serially every 3 mm starting at the caudal extremity of each caudal lung. The lung slices were designated 0, 1, 2, 3, etc., starting with the caudalmost lung slice. After postfixation overnight in 4% formaldehyde–1% glutaraldehyde, the lesions were delineated grossly and areas of pneumatic lung were determined by using a computerized image analysis system (Bioquant II; R and M Biometrics, Nashville, Tenn.) as described previously (13, 14a). For each calf, the volumes of pneumatic lung in each lung slice (0, 1, 2, 3, etc.) from the left and right sides were compared by the paired *t* test (one tailed). In addition, the paired *t* test (one tailed) was used to compare the total volumes of pneumatic lung for each treatment for all four calves.

Slices of all grades of gross lesions were embedded in paraffin, sectioned at 5 μm, mounted on glass slides, and stained with hematoxylin and eosin; on selected occasions, sections were stained by the method of Gram, as modified by Brown and Hops (23).

**Isolation of bacterial outer membrane-enriched fractions.** After 18 h of growth on Columbia blood agar plates, the first subculture of *H. somnus* was inoculated into brain heart infusion broth (Difco) containing 1 mg of Trizma base per ml and 10 μg of thiamine monophosphate (Sigma Chemical Co., St. Louis, Mo.) per ml and grown overnight at 37°C in a shaking water bath to a density of 5 x 10^8 CFU/ml. The bacteria were washed twice in 0.05 M Tris hydrochloride (pH 7.8) at 4°C, suspended in the same buffer containing 2 mM MOPS (McKernan and sonicated (Biosonik IV; VWR Scientific, San Francisco, Calif.) for 3 min in 15-s bursts (80 W) on ice. Cellulase debris was removed by centrifugation at 10,000 x g for 15 min at 4°C. The supernatant was centrifuged at 230,000 x g for 70 min. The pellet was suspended in 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer at pH 7.4 and extracted with an equal volume of 20% N-lauroylsarcosine (sarcosyl; Sigma) (3) in 10 mM HEPES for 20 min at room temperature. After centrifugation at 175,000 x g for 110 min, the insoluble pellet was resus-
pended in 10 mM HEPES at pH 7.4. Protein concentrations were determined by using the Bio-Rad protein assay (Bio-
Rad Laboratories, Richmond, Calif.) with bovine plasma albumin (Bio-Rad) as a standard.

**Transmission electron microscopy.** Sarcosyl-insoluble pel-
et were also fixed overnight in 2% glutaraldehyde. Samples were rinsed in 0.1 M cacodylate buffer, postfixed in 1% osmium tetroxide, dehydrated in alcohols, cleared in propylene oxide, and embedded in epoxy resin. Ultrathin sections were cut and stained with uranyl acetate and lead citrate.

**LPS isolation.** *H. somnus* lipopolysaccharide (LPS) was extracted with hot 45% phenol, as described by Westphal and Jann (38). Results in our laboratory have shown that this

**SDS-polyacrylamide gel electrophoresis.** For sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, outer membrane-enriched fractions (about 1 mg of protein per ml) or whole bacteria (3 × 10⁹/ml) were solubilized by boiling for 5 min in SDS-gel sample buffer (21), and approximately 5 μg of outer membrane-enriched fraction or 10⁷ bacteria per lane were resolved in 7.5 to 17.5% (wt/vol) polyacrylamide gradient slab gels run under reducing conditions with the buffer system of Laemmli (21) at 35 mA per gel until the dye front reached the bottom of the gel. Protein standards used for molecular weight comparisons consisted of lysozyme (14.4 kilodaltons [kDa]), soybean trypsin inhibitor (21.5 kDa), carboxylic anhydrase (31 kDa), ovalbumin (45 kDa), bovine serum albumin (66.2 kDa), and phosphorylase b (92.5 kDa) (Bio-Rad).

A similar discontinuous gel system (21) with a 15% polyacrylamide running gel incorporating 3 M urea was used to resolve LPS. Purified LPS from *Escherichia coli* O111:B4 and *E. coli* J5 (Sigma) were used as controls. Samples containing 1 to 10 μg of LPS were prepared as described previously (18). LPS was visualized in gels by silver staining (37) or electroblotted onto nitrocellulose.

**Western blots.** After SDS-polyacrylamide gel electrophoresis, the gel was equilibrated for 30 min in transfer buffer (0.025 M Tris base, 0.192 M glycine, 20% methanol), and outer membrane-enriched fraction or LPS was electrophoretically transferred to nitrocellulose paper (pore size, 0.45 μm; Bio-Rad) (36) in a Trans-Blot chamber (Bio-Rad) overnight at 0.1 A (25 V) followed by 70 V for 1 h. Successful transfer of proteins was verified by amido black staining (0.1% in 25% isopropl acetic acid) of a nitrocellulose strip containing the blotted protein standards. The rest of the nitrocellulose was rinsed in TBS-Tween (0.02 M Tris hydrochloride, 0.5 M NaCl, 0.05% [vol/vol] Tween-20 [pH 7.5]) and then incubated in a 1:1,150 dilution of convalescent or preimmune serum (1:1,000 dilutions for LPS blots) or with a 1:30 dilution of convalescent or preimmune eluate in TBS-Tween for 1.5 h at room temperature. After being rinsed twice in TBS-Tween, the nitrocellulose was incubated in a 1:2,000 dilution of porous-conjugated, rabbit anti-bovine IgG (heavy and light chain specific) (Cooper Biomedical, West Chester, Pa.) for 1.5 h at room temperature. Blots were rinsed in distilled water and washed twice in TBS. Reactivity was visualized with 60 mg of 4-chloro-1-naphthol (Bio-Rad) in 20 ml of methanol, added to 100 ml of TBS containing 0.018% hydrogen peroxide added immediately before use. The reaction was allowed to proceed for 3 min (20 min for LPS) before the blots were rinsed in distilled water. The immunoblots were overstained with amido black to stain molecular weight standards or to identify nonimmunoreactive protein bands.

**Affinity purification of antibodies directed against the bacterial surface.** Bacteria were prepared as described above for outer membrane preparations except that broths were harvested during the log phase after 5 h of incubation (approximately 10⁸ CFU/ml). Pellets of live bacteria (approximately 2.5 × 10¹⁰ CFU) were washed twice in sterile saline and suspended in 2 ml of a 1:8 dilution of heat-inactivated (56°C for 30 min) preimmune or convalescent bovine serum for 1 h at room temperature with gentle agitation. After centrifugation at 10,000 × g for 15 min at 4°C, the pellet was washed three times to remove any unattached antibody. The bacterial pellet was suspended in 2 ml of cold (4°C) glycine hydrochloride (1.5 ml of 0.2 M glycine, 0.5 ml of 0.2 M HCl, 58.6 mg of NaCl [pH 2.2]) and centrifuged immediately at 10,000 × g for 15 min at 4°C. The supernatant was recovered and added to 30 μl of 1.5 M Tris hydrochloride (pH 7.4) before dialysis in phosphate-buffered saline at 4°C. The dialyzed supernatant is referred to as the preimmune or convalescent eluate and was diluted 1:30 with TBS-Tween (pH 7.5) for use in immunoblots.

**RESULTS**

Calves with chronic experimental *H. somnus* pneumonia. Calves with chronic experimental pneumonia were mildly depressed for a few days after inoculation, coughed occasionally, and had a mild febrile response. Titers of antibody to *H. somnus* in serum in the two chronically infected calves were almost always within one doubling dilution of each other. IgM titers were highest during the first 2 weeks, whereas IgG1 and IgG2 titers increased more gradually, stabilizing at high antibody concentrations by weeks 2 and 3.

**TABLE 1. Recovery of *H. somnus* from bronchoalveolar lavage fluid of calves with experimental *H. somnus* pneumonia**

<table>
<thead>
<tr>
<th>Wk</th>
<th>No. (log₁₀ CFU) of viable bacteria</th>
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<tbody>
<tr>
<td></td>
<td>Calf E5</td>
</tr>
<tr>
<td>0</td>
<td>NG</td>
</tr>
<tr>
<td>1</td>
<td>NG</td>
</tr>
<tr>
<td>2</td>
<td>0.35</td>
</tr>
<tr>
<td>3</td>
<td>3.23</td>
</tr>
<tr>
<td>4</td>
<td>3.76</td>
</tr>
<tr>
<td>5</td>
<td>1.01</td>
</tr>
<tr>
<td>6</td>
<td>NG</td>
</tr>
<tr>
<td></td>
<td>Calf E7</td>
</tr>
<tr>
<td>0</td>
<td>NG</td>
</tr>
<tr>
<td>1</td>
<td>5.02</td>
</tr>
<tr>
<td>2</td>
<td>5.78</td>
</tr>
<tr>
<td>3</td>
<td>4.27</td>
</tr>
<tr>
<td>4</td>
<td>1.52</td>
</tr>
<tr>
<td>5</td>
<td>3.10</td>
</tr>
<tr>
<td>6</td>
<td>NG</td>
</tr>
</tbody>
</table>

* CFU of *H. somnus* per milliliter of alveolar lavage fluid.
* NG, No growth.

FIG. 1. Geometric mean (n = 2) isotypic serum antibody titers to *H. somnus* in calves with experimental chronic *H. somnus* pneumonia. Symbols: ☐, IgG1; ◊, IgG2; △, IgM.
**FIG. 2.** Passive protection against *H. somnus* pneumonia. Severe pneumonia (arrow) was present in the caudal lung inoculated with preimmune serum and *H. somnus*, whereas the contralateral lung, inoculated with convalescent serum and bacteria, was essentially normal.

(*Fig. 1*) *H. somnus* was recovered by lavage during weeks 1 or 2 through week 5 (Table 1). In calf E7, *Pasteurella multocida* was also isolated during weeks 1, 2, 3, and 5 but always in lower numbers than *H. somnus*. In calf E5, *P. multocida* was recovered in low numbers (10^2 CFU) during week 5 and in high numbers (10^6 CFU) during week 6 (at necropsy). *H. somnus* was not recovered from either calf at necropsy 6 weeks postinoculation. No mycoplasmas, respiratory viruses, or other bacterial pathogens were cultured.

Gross pulmonary changes in both calves were minimal and limited to subpleural and interlobular fibrosis in the caudal aspect of the right caudal lung lobes, correlating with the site of inoculation. In cross sections of lung from these areas, there were occasional, well-demarcated, brown-white firm lobules. Histologically, the grossly affected areas corresponded to areas of fibrosis and neovascularization of subpleural and interlobular septa, with foci of plasma cells, lymphocytes, and macrophages scattered throughout the areas of fibrosis. Bronchial-associated lymphoid tissue was markedly hyperplastic. In one calf (E5) there was a focally extensive abscess and marked, regional, neutrophilic bronchiolitis.

**Passive protection.** The rationale for use of an inoculum dose of 10^7 CFU in the passive protection experiments was based on previous results showing that intrabronchial inoculation of 10^7 CFU of *H. somnus* in the caudal lung of calves induced locally extensive, well-demarcated pneumonia 24 h postinoculation (14a). By use of bronchosopic techniques, each caudal lobe could be inoculated separately, allowing controlled comparison of preimmune serum and convalescent serum protection in the same animal, thereby overcoming the problem of variation between animals and minimizing the number of animals required in experiments. This experimental protocol allows effective definition of the role of immune serum protection against *H. somnus* pneumonia. The bacteria were incubated with serum for only 5 min to allow primary antigen-antibody interactions but minimize secondary antigen-antibody reactions. Bacterial counts in each bacterial suspension were essentially the same before and after opsonization with the sera, verifying that agglutination had not occurred.

During the 24-h course of infection, the animals became moderately depressed, coughed occasionally, and became mildly febrile (mean body temperature increased from 39.1 to 40.1°C). At necropsy, 24 h after inoculation, almost no pneumonia was present in lungs inoculated with bacteria incubated with convalescent serum, whereas moderate-to-severe pneumonia was present in lungs inoculated with bacteria incubated with preimmune serum (Fig. 2). For each calf, the lesion volume in each lung slice of protected lung was significantly different (*P < 0.0005*) from the lesion volume on the unprotected side (Table 2). The difference in the total volumes of pneumatic lung for each treatment for all four calves was also statistically significant (*P < 0.05*).

Occasional, small areas of firm crimson tissue were present in the right cranial lobes of two of the four calves at the site of deposition of the diluent control. *H. somnus* was recovered from swabs of the anterior bronchi in the calves with mild anterior lesions but not from the other two calves. Histologically, typical lesions in protected lungs contained mild, focal exudation of neutrophils into alveoli and bronchioles, as opposed to marked, well-demarcated, fibrinous bronchopneumonia in unprotected lungs (Fig. 3). The latter were characterized by suppurative necrotizing bronchiolitis, vasculitis, lymphatic dilatation, lobular necrosis, hemorrhage, and degeneration of alveolar macrophages. Gram-negative bacteria with the morphology of *H. somnus* were present in alveoli of severely affected areas of unprotected lung in all calves.

The only pathogenic bacterium isolated from the lungs of all four calves was *H. somnus*. Since it is not possible to quantitate bacterial numbers in pulmonary lesions and concurrently perform morphometric analysis, we estimated bacterial numbers in bronchoalveolar lavage samples taken at necropsy from protected and unprotected sides of the lungs. Significantly greater numbers of *H. somnus* were recovered from postmortem lavage of unprotected lungs than from lavage of protected lungs (*P < 0.05*) (Table 3). Viruses, mycoplasmas, and bacterial pathogens other than *H. somnus* were not recovered from the lungs of any calf in this acute experiment.

**TABLE 2. Volumes of pneumatic lung from calves in passive protection experiment**

<table>
<thead>
<tr>
<th>Calf</th>
<th>Vol (cm³) with serum²</th>
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<tbody>
<tr>
<td></td>
<td>Preimmune</td>
<td>Convalescent</td>
</tr>
<tr>
<td>1</td>
<td>19.35</td>
<td>0.03</td>
</tr>
<tr>
<td>2</td>
<td>12.01</td>
<td>0.52</td>
</tr>
<tr>
<td>3</td>
<td>71.19</td>
<td>1.36</td>
</tr>
<tr>
<td>4</td>
<td>97.59</td>
<td>1.89</td>
</tr>
</tbody>
</table>

*Inoculum was incubated with preimmune or convalescent serum. There was a statistically significant difference between the preimmune and convalescent sera when volumes of pneumatic lung were compared for each calf at each level of the lungs; *P < 0.0005* (one-tailed, paired *t* test). There was also a statistically significant difference between the preimmune and convalescent sera when total volumes of pneumatic lung were compared for each treatment for all four calves; *P < 0.05* (one-tailed, paired *t* test).
FIG. 3. Representative slices from protected and unprotected lungs. (A) Protected lung inoculated with *H. somnus* and convalescent serum. Note the small focus of tan firm tissue around a small bronchus (arrows). (B) Unprotected lung inoculated with *H. somnus* and preimmune serum. Locally extensive, well-demarcated, brown and red-gray firm tissue (arrows) is apparent. Note the marked distension of the interlobular septa and fibrin accumulation in the affected lung but not in the protected lung.

**Electron microscopy.** Sarcosyl-insoluble pellets were composed of abundant vesicles and ribbons of bilaminar membranes characteristic of the outer membranes of gram-negative bacteria (Fig. 4).

**Western blots of bacterial lysates and outer membrane-enriched fractions.** Immunoblots of whole bacterial lysates had essentially no reactivity with preimmune serum, but with convalescent serum there was intense reactivity at 78 and 40 kDa and weaker reactivity at 60, 38, 34, 31, 18, and 15 kDa (Fig. 5). More than 40 nonimmunoreactive protein bands were visualized after counterstaining of replicate blots with amido black (Fig. 5). Interestingly, the 41-kDa predominant outer membrane protein was not immunoreactive (Fig. 6).

With immunoblots of outer membrane-enriched fractions, no reactivity was detected with preimmune serum at 1:1,500 dilution, but with convalescent serum there was intense reactivity at 78 and 40 kDa and weaker reactivity at 60, 34, 31, 29, 18, and 15 kDa (Fig. 6). If blots of outer membrane-enriched fractions were developed for a longer time, faint bands were recognized by convalescent serum at 38 and 27 kDa (data not shown). The reactivity observed with preimmune and convalescent eluates was almost identical to that observed with the corresponding homologous antisera (Fig.
6). Convalescent sera and eluates from both calves produced essentially identical results in Western blots (data for one calf are shown in Fig. 6).

**LPS electrophoretic profile and Western blotting.** In SDS-polyacrylamide gels *H. somnus* LPS (1 to 2 µg) contained five closely spaced bands of slightly higher molecular weight than that of LPS from the rough mutant *E. coli* 15 and lacked the extensive heterogeneity of LPS from *E. coli* 0111:B4 (Fig. 7). In Western blots (2 µg of LPS per well), convalescent serum reacted intensely with two bands at serum dilutions for which no reactivity was observed with preimmune serum (Fig. 6).

**DISCUSSION**

The initial objective of this study was to determine whether convalescent serum protects calves against *H. somnus* pneumonia. Serum samples used in this study were collected from calves convalescing from *H. somnus* pneumonia, as indicated by the presence of mild, clinical respiratory disease in the inoculated calves, the ability to isolate *H. somnus* from bronchoalveolar lavage fluid of infected calves for 5 weeks after inoculation, the marked elevations of serum IgG1 and IgG2 against *H. somnus* in infected calves, and the presence of mild, resolving, chronic pneumonia at the site of inoculation, as determined at necropsy. These findings indicate that *H. somnus* was not cleared rapidly from the lungs and was likely the primary cause of the pneumonia observed at necropsy. The contribution of *P. multocida*, which was recovered from both calves, is not clear; however, *P. multocida* has been recovered in association with *H. somnus* in naturally occurring bovine pneumonia (1,31) and can cause lesions similar to those observed in naturally occurring *H. somnus* pneumonia (2). In both calves with chronic pneumonia, when *P. multocida* was isolated from bronchoalveolar lavage fluid in the first 5 weeks after inoculation, it was always recovered in lower numbers than was *H. somnus*, suggesting that *H. somnus* is likely to have been the major pathogen. Since *H. somnus* is known to be toxic for alveolar macrophages (H. D. Liggett, L. Huston, and L. B. Corbeil, Abstr. Annu. Meet. Conf. Res. Work. Anim. Dis. 1984, 174, p. 31) and impairs bovine neutrophil function (16), it may predispose calves to secondary infection with *P. multocida*.

Convalescent sera protected calves against *H. somnus* pneumonia, when they were examined 24 h postinoculation.

![FIG. 4. Transmission electron micrograph of outer membrane-enriched fraction of *H. somnus*. Note abundant vesicles and ribbons of bilaminar membranes. Magnification, ×160,000.](http://iai.asm.org/)

![FIG. 5. Western blots of whole bacterial lysates reacted with preimmune and convalescent sera. (A) Immunoreactivity; (B) immunoreactivity plus proteins overstained with amido black. Lanes: 1 and 3, preimmune serum (1:1,500); 2 and 4, convalescent serum (1:1,500). Note that the convalescent serum recognized multiple antigens but that no immunoreactivity was detected with preimmune serum (panel A). In excess of 40 nonimmunoreactive protein bands were detected when proteins were overstained with amido black (panel B).](http://iai.asm.org/)
Recovery of *H. somnus* as the sole microbial (bacterial or viral) pulmonary pathogen from lungs in passive protection experiments confirmed that the pneumonia was caused by *H. somnus*. The protective capacity of convalescent serum compared with preimmune serum was correlated with increased titers of IgG1 and IgG2 antibody to *H. somnus*. This is consistent with our previous finding that antibody is important in protection against calfhood pneumonia, in that high susceptibility to pneumonia correlates with the lowest IgG1, IgG2, and IgA concentrations in serum and in nasal secretions (10). In pneumococcal disease, specific antcapsular antibody is protective (8), but a capsule has not been identified for *H. somnus* (32); therefore, outer membrane protein and LPS are likely candidate antigens for inducing protection against *H. somnus* pneumonia. There are many examples of gram-negative bacterial infections in which antibodies to LPS or outer membrane protein conferred protection. Antibodies prepared against whole boiled *E. coli* J5 (an O-polysaccharide-deficient mutant) have been shown to confer protection against experimental rabbit endotoxemia induced by purified endotoxins from *E. coli* and *Salmonella typhimurium* (5, 6) and experimental *Haemophilus influenzae* type b septicemia in mice (24). In the last study, antisera prepared against purified *E. coli* J5 LPS gave protection just as potent as that observed with antiserum prepared against whole boiled *E. coli*. More recently, human monoclonal IgM antibodies reactive against a wide range of bacterial LPSs provided protection against a range of gram-negative bacteria in mice (34). Antibodies specific for homologous LPSs protect against *P. aeruginosa* infection in rats (30) and guinea pigs (28), *S. typhimurium* infection in mice (22), and *E. coli* K1 infection in neonatal rats (29). Antibodies to outer membrane proteins also protect against many experimental infections including *S. typhimurium* (20), *H. influenzae* (19), and *Neisseria meningitidis* (14).

Antibody may prevent disease by interacting with host effector functions to eliminate bacteria from the infection site and by neutralizing toxins. Comparison of bacterial numbers recovered from each inoculated lung lobe revealed significantly reduced bacterial numbers in the lobes protected by convalescent serum, suggesting that bacterial elimination is important in protection. In vitro studies have shown that *H. somnus* is toxic for endothelial cells (35) and alveolar macrophages (Liggitt et al., Abstr. Annu. Meet. Conf. Res. Work. Anim. Dis. 1984). In the present in vivo study, pneumonic lesions in protected and unprotected lungs differed quantitatively and qualitatively. Marked serofibrinous exudation, vasculitis, and degeneration of alveolar macrophages were prominent in unprotected lungs 24 h after inoculation but were not present in protected lungs. Serofibrinous exudation is compatible with widespread endothelial injury; therefore, *H. somnus* antibody may mediate endothelial protection. Additionally, the lack of alveolar macrophage degeneration in protected lungs may be associated with antitoxic antibody.

Determination of the specificity of the protective serum would permit identification of antigens potentially useful in prophylaxis. Antigens involved in endothelial or macrophage toxicity or both might also be identified. Relatively few immunodominant antigens were recognized by protective convalescent serum at a dilution of 1:1,500, as shown in...
Western blots against whole bacteria. The predominant serum antibody response was directed to outer membrane antigens of \textit{H. somnus}, since the reactivity for whole bacteria and outer membrane-enriched fractions of \textit{H. somnus} was essentially the same. By adsorbing convalescent serum with live \textit{H. somnus} and eluting the adsorbed antibody, we showed that the predominant antibody reactivity in protective convalescent serum was directed to antigens exposed and accessible on the bacterial surface. This provides added reason to suspect that these antigens may be important in protection, since the bacterial surface is the primary target of host effector functions. In addition, the antibodies in convalescent serum eluted from live \textit{H. somnus} are likely to react predominantly with epitopes that are conserved after solubilization of bacterial antigens, since convalescent serum and eluates reacted almost identically in Western blots of \textit{H. somnus} outer membrane-enriched fractions. The intense reactivity of convalescent serum in Western blots against purified \textit{H. somnus} LPS confirmed that there is a strong response to LPS of the bacterial outer membrane in experimental, chronic \textit{H. somnus} pneumonia.

In summary, convalescent sera obtained from calves with experimental \textit{H. somnus} pneumonia protected calves against acute \textit{H. somnus} pneumonia. The specificity of this protection was directed primarily against surface-accessible antigens of the bacterial outer membrane. Intense reactivity was detected against LPS as well as 78- and 40-kDa outer membrane antigens, with weaker reactions against 60-, 34-, 31-, 29-, 18-, and 15-kDa antigens. It is likely that one or more of these antigens may be useful prophylactically. These antigens may also be useful in serologic diagnosis of \textit{H. somnus} infection, since convalescent calves had high IgG1 and IgG2 titers to \textit{H. somnus} over several weeks.

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LITERATURE CITED


