Protective Ability of Antibodies against 78- and 40-Kilodalton Outer Membrane Antigens of *Haemophilus somnus*

RONALD P. GOGOLEWSKI,[1] STEPHEN A. KANIA,[2] H. DENNY LIGGITT,[1,3] AND LYNETTE B. CORBEIL[1,2]*

Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, Washington 99164-7040[1]; Department of Pathology H811F, University of California at San Diego Medical Center, 225 Dickinson Street, San Diego, California 92103[2]; and Genentech Inc., South San Francisco, California 94080[3]

The ability of concentrated antibody against the 78- or 40-kilodalton (kDa) outer membrane protein (OMP) of *Haemophilus somnus* to passively protect calves against *H. somnus*-induced pneumonia was determined. The 78- and 40-kDa OMPs were evaluated in passive protection experiments, because results of previous studies demonstrated their (i) immunogenicity for cattle, (ii) intense reactivity with convalescent-phase sera which passively protected calves against experimental *H. somnus* pneumonia, (iii) surface location and accessibility to antibody, and (iv) conservation among a wide range of *H. somnus* isolates obtained from animals with different diseases and from different geographic locations. The specificity of the two antisera evaluated in this study was verified by (i) immunoblots in which reactivity against the 78- or 40-kDa OMP was present in postimmunization but not preimmunization serum and (ii) immunoblots in which affinity-purified, surface-reactive antibodies in each antisera were used, which demonstrated that essentially only antibody to the 78- or 40-kDa OMP was reactive with the surface of *H. somnus*. In enzyme-linked immunosorbent assays, the antiserum against the 40-kDA OMP contained immunoglobulin G1 (IgG1), IgG2, and IgM against *H. somnus*, while the antiserum against the 78-kDa OMP contained IgG1 and IgM but no IgG2 against *H. somnus*. The antiserum against the 40-kDa OMP contained IgG1 and IgG2 specific for the 40-kDa OMP, as determined by Western blot analysis. Slight reactivity against *H. somnus* lipopolysaccharide was detected by enzyme-linked immunosorbent assay but not by Western blot analysis. In passive protection experiments, preincubation of bacteria with antibody against the 40-kDa OMP protected calves (*P* < 0.025) against *H. somnus* pneumonia, while antibody against the 78-kDa OMP failed to protect calves against *H. somnus* pneumonia. Determination of the potential protective capacity of the 78-kDa OMP awaits resolution of the role of anti-78-kDa IgG2 in protection against *H. somnus* pneumonia. The 40-kDa OMP is, however, a good candidate antigen for evaluation of protective ability against *H. somnus* pneumonia following active immunization.

Haemophilus somnus is a bovine mucosal and systemic bacterial pathogen which can also colonize the nasal and genital mucosa asymptomatically (10, 20–22). Respiratory disease (1, 22), reproductive failure (6, 22), and thromboembolic meningoencephalitis (22, 41) are the predominant diseases associated with *H. somnus* infections. Losses caused by *H. somnus* pneumonia occur worldwide, with reports of disease from North America (5), Europe (35, 39, 44), and Australia (29). Pneumonia is one of the most important health problems facing the American cattle industry, with annual losses in excess of $500 million (34), and the contribution of *H. somnus* to these losses is only beginning to be recognized (49). In a case-control study in southern Ontario, *H. somnus* was isolated from 28.8% of cases of bovine pneumonia in animals under 1 year of age (S. C. Groom and P. B. Little, Conf. Res. Workers Anim. Dis., abstr. 240, p. 44, 1985). In the midwestern United States, Andrews et al. (1) found that *H. somnus* isolations from cattle with pneumonic lungs were far more frequent than isolations from cattle with thromboembolic meningoencephalitis (162 isolates compared with 21 isolates, respectively, over a 5-year period). This allowed them to conclude that the role of *H. somnus* as an etiologic agent of pneumonia in the United States may be underestimated and often unrecognized. Additionally, the fastidious nature of *H. somnus*, its relatively slow growth in vitro with resultant overgrowth by copathogens or contaminants, and the problem of isolating *H.*

somnus following antibiotic therapy (Groom and Little, Conf. Res. Workers Anim. Dis., abstr. 240) contribute to the probable underestimation of the importance of *H. somnus* pneumonia, since bacterial isolation is generally required for a definitive diagnosis.

Although killed *H. somnus* vaccines are commercially available, their efficacy for the prevention of pneumonia could be improved (18, 32). Antigenic competition is thought to be one possible explanation for the low level of protection induced by many killed vaccines (3). Subunit vaccines (2, 23) or monoclonal antibodies (38) have been shown to protect against bacteria which cause pneumonia in other species, so we directed our investigation of *H. somnus* pneumonia toward prophylaxis with purified antigens that may be important in eliciting protective immunity.

Initially, we produced acute *H. somnus* pneumonia in calves and characterized changes both qualitatively and quantitatively (16). Subsequently, we demonstrated passive protection with serum from calves that had recovered from *H. somnus* pneumonia. This was done by preincubation of bacteria with convalescent-phase serum prior to intrabronchial inoculation into recipient calves (15). Analysis of the specificity of this protective convalescent-phase serum by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) of *H. somnus* outer membrane-enriched fractions or whole bacteria followed by Western blotting revealed that the predominant response was directed against *H. somnus* outer membrane proteins (OMPs) with apparent molecular masses of 78 and 40 kilodaltons (kDa) (15). These

---

antigens were shown to be surface exposed and accessible to antibody (15) and to be conserved among a wide range of *H. somnus* isolates from animals with different diseases and from different geographic locations (7; S. A. Kania, M. S. thesis, Washington State University, Pullman, 1987). They were thus considered to be good candidate antigens for a subunit vaccine against *H. somnus* pneumonia. Furthermore, Stephens et al. (41) have shown that outer membrane complexes protect against *H. somnus*-induced thromboembolic meningoencephalitis. These data provide a basis for the hypothesis that antibodies against the 78-kDa OMP or the 40-kDa OMP (or both) of *H. somnus* protect against *H. somnus* pneumonia.

We report here that preincubation of bacteria with an immunoglobulin fraction containing immunoglobulin G1 (IgG1) and IgG2 antibody against the 40-kDa OMP passively protects calves against *H. somnus* pneumonia, while an immunoglobulin fraction containing IgG1 antibody against the 78-kDa OMP does not passively protect calves against *H. somnus* pneumonia.

MATERIALS AND METHODS

Bacteria. *H. somnus* 2336 was the only pathogen isolated at the Washington Animal Disease Diagnostic Laboratory (Pullman, Wash.) from the lungs of a 5-week-old vealer calf during an outbreak of *H. somnus* pneumonia. Isolates from the primary culture were stored at $-70°C$ in 40% phosphate-buffered saline (PBS)–60% glycerol. This isolate was thawed and passaged once in a calf, and the calf-passaged isolate of strain 2336 was stored at $-70°C$ for use in this study. For enzyme-linked immunosorbent assays (ELISAs) and SDS-PAGE, *H. somnus* was grown overnight at 37°C in 10% CO_2 on Columbia blood agar plates containing 10% bovine blood. These cultures were then used to seed brain heart infusion broth containing 0.1% Tris base and 0.001% thiamine monophosphate (24). Broth cultures were grown to the late log phase (5 to 6 h) in a shaking water bath at 37°C, yielding approximately 10^9 bacteria per ml. For passive protection experiments, bacteria were subcultured once and grown on Columbia blood agar plates containing 10% fetal bovine serum (HyClone Laboratories, Logan, Utah) for 18 h.

Animals. Holstein bull calves were purchased from dairies in which there was no evidence of *H. somnus* disease. While under study, the calves were housed indoors on rubber mats and were fed grain and alfalfa hay or pellets.

Sera. (i) Convalescent-phase serum. Convalescent-phase serum was prepared as described previously (15). Briefly, a 12-week-old male Holstein calf (E7) was inoculated intrabronchially with 10^7 CFU of *H. somnus*. Preimmune serum was collected before inoculation, and convalescent-phase serum was collected 6 weeks after inoculation. Clinical signs of mild pneumonia developed, and *H. somnus* was recovered by bronchoalveolar lavage on weeks 1, 2, 3, 4, and 5 after inoculation. At necropsy at 6 weeks after inoculation, there was focal, mild, resolving pneumonia at the site of inoculation; and there were marked elevations of IgG1 and IgG2 serum antibodies specific for *H. somnus*, as determined by ELISA (15).

(ii) Antiserum against the 78-kDa OMP. Antiserum against the 78-kDa OMP was prepared as described by Kania (M.S. thesis). In brief, *H. somnus* outer membrane-enriched fractions were obtained by *N*-lauroyl-sarcosine (Sigma Chemical Co., St. Louis, Mo.) solubilization of cytoplasmic membrane components as described previously (15), except that bacteria were disrupted by using a French press at a pressure of

15,000 lb/in^2. Outer membrane-enriched fractions were solubilized in 2% SDS (Sigma) and chromatographed over Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden). Fractions containing the 78-kDa OMP exclusively were identified by SDS-PAGE, followed by Western blots (as described below) reacted with convalescent-phase bovine serum (calf E7). Chromatographically isolated 78-kDa OMP was dialyzed extensively against distilled water for immunization of an 11-week-old calf. The calf was immunized intramuscularly with approximately 200 µg of the 78-kDa OMP emulsified in Freund incomplete adjuvant on weeks 0, 4, and 7. Serum was obtained from this calf prior to immunization and 7 days following the final immunization. The reactivity of the serum was determined by Western blot analysis with whole cells of *H. somnus* 2336 solubilized by boiling in SDS sample-gel buffer (28).

(iii) Antiserum against the 40-kDa OMP. Antiserum against the 40-kDa OMP was prepared by immunizing a 16-week-old calf with gel-purified 40-kDa OMP from SDS-polyacrylamide gels of an *N*-lauroyl-sarcosine-insoluble, *H. somnus* outer membrane-enriched fraction. For each immunization approximately 200 µg of the outer membrane-enriched fraction was solubilized in SDS-gel sample buffer (28) and loaded onto each of two 10% SDS-polyacrylamide slab gels (150 by 120 by 3 mm). Electrophoresis was continued at 70 mA per gel until the dye front reached the bottom of the gel. To confirm the identity of the 40-kDa OMP to be excised from the gel, 5-mm-wide strips were excised from the lateral margins of each SDS-polyacrylamide gel, electroblotted onto nitrocellulose, probed with convalescent-phase bovine serum (calf E7), and overstained with amido black (0.1% in 25% isopropyl acetic acid). Western blots were then aligned with the remained of the SDS-polyacrylamide gel, which was lightly stained with Coomassie brilliant blue (0.075% [wt/vol] in distilled water-methanol-acetic acid [6:4:1]) to identify unequivocally the 40-kDa OMP as well as the 41-kDa major OMP band which did not react with the convalescent-phase serum. An approximately 1-mm-wide strip of SDS-polyacrylamide gel containing the 40-kDa OMP was excised with a scalpel blade immediately below the 41-kDa band. The strip was pulverized with a mortar and pestle containing 1 ml of PBS (pH 7.2). The resultant slurry was emulsified with 1 ml of Freund incomplete adjuvant; and a calf was immunized subcutaneously on weeks 0, 4, 6, 8, 10, 12, and 16. Serum was obtained prior to the first immunization and 7 days following the last immunization. The reactivity of the serum was determined by Western blot analysis with whole *H. somnus* 2336.

Concentration of antibodies against the 78- and 40-kDa OMPs. Serum fractionation was done by adding 82 ml of saturated ammonium sulfate solution to 100 ml of each antiserum sample (19) by gentle mixing at room temperature for 30 min, centrifugation at 10,000 $\times$ *g* for 15 min, and resuspension of the precipitates to a final volume of approximately 25 ml in sterile PBS. This suspension was dialyzed against multiple changes of PBS at 4°C and cultured bacteriologically to verify sterility. Reactivity of the immunoglobulin fraction for *H. somnus* was assessed by ELISA and Western blots. Densitometer tracings (scanning densitometer; model 1650; Bio-Rad Laboratories, Richmond, Calif.) of Western blots were done to compare the degree of reactivity of the antisera and the immunoglobulin fractions.

ELISA. Briefly, antigen consisted of Formalin-treated *H. somnus* (approximately 10^7 CFU per well) from the calf-passaged isolate of strain 2336, and antibodies were detected with bovine isotype-specific (IgG1, IgG2, and IgM) mono-

clonal 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 (heavy chain) (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.). Reactions were developed with hydrogen peroxide in 5-aminosalicylic acid, and plates were read in the dual-wavelength mode (630 and 490 nm) of an ELISA reader (Dynatech Laboratories, Inc., Alexandria, Va.). Titers for each serum sample were derived by fitting regression lines of the optical density (OD) against the serum dilution, and from these lines the reciprocal of the serum dilution was determined at an OD of 0.2. Variation between plates was controlled by including a high-titered positive control serum sample on each plate and correcting the serum endpoint as follows: corrected sample endpoint = (sample endpoint for place X × mean high-titered serum endpoint of all samples)/high-titered serum endpoint for plate X. ELISAs to detect antibody to *H. somnus* lipopolysaccharide (LPS) were done as described above, except that wells were coated with 1 μg of *H. somnus* LPS that was purified by hot phenol-water extraction as described previously (15). If mean backgrounds in the LPS ELISAs were higher than an OD of 0.1, endpoints were taken at twice the mean background level.

SDS-PAGE. Discontinuous SDS-PAGE was done by using 1.5-mm-thick slab gels with stacking and gradient gels of 5% and 7.5 to 17.5% (wt/vol) polyacrylamide, respectively, unless indicated otherwise. Whole bacteria (~3 × 10^9/ml) were solubilized by boiling them for 5 min in SDS-gel sample buffer (28), and approximately 10^8 bacteria per lane were electrophoresed under reducing conditions with the buffer system of Laemmli (28) at 35 mA per gel until the dye front reached the bottom of the gel. Protein standards used for molecular mass comparisons consisted of lysozyme (14.4 kDa), soybean trypsin inhibitor (21.5 kDa), carbonic anhydrase (31 kDa), ovalbumin (45 kDa), bovine serum albumin (66.2 kDa), and phosphorylase *b* (92.5 kDa) (Bio-Rad).

Western blots. After SDS-PAGE, the gel was equilibrated for 30 min in transfer buffer (0.025 M Tris base and 0.192 M glycine [pH 8.3] with 20% methanol) and electrotransferred to nitrocellulose (pore size, 0.45 mm; Bio-Rad) (43) in a Trans-Blot Cell (Bio-Rad) overnight at 0.1 A (30 V) followed by 70 V for 1 h. Successful transfer of proteins was verified by amido black staining (0.1% in 25% isopropyl acetic acid) of a portion of the blot. The rest of the blot was rinsed in 0.02 M Tris hydrochloride–0.5 M NaCl–0.05% (vol/vol) Tween 20 (pH 7.5; Tris-buffered saline [TBS]–Tween) and then incubated for 1.5 h at room temperature in bovine serum that was diluted in TBS-Tween. After being rinsed twice in TBS-Tween, the nitrocellulose was incubated in a 1:2,000 dilution of peroxidase-conjugated, affinity-purified, goat anti-bovine IgG (heavy and light chain specific; Kirkegaard and Perry) for 1.5 h at room temperature. In some cases the goat anti-bovine IgG conjugate was replaced with protein A-peroxidase (Kirkegaard and Perry) or monoclonal mouse anti-bovine IgG1 and IgG2, followed by anti-mouse IgG peroxidase, as described above for the ELISA. Blots were rinsed in distilled water and washed twice in TBS. Immunoreactivity was visualized with 60 mg of 4-chloro-1-naphthol (Bio-Rad) in 20 ml of methanol, which was added to 100 ml of TBS with 0.018% hydrogen peroxide (added immediately before use). The reaction was allowed to proceed for 5 min before the blots were rinsed repeatedly in distilled water and dried.

Monoclonal antibodies against *H. somnus* LPS. BALB/c mice were immunized intraperitoneally with 5 × 10^7 live *H. somnus* and boosted intraperitoneally 3 weeks later with 1 × 10^8 bacteria. Three days later, cell fusions were done. Briefly, 1 × 10^8 spleen cells were mixed with 5 × 10^7 P3U1 cells (a gift from Gerald Schwaber, Boston Children's Hospital, Boston, Mass.) in polyethylene glycol 1500 (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and centrifuged at 150 × g for 3 min. At 8 min after the addition of polyethylene glycol, the cells were washed and suspended in Dulbecco modified Eagle medium containing hypoxanthine-aminopterin-thymidine (Hazelton Research Products, Denver, Penn.) (28) supplemented with 20% fetal bovine serum (Hazelton). The cells were grown in 96-well tissue culture plates. Monoclonal antibodies were detected by ELISA by using *H. somnus* LPS as the antigen. Purified LPS from *H. somnus* was obtained by hot phenol extraction (47), followed by repeated ultracentrifugation of supernatants for 4 h at 100,000 × g, which was repeated until the OD of the supernatant was less than 0.005 at 260 and 280 nm. Nonspecific monoclonal antibodies were identified by ELISA by using uncoated, washed plates and were eliminated from the study. Hybridoma cell lines of interest were cloned by limiting dilution, and supernatants were used as the antibody source.

Surface reactivity of antibodies against the 78- and 40-kDa OMPs. Broth-grown bacteria were harvested in the logarithmic phase after about 4 to 5 h of incubation (approximately 10^8 CFU/ml). Pellets of live bacteria (approximately 1.5 × 10^{10} CFU) were washed twice in sterile saline and suspended in 4 ml of a 1:8 dilution of heat-inactivated (56°C for 30 min) pre- or postimmunization antiserum for 1 h at 4°C with gentle agitation. After centrifugation at 10,000 × g for 15 min at 4°C, the pellet was washed 3 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, and 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 restored to pH 7.4 with 1.5 M Tris hydrochloride (pH 7.4) before dialysis in PBS. The dialyzed supernatant is hereafter referred to as the preimmunization or postimmunization eluate. These eluates were diluted in TBS-Tween (pH 7.5) for use in immunoblots.

Passive protection experiments. The immunoglobulin fractions of antisera (78 or 40 kDa) and their respective preimmunization sera were each heated for 30 min at 56°C. Bacteria, which were suspended in sterile saline (10^7 CFU/ 100 ml), were mixed with immunoglobulin fractions (1.9 ml) and incubated for 15 min at room temperature, before intrabronchial inoculation of each reaction mixture (2 ml). Bacterial counts were done in duplicate by the drop method (8) before and after incubation of bacteria and antibody, to determine whether agglutination occurred. Male Holstein calves (7 to 8 weeks old) were used in each protection experiment. Mean ELISA titers of preinoculation sera to whole *H. somnus* antigens were ≤10 for IgG2, 71 for IgM (range, 32 to 127), and 154 for IgG1 (range, <10 to 289). Calves were sedated with xylazine (Rompun; Haver-Lockhart, Shawnee, Kans.) and were inoculated with a flexible fiber-optic bronchoscope (diameter, 6 mm; Machida, Norwood, N.J.). Bacteria incubated with antibodies against OMPs were inoculated into one caudal lung, and bacteria incubated with the immunoglobulins from the respective preimmunization serum were inoculated into the contralateral lung. Use of the left and right lungs was reversed in each successive calf. A vehicle control of 2 ml of preimmune serum with no bacteria (100 ml of sterile saline and 1.9 ml of

serum) was inoculated into the anterior bronchus of each calf. Twenty-four hours postinoculation, the calves were heparinized (injection of 0.750 IU; of intravenous heparin sodium [Ilkins-Sinn, Inc., Cherry Hill, N.J.] per kg) and then killed with an intravenous overdose of sodium pentobarbitone (Veterinary Laboratories, Inc., Lenexa, Kans.). Swabs were collected from caudal lung lobes and the right cranial lobe and were submitted to the Washington Animal Disease Diagnostic Laboratory for routine bacteriologic culture, including mycoplasmal culture and viral culture for bovine respiratory viruses (parainfluenza type 3 virus, infectious bovine rhinotracheitis virus, and bovine virus diarrhea virus). The lungs were perfused with normal saline via the pulmonary artery before swabs of caudal lungs were taken for bacteriologic and virologic culture. Lungs were fixed by vascular perfusion with 4% formaldehyde–1% glutaraldehyde, as described previously (16). The fixed lungs were sectioned serially every 3 mm starting at the caudal extremity of each caudal lung. After postfixation overnight in 4% formaldehyde–1% glutaraldehyde, the lesions were delineated grossly and areas of pneumonic lung were determined by using a computerized image analysis system (Bioquant IV: R and M Biometrics, Nashville, Tenn.) as described previously (13, 16). The paired student t test (one-tailed) was used to determine whether lungs inoculated with *H. somnus* incubated with each postimmunization immunoglobulin fraction had less pneumonia than lungs inoculated with *H. somnus* incubated with the homologous preimmunization immunoglobulin fraction. Histologic sections were prepared from all grades of gross lesions and were stained with hematoxylin-eosin.

RESULTS

Antisera against the 78- and 40-kDa OMPs. The specificity of antisera against the 78- and 40-kDa OMPs is demonstrated in Western blots (Fig. 1). Preimmunization serum (1:1,500) against the 78- or 40-kDa OMPs did not contain detectable antibody to *H. somnus*, while postimmunization sera (1:1,500) contained reactivity only to the 78- or the 40-kDa OMP. ELISA with whole *H. somnus* cells used as the antigen showed that antiserum against the 78-kDa OMP had a 16-fold increase in IgG1 titer compared with the preimmunization serum, but there was essentially no change in the IgM titer and there was no detectable IgG2 titer (Table 1). In contrast, although antiserum against the 40-kDa OMP had a moderate increase in IgG1 titer (6-fold) and essentially no change in IgM titer, there was a substantial increase in the IgG2 titer (45-fold) compared with the respective preimmunization serum from the same calf (Table 1). In Western blots of solubilized whole *H. somnus* antigens, both IgG1 and IgG2 antibodies were shown to react with the 40-kDa OMP whether the IgG2 was detected by monoclonal anti-IgG2 or protein A peroxidase (Fig. 2). The latter has been shown to react with bovine IgG2 (30; R. P. Gogolewski, unpublished data). The concentration of antibody against the 78- and 40-kDa OMPs generally resulted in two- to threefold increases of each immunoglobulin isotype tested, as determined by ELISA of *H. somnus* (Table 1). In parallel Western blot and densitometry studies (Fig. 3) of unconcentrated and concentrated antibodies against the 78- and 40-kDa OMPs, reactivity was detected with concentrated antibody at one threefold dilution higher than that detected with the homologous unconcentrated serum.

Antisera against both the 78- and 40-kDa OMPs contained antibody against *H. somnus* LPS, as determined by ELISA
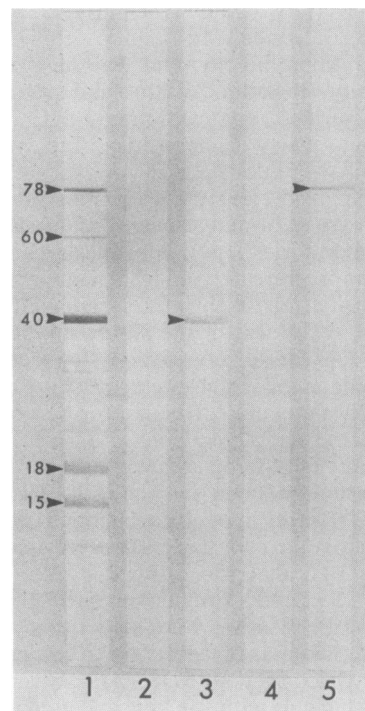

FIG. 1. Western blots of whole *H. somnus* cells reacted with bovine convalescent-phase serum and antisera against the 78- and 40-kDa OMPs. Lane 1, convalescent serum (1:1,500); lane 2, preimmunization 40-kDa antiserum (1:1,500); lane 3, postimmunization 40-kDa antiserum (1:1,500); lane 4, preimmunization 78-kDa antiserum (1:1,500); lane 5, postimmunization 78-kDa antiserum (1:1,500). Note that the 40- or the 78-kDa antiserum react solely with the 40- or 78-kDa OMP, respectively, while the homologous preimmunization serum failed to react. Convalescent-phase serum collected from a calf 6 weeks after intrabronchial inoculation of *H. somnus* reacted intensely with both the 78- and 40-kDa OMPs.

by using purified *H. somnus* LPS as the antigen (Table 2). There was not more than a twofold increase in anti-LPS titer with any of the isotypes tested (IgG1, IgG2, and IgM) in the antiserum against the 78-kDa OMP or with IgM antibodies in the antiserum against the 40-kDa OMP. This was considered to be within the error limits of the assay. In the antiserum against the 40-kDa OMP, IgG1 and IgG2 anti-LPS titers increased about 12- and 4-fold, respectively (Table 2). Antisera against the 78- or 40-kDa OMP at the lowest dilution tested (1:1,500) failed to react with LPS in Western blots of whole cells (Fig. 1) or purified LPS (data not shown). Convalescent-phase serum, however, did react with purified LPS in Western blots at dilutions to 1:2,000, as we have shown previously (15). In order to determine the reason for the increase in antibody against *H. somnus* LPS in the antiserum against the 40-kDa OMP, we addressed the possibility that the gel-purified 40-kDa OMP used for immunizations may have been contaminated with LPS. Western blots of *H. somnus* outer membranes (15) were reacted with monoclonal antibodies against *H. somnus* LPS. Although these monoclonal antibodies reacted with LPS (at a relative molecular mass of 10 to 14 kDa) in blots of outer membranes (Fig. 4), there was no reactivity detected at the level of the 40-kDa OMP, which corresponded to the band cut from the SDS-polyacrylamide gel of outer membranes for emulsification with adjuvant for immunization.

TABLE 1. Titers of antisera determined by ELISA with whole *H. somnus* antigen

| Serum | | Titers of the following antisera: | | | | | |
| | | Preimmunization | | | Postimmunization | | |
Specificity	Treatment	IgG1	IgG2	IgM	IgG1	IgG2	IgM
78-kDa OMP	Whole serum	303	<10	157	5,048	<10	118
78-kDa OMP	Immunoglobulin fraction	1,116	<10	319	9,751	<10	306
40-kDa OMP	Whole serum	224	<21	295	1,526	909	341
40-kDa OMP	Immunoglobulin fraction	594	25	908	4,129	1,965	845

Surface reactivity of antisera against the 78- and 40-kDa OMPs. In order to determine the surface reactivity of antibodies in the pre- and postimmunization antisera, antisera against the 78- and 40-kDa OMPs were adsorbed with live, log-phase *H. somnus*. Adsorptions were done for 1 h at 4°C in order to ensure that the bacteria remained alive during the procedure. Surface-reactive antibodies were eluted from the bacterial surface and are referred to as pre- and postimmunization eluates. In Western blots, postimmunization 78- and 40-kDa eluates reacted intensely with the 78- and 40-kDa OMPs, respectively, while the preimmunization eluates failed to react (Fig. 5). The reactivity observed with pre- and postimmunization eluates was almost identical to that observed with the corresponding homologous antisera. Additional bands with faint reactivities were detected in the 78-kDa pre- and postimmunization eluates but were not detected in blots when the homologous sera were used (1: 1,500). These additional bands stained faintly in blots of both pre- and postimmunization eluates, and therefore, they appeared to be due to nonspecific reactivity. No bands were apparent at relative molecular masses between 10 and 14 kDa, which would be characteristic of *H. somnus* LPS (Fig. 5).

Passive protection. The rationale for conducting passive protection experiments with an inoculum dose of 10^7 CFU of *H. somnus* and bronchoscopic techniques to inoculate each caudal lung lobe in an animal separately was based on results of our prior studies, in which we showed that an inoculum dose of 10^7 CFU of *H. somnus* 2336 reliably produces a focally extensive, well-demarcated pneumonia in calves (15, 16). The bronchoscopic technique permits evaluation of the protective capacity of pre- and postimmunization sera in the same animal, thereby overcoming problems of variation between animals and resulting in the use of a minimal number of experimental animals. In preliminary experiments neither unconcentrated antiserum sample against the 78- or 40-kDa OMP protected passively against *H. somnus* pneumonia; therefore, we decided to fractionate and concentrate immunoglobulin from each of the antisera prior to use in the experiments reported here.

In experiments with concentrated immunoglobulin, bacterial counts of each inoculum sample were essentially the same before and after incubation with pre- or postimmunization immunoglobulins, verifying that bacterial agglutination was not a factor. After inoculation all calves were mildly or moderately depressed, coughed occasionally, and were febrile (mean temperatures increased from 39.2 ± 0.3°C to 40.2 ± 0.5°C). At necropsy 24 h after inoculation, *H. somnus* was recovered from left and right lung lobes and anterior bronchi of all calves. No viruses or other bacterial pathogens, including mycoplasmas, were isolated. The volume of pneumonic lung was significantly ($P < 0.025$) greater in lungs inoculated with bacteria incubated with preimmunization immunoglobulin against the 40-kDa OMP compared with lungs inoculated with bacteria incubated with postimmunization antibody (Table 3). Conversely, although there appeared to be more pneumonia in the presence of antibody to the 78-kDa OMP, no significant difference ($P > 0.05$) in the volume of pneumonia was detected when lungs inoculated with bacteria incubated with pre- or postimmunization immunoglobulin against the 78-kDa OMP were compared (Table 3). Although quantitative differences were identified in volumes of pneumonic lung in sides inoculated with pre- or postimmunization antibody against the 40-kDa OMP, gross lesions were qualitatively similar (Fig. 6). Histologically varying degrees of suppurative bronchopneumonia,
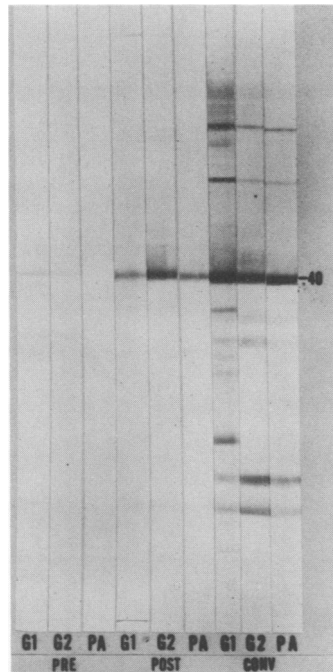

FIG. 2. Western blot of whole *H. somnus* cells (10% SDS-PAGE) reacted with preimmunization (PRE) and postimmunization (POST) serum against the 40-kDa OMP or with convalescent-phase (CONV) serum, all at a dilution of 1:500, and then with mouse monoclonal antibody against bovine IgG1 (G1), bovine IgG2 (G2), or protein A peroxidase (PA); the latter was used as a correlate of IgG2 activity. The mouse monoclonal antibodies were reacted with goat anti-mouse IgG peroxidase. Note that the IgG1 and IgG2 antibodies in convalescent-phase serum reacted with many *H. somnus* antigens, including the immunodominant 40-kDa OMP. The preimmunization serum had minimal reactivity, whereas the postimmunization serum had intense reactivity against the 40-kDa OMP in both the IgG1 and IgG2 subclasses.
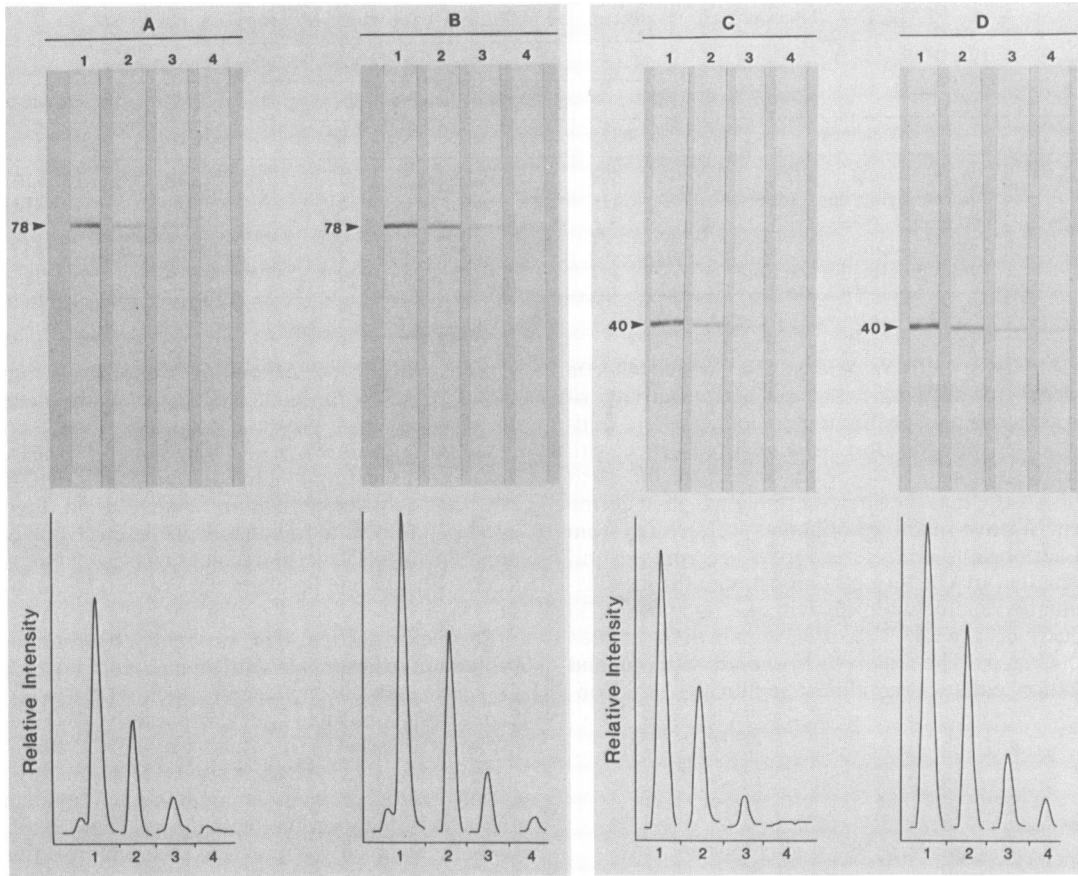
FIG. 3. Western blots and densitometer tracings of whole *H. somnus* cells reacted with 78- and 40-kDa antisera and concentrated immunoglobulin fractions. (A and C) Immunoreactivity of the 78- and 40-kDa antisera, respectively; (B and D) immunoreactivity of concentrated immunoglobulin fractions against the 78- and 40-kDa OMPs, respectively. Lanes 1, 1:2,000; lanes 2, 1:6,000; lanes 3, 1:18,000; lanes 4, 1:54,000. Each densitometer tracing corresponds to the Western blot immediately above it. Note that both the concentrated fractions of antibody against the 78- and 40-kDa OMPs titrated out approximately one threefold dilution further than the homologous serum.

necrotizing bronchiolitis, lobular necrosis, neutrophilic to fibrinoid vasculitis sometimes associated with thrombosis, degeneration of alveolar macrophages, and dilation and thrombosis of lymphatic tissues were present in protected and unprotected caudal lung lobes of all calves 24 h post-inoculation. The cranial lung lobes (which were inoculated with preimmune serum but not with bacteria) were either normal or contained occasional small focal areas of neutrophilic bronchiolitis and alveolitis.

DISCUSSION

In this study we showed that bovine antibody against the 40-kDa OMP protected, but antibody against the 78-kDa

TABLE 2. Titers of antisera determined by ELISA with purified LPS from *H. somnus* used as the coating antigen

Serum specificity	Serum titers[a]					
	Preimmunization			Postimmunization		
	IgG1	IgG2	IgM	IgG1	IgG2	IgM
78-kDa OMP	<10	<10	51	<10	<10	85
40-kDa OMP	10	<10	141	126	37	264

[a] Unconcentrated serum.

FIG. 4. Western blot of *N*-lauroyl-sarcosine-insoluble outer membrane-enriched fraction of *H. somnus* (~5 µg per lane) reacted with two mouse monoclonal antibodies against *H. somnus* LPS. Lane A, monoclonal antibody 2E7; lane B, monoclonal antibody 2E9. Note that the monoclonal antibodies reacted with antigens with low relative molecular masses, which is characteristic of *H. somnus* LPS (15), but not with the 40-kDa OMP.
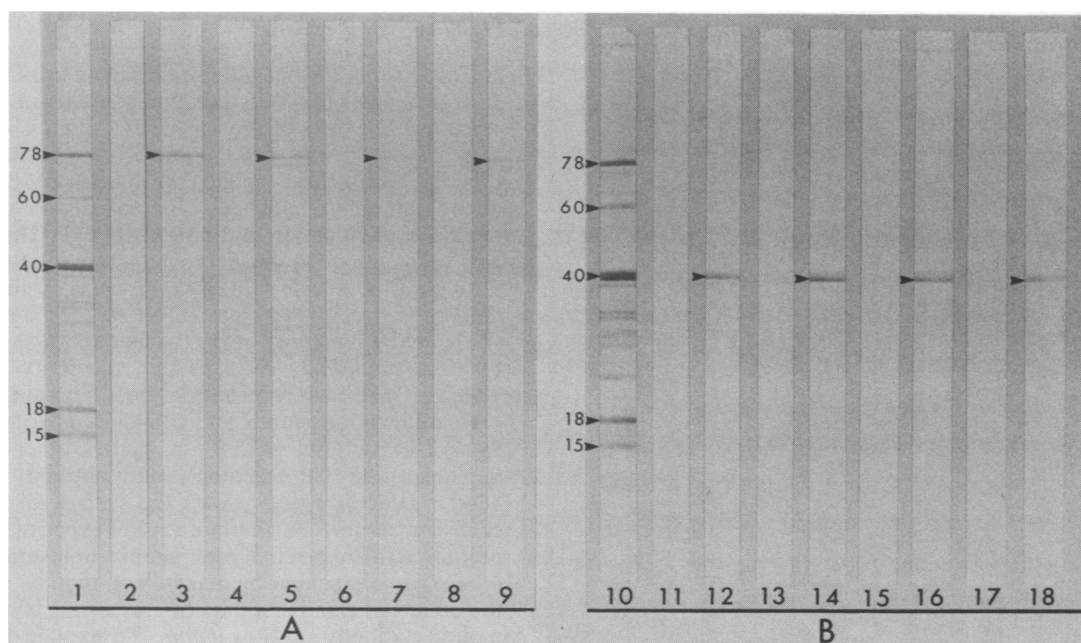
FIG. 5. Western blots of whole *H. somnus* cells reacted with bovine convalescent serum or pre- and postimmunization 78- or 40-kDa antiserum and eluates. (A) The 78-kDa antiserum and eluates; (B) the 40-kDa antiserum and eluates. Lanes 1 and 10, convalescent-phase *H. somnus* serum (1:1,500); lanes 2 and 11, preimmunization antisera (1:1,500); lanes 3 and 12, postimmunization antisera (1:1,500); lanes 4, 6, and 8, preimmunization eluates (1:5, 1:10, and 1:20, respectively); lanes 5, 7, and 9, postimmunization eluates (1:5, 1:10, and 1:20, respectively); lanes 13, 15, and 17, preimmunization eluates (1:20, 1:30, and 1:40, respectively); lanes 14, 16, and 18, postimmunization eluates (1:20, 1:30, and 1:40, respectively). Note that respective postimmunization eluates and postimmunization antisera have similar immunore-activities.

OMP did not protect, calves against *H. somnus* pneumonia. Although the antisera prepared against the 78- and 40-kDa OMPs were essentially monospecific, anti-LPS antibody was present in both sera. The increase in anti-LPS activity was twofold or less for all three isotypes of anti-78-kDa antibody. Since the preimmunization serum did not protect against infection and the difference in titer between pre- and post-immunization sera was within the error of the tests, we concluded that this anti-LPS antibody could be discounted. In the antiserum against the 40-kDa OMP, there was no

TABLE 3. Volumes of pneumonic lung from calves in passive protection experiments

Monospecific antiserum specificity	Calf no.	Vol (cm³) with the following antibodies[a]:	
		Preimmunization	Postimmunization
78-kDa OMP	1	43.78	46.09
	2	36.49	79.73
	3	48.75	68.04
	4	17.62	147.18
40-kDa OMP	5	34.95	18.61
	6	41.59	5.37
	7	71.84	16.10
	8	25.03	10.01

[a] Inoculum was preincubated for 15 min at room temperature with pre- or postimmunization antibody. There was a statistically significant difference between pre- and postimmunization 40-kDa antibody when total volumes of pneumonic lung were compared for calves 5 to 8; $P < 0.025$ (one-tailed, paired Student t test). No significant difference ($P < 0.05$, one-tailed, paired Student t test) was detected between pre- and postimmunization 78-kDa antiserum when total volumes of pneumonic lung were compared for calves 1 to 4.

increase in anti-LPS IgM, but there was approximately a 12-fold increase in IgG1 and a 4-fold increase in IgG2 anti-LPS antibodies in the postimmunization serum titers, as detected by ELISA against purified *H. somnus* LPS. Since anti-LPS titers were much lower than titers against whole formalinized *H. somnus*, it appears that antibodies of other specificities may predominate. This assumption was upheld in Western blots of whole *H. somnus* in which a 1:1,500 dilution of antiserum against the 40-kDa OMP showed intense reactivity with the 40-kDa OMP but no detectable reactivity with the *H. somnus* LPS. Also, the antiserum against the 40-kDa OMP did not react with purified LPS in Western blots (data not shown), although two or more bands were detected with convalescent-phase serum (15). Thus, it appears that there is much less anti-LPS reactivity compared with anti-40-kDa OMP reactivity in the antiserum against the 40-kDa OMP.

In order to determine whether the slight increase in antibody to *H. somnus* LPS was due to contamination of the 40-kDa OMP that was used for immunization, Western blots of *H. somnus* outer membranes were reacted with either convalescent-phase serum or monoclonal antibodies to *H. somnus* LPS. Although the monoclonal antibodies detected several closely spaced low-molecular-weight bands when reacted against LPS in Western blots, no reactivity of the anti-LPS monoclonal antibodies against the 40-kDa band was recognized in blots of outer membranes. In a parallel strip of blotted outer membrane antigens, convalescent-phase serum detected an intense band at 40-kDa and low-molecular-mass bands at 10 to 14 kDa. Since the 40-kDa antigen used for immunization was prepared by excising the 40-kDa band from an SDS-polyacrylamide gel of outer
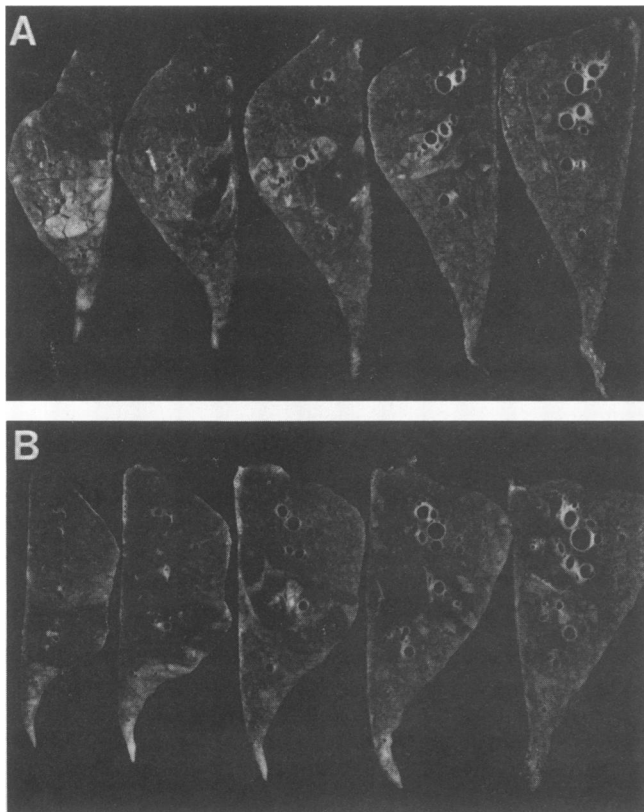
FIG. 6. Representative slices of protected and unprotected lungs inoculated with *H. somnus* and pre- or postimmunization 40-kDa antibody. (A) Protected lung inoculated with *H. somnus* and post-immunization 40-kDa OMP antibody. (B) Unprotected lung inoculated with *H. somnus* and preimmunization 40-kDa OMP serum. Note the qualitative similarities in pneumonic lesions but the marked quantitative differences in lesion areas.

membranes, the lack of reactivity of this band with mono-clonal antibodies to *H. somnus* LPS suggests that LPS contamination of the gel-purified band was unlikely. Further-more, we have demonstrated that it is very difficult to immunize cattle with purified *H. somnus* LPS, even when 100 μg of LPS emulsified in Freund incomplete adjuvant is given intramuscularly 6 times over 4 months (unpublished data). This is perhaps due to the lack of O side chains of *H. somnus* LPS (T. J. Inzana, R. Gogolewski, and L. B. Corbeil, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, B183, p. 54). Thus, it is unlikely that undetectable amounts of LPS in the gel-purified 40-kDa OMP would account for increases in anti-LPS antibodies in the postimmunization sera. This result is in contrast to reports of LPS contamina-tion of OMP preparations in protection studies with *Hae-mophilus influenzae* type b (17) and *Salmonella typhimurium* (40). The 39-kDa OMP of *H. influenzae* type b, which was purified by differential solubility in detergents, was found to contain LPS and to elicit anti-LPS antibody following immu-nization of rats (17). In studies with purified porins from *S. typhimurium*, protection observed in a mouse model was originally reported to be due to anti-porin antibodies (27), but was later shown to be due to antibodies directed against LPS contaminants of the porin protein preparation (40). Results of the latter study are different from those of our study, in that *Salmonella* LPS has long O side chains (42), making *Salmonella* LPS much more immunogenic than *H.*

somnus LPS. The major reason for increasing anti-LPS titers in the calf immunized with the 40-kDa OMP may be the age of the calf, since young calves have very low natural antibody titers (9) but older cattle have cross-reacting anti-LPS antibodies (12). Postimmunization serum was collected at 19 and 33 weeks of age for the 78- and the 40-kDa OMPs, respectively. Therefore, the small increase in anti-LPS ac-tivity in the antiserum against the 40-kDa OMP was probably due to natural antibodies that increased with age rather than to LPS contamination of the 40-kDa OMP. The low levels of anti-LPS antibody, however, may have contributed to the protection observed with the antiserum against the 40-kDa OMP.

The major reactivity of the two antisera was demonstrated by (i) immunoblots showing that reactivity to the 40- or 78-kDa OMP was present in postimmunization but not preimmunization serum and (ii) immunoblots with eluates from live bacterial adsorption studies of each antiserum, demonstrating that essentially only antibody to the 40- or 78-kDa OMP is reactive with the surface of *H. somnus* in each antiserum. Furthermore, the experimental protocol permitted the respective preimmunization sera to be used as controls in each experimental animal, thereby controlling for the effects of small amounts of preexisting cross-reactive or natural antibody to *H. somnus*. To explain the protection with the antiserum to the 40-kDa OMP but not the antiserum to the 78-kDa OMP, it is necessary to address several important characteristics of the antisera, including (i) isotype distribution, (ii)antibody specificity, and (iii) antibody titer.

These two antisera were markedly different in their isotype distributions of antibodies against *H. somnus*, in that the protective immunoglobulin fraction contained IgG1, IgG2, and IgM antibodies, while the nonprotective immuno-globulin fraction against the 78-kDa OMP contained only IgG1 and IgM but no detectable IgG2 against *H. somnus*, as determined by ELISA. Pre- and postimmunization immuno-globulin fractions contained similar IgM titers against *H. somnus*, but the preimmunization immunoglobulin fraction did not afford protection in either case, indicating that IgM antibodies against the OMPs do not play a role in protection. Since both protective and nonprotective immunoglobulin fractions had IgG1 antibodies to the selected OMPs, whereas only the protective antiserum had IgG2 antibodies, it ap-pears that IgG2 may be important in providing protection against *H. somnus* pneumonia. The fact that the IgG2 antibodies against the 40-kDa OMP reacted only with the 40-kDa antigen in Western blots strengthens the hypothesis that IgG2 antibodies to OMP may play a significant role. Several lines of evidence indicate that IgG2 is important in offering protection against several bacterial diseases in cat-tle. Red Danish milk cattle that are deficient in IgG2 have reduced resistance to pyogenic infections (37). Of the IgG2-deficient animals, 36% (8 of 22) had bronchopneumonia (37). Furthermore, protection against staphylococcal mastitis in cattle and sheep is principally correlated with levels of anti-staphylococcal IgG2 in serum (45, 46). Receptors on neutrophils for the Fc region of IgG2 transport cytophilic IgG2 (4) into the mammary secretion, where the opsonizing activity of cytophilic IgG2 of appropriate specificity can increase the clearance of *Staphylococcus aureus*. Similarly, ovine IgG2 specific for the surface of *Bacteroides nodosus* is a strong opsonin for neutrophils and therefore is thought to be important in protection (14). Since neutrophilic infiltrates characterize acute *H. somnus* pneumonia (16), similar IgG2-mediated mechanisms may be important in protecting calves against *H. somnus* pneumonia. Recently, Czuprynski and

Hamilton (11) have shown that bovine peripheral blood neutrophils ingest but do not kill *H. somnus* in vitro. However, the *H. somnus* antiserum used in those experiments was prepared by subcutaneous injection of Formalin-killed bacteria, and there was no information on the distribution of immunoglobulin isotypes in the antiserum. It may be that Formalin-killed *H. somnus* did not stimulate IgG2 antibodies, since vaccination of sheep and cattle with killed *S. aureus* has been shown to produce predominantly an IgG1 response, whereas immunization with live *S. aureus* stimulates a substantial IgG2 response (25, 45, 46). We have shown that convalescent-phase serum from calves with *H. somnus* pneumonia is passively protective in vivo (15). This protective convalescent-phase serum had both IgG1 and IgG2 antibodies against both the 78- and 40-kDa antigens (unpublished data). Hence, it is possible that cytophilic IgG2 with an appropriate specificity may be important in the opsonization and killing of *H. somnus* by bovine neutrophils in the lungs. Earlier, Corbeil et al. (9) have shown that immunoglobulin concentrations in serum and nasal secretions of young calves correlated inversely with the occurrence of pneumonia. In that study, the peak onset of pneumonia occurred when the IgG1, IgG2, and IgA concentrations in the sera of calves were lowest; and as IgG2 concentrations increased, fewer calves developed pneumonia, suggesting a role for IgG2 in protection against pneumonia. It has also been shown that bovine IgG2 is more opsonic than IgG1 (33). Hence, if opsonization is important in the clearance of *H. somnus*, then specific IgG2 against *H. somnus* is likely to be protective. In our studies of experimental *H. somnus* abortion in cattle, the greatest and most persistent specific serum antibody response was within the IgG2 subclass; and prechallenge *H. somnus* IgG2 titers were lower in animals that aborted than in animals that calved normally, suggesting that IgG2 antibodies against *H. somnus* may limit hematogenous dissemination of *H. somnus* (48). Therefore, many lines of evidence indicate that IgG2 plays a potentially important role in offering protection against bacterial diseases in cattle in general and against *H. somnus* in particular. The lack of protection offered by the antibodies against the 78-kDa OMP may have been due to its lack of specific IgG2 antibody. Studies are in progress to elicit an IgG2 response against the purified 78-kDa OMP, since we know that infected cattle produce IgG2 antibodies against the 78-kDa OMP (unpublished data). Then, we will be able to determine whether antibodies of this isotype, which are specific for the 78-kDa antigen, are protective. The protection demonstrated with the antibodies against the 40-kDa OMP may be due, in part at least, to the presence of IgG2 antibodies that are specific for the 40-kDa OMP.

The importance of antibody specificity in protection also has been highlighted by numerous studies involving a diverse range of bacteria, which indicate differences among OMPs in their ability to induce protective immunity. For example, polyclonal antibodies to the P5 OMP of *H. influenzae* type b (36) and monoclonal antibody to the porin OMP of *Pseudomonas aeruginosa* (38) are not protective in respective animal models of disease, while monoclonal antibody to the 98-kDa OMP of *H. influenzae* type b (26) and polyclonal antibodies to P6 (36) or Pl (protein a) (31) OMP of *H. influenzae* type b are protective in their respective animal models of disease. Thus, some antibody specificities to OMPs protect and others do not.

Antibody titer is also likely to be an important factor to consider in protection. In preliminary experiments, neither unconcentrated antiserum against the 78- or 40-kDa OMP

passively protected animals against *H. somnus* pneumonia; therefore, we decided to concentrate the respective antibodies before we used them in full-scale experiments. Fractionation of antisera and concentration of the immunoglobulins proved decisive for the antibody against the 40-kDa OMP. Protection was demonstrated with concentrated antibody against the 40-kDa OMP but not with concentrated antibody against the 78-kDa OMP, even through the IgG1-specific ELISA titer of the latter was higher than the IgG1 titer of the protective 40-kDa antibody preparation. The lack of protection afforded by the antibody against the 78-kDa OMP may have been due to (i) a lack of anti-78-kDa IgG2, (ii) the fact that the 78-kDa OMP did not have a protective specificity, (iii) insufficient total anti-78-kDa antibody; or (iv) a combination of these three possibilities. Resolution of this question is dependent on results of further studies.

In summary, results of this study indicate that preincubation of bacteria with IgG1 antibody against the 78-kDa OMP fails to protect calves passively against *H. somnus* pneumonia, while IgG1 and IgG2 antibodies against the 40-kDa OMP protect calves passively against *H. somnus* pneumonia. Therefore, the vaccinogenic potential of the 78-kDa OMP is not clear. However, the 40-kDa OMP is a good candidate antigen for active immunization against *H. somnus* pneumonia.

LITERATURE CITED

1. **Andrews, J. J., T. D. Anderson, L. N. Slife, and G. W. Stevenson.** 1985. Microscopic lesions associated with the isolation of *Haemophilus somnus* from pneumonic lungs. Vet. Pathol. **22**:131–136.
2. **Austrian, R., R. M. Douglas, and G. Schiffman.** 1976. Prevention of pneumonococcal pneumonia by vaccination. Trans. Assoc. Am. Physiol. **89**:184–194.
3. **Bjornson, A. B., and J. G. Michael.** 1970. Biological activities of rabbit immunoglobulin M and immunoglobulin G antibodies to *Pseudomonas aeruginosa* in mice. Infect. Immun. **2**:453–461.
4. **Brandon, M. R., D. L. Watson, and A. K. Lascelles.** 1971. The mechanism of transfer of immunoglobulin into mammary secretion of cows. Aust. J. Exp. Biol. Med. Sci. **49**:613–623.
5. **Brown, L. N., R. C. Dillman, and R. E. Dierks.** 1970. The *Haemophilus somnus* complex. Proc. U.S. Anim. Health Assoc. **74**:94–108.
6. **Chladek, D. W.** 1975. Bovine abortion associated with *Haemophilus somnus* Am. J. Vet. Res. **36**:1041.
7. **Corbeil, L. B., J. E. Arthur, P. R. Widders, J. W. Smith, and A. F. Barbet.** 1987. Antigenic specificity of convalescent serum from cattle with *Haemophilus somnus*-induced abortion. Infect. Immun. **55**:1381–1386.
8. **Corbeil, L. B., K. Blau, D. J. Prieur, and A. C. S. Ward.** 1985. Serum susceptibility of *Haemophilus somnus* from bovine clinical cases and carriers. J. Clin. Microbiol. **22**:192–198.
9. **Corbeil, L. B., B. Watt, R. R. Corbeil, T. G. Betzen, R. K. Brownson, and J. L. Morril.** 1984. Immunoglobulin concentrations in serum and nasal secretions of calves at the onset of pneumonia. Am. J. Vet. Res. **45**:773–778.
10. **Corbeil, L. B., P. R. Widders, R. Gogolewski, J. Arthur, T. J. Inzana, and A. C. S. Ward.** 1986. *Haemophilus somnus*: bovine reproductive and respiratory disease. Can. Vet. J. **27**:90–94.
11. **Czuprynski, C. J., and H. L. Hamilton.** 1985. Bovine neutrophils

ingest but do not kill *Haemophilus somnus* in vitro. Infect. Immun. **50**:431–436.

12. **Duncan, J. R., B. N. Wilkie, and A. J. Winter.** 1972. Natural and immune antibodies for *Vibrio fetus* in serum and secretions of cattle. Infect. Immun. **5**:728–733.

13. **Dunnill, M. S.** 1968. Quantitative methods in histology, p. 401–416. *In* S. C. Dyke (ed.), Recent advances in clinical pathology: series V. J & A Churchill Ltd., London.

14. **Emery, D. L., and D. J. Stewart.** 1984. Phagocytosis of *Bacteroides nodosus* by ovine peripheral blood leukocytes. Vet. Microbiol. **9**:169–179.

15. **Gogolewski, R. P., S. A. Kania, T. J. Inzana, P. R. Widders, H. D. Liggitt, and L. B. Corbeil.** 1987. Protective ability and specificity of convalescent serum from calves with *Haemophilus somnus* pneumonia. Infect. Immun. **55**:1403–1411.

16. **Gogolewski, R. P., C. W. Leathers, H. D. Liggitt, and L. B. Corbeil.** 1987. Experimental *Haemophilus somnus* pneumonia in calves and immunoperoxidase localization of bacteria. Vet Pathol. **24**:250–256.

17. **Gulig, P. A., and E. J. Hansen.** 1985. Coprecipitation of lipopolysaccharide and the 39,000-molecular-weight major outer membrane protein of *Haemophilus influenzae* type b by lipopolysaccharide-directed monoclonal antibody. Infect. Immun. **49**:819–827.

18. **Hall, R. F., J. M. Williams, and G. L. Smith.** 1977. Field evaluation of *Haemophilus somnus* bacterin. Vet. Med. Small Anim. Clin. **72**:1368–1370.

19. **Hudson, L., and F. C. Hay.** 1980. Initial preparations, p. 1–2. *In* Practical immunology. Blackwell Scientific Publications Ltd., Oxford.

20. **Humphrey, J. D., P. B. Little, D. A. Barnum, P. A. Doig, L. R. Stephens, and J. Thorsen.** 1982. Occurrence of *Haemophilus somnus* in bovine semen and in the prepuce of bulls and steers. Can. J. Comp. Med. **46**:215–217.

21. **Humphrey, J. D., P. B. Little, L. R. Stephens, D. A. Barnum, P. A. Doig, and J. Thorsen.** 1982. Prevalence and distribution of *Haemophilus somnus* in the male reproductive tract. Am. J. Vet. Res. **43**:791–794.

22. **Humphrey, J. D., and L. R. Stephens.** 1983. *Haemophilus somnus*: a review. Vet. Bull. **53**:987–1004.

23. **Immunization Practices Advisory Committee.** 1985. Recommendation of the Immunization Practices Advisory Committee (ACIP): polysaccharide vaccine for prevention of *Haemophilus influenzae* type b disease. Morbid. Mortal. Weekly Rep. **34**:201–205.

24. **Inzana, T. J., and L. B. Corbeil.** 1987. Development of a defined medium for *Haemophilus somnus* isolated from cattle. Am. J. Vet. Res. **48**:366–369.

25. **Kennedy, J. W., and D. L. Watson.** 1982. Cellular basis for differences in humoral immune responses of sheep immunized with living or killed *Staphylococcus aureus* vaccines. Aust. J. Exp. Biol. Med. Sci. **60**:643–654.

26. **Kimura, A., P. A. Gulig, G. H. McCracken, Jr., T. A. Loftus, and E. J. Hansen.** 1985. A minor high-molecular-weight outer membrane protein of *Haemophilus influenzae* type b is a protective antigen. Infect. Immun. **47**:253–259.

27. **Kuusi, N., M. Nurminen, H. Saxen, M. Valtonen, and P. H. Mäkelä.** 1979. Immunization with outer membrane proteins in experimental salmonellosis of mice. Infect. immun. **25**:857–862.

28. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.

29. **Lancaster, M. J., D. J. McGillivery, R. M. Patterson, and S. Irwin.** 1984. Pneumonia associated with *Haemophilus somnus* in a calf. Aust. Vet. J. **61**:269.

30. **Lawman, G. J. P., D. R. Ball, E. M. Hoffmann, L. E. DesJardin,**

and M. D. P. Boyle. 1986. Production of *Brucella abortus* specific protein A-reactive antibodies (IgG2) in infected and vaccinated cattle. Vet. Microbiol. **12**:42–53.

31. **Loeb, M. R.** 1987. Protection of infant rats from *Haemophilus influenzae* type b infection by antiserum to purified outer membrane protein a. Infect. Immun. **55**:2612–2618.

32. **Martin, W., S. Acres, E. Janzen, P. Wilson, and B. Allen.** 1984. A field trial of preshipment vaccination of calves. Can. Vet. J. **25**:145–147.

33. **McGuire, T. C., and A. J. Musoke.** 1981. Biologic activities of bovine IgG subclasses. Adv. Exp. Med. Biol. **137**:359–366.

34. **McMillan, C. W.** 1985. Working together, sharing knowledge, p. 64–79. *In* R. W. Loan (ed.), Bovine respiratory disease, a symposium. Texas A&M University Press, College Station, Tex.

35. **Molenda, J., and J. Krozyrczak.** 1980. *Haemophilus somnus* as an etiological agent of pneumonia in calves. Medycyna Wet. **36**:209–212.

36. **Munson, R. S., Jr., and D. M. Granoff.** 1985. Purification and partial characterization of outer membrane proteins P5 and P6 from *Haemophilus influenzae* type b. Infect. Immun. **49**:544–549.

37. **Nanssen, P.** 1972. Selective immunoglobulin deficiency in cattle and susceptibility to infection. Acta Pathol. Microbiol. Scand. Sect. B **80**:49–54.

38. **Pennington, J. E., G. J. Small, M. E. Lostrom, and G. B. Pier.** 1986. Polyclonal and monoclonal antibody therapy for experimental *Pseudomonas aeruginosa* pneumonia. Infect. Immun. **54**:239–244.

39. **Pritchard, D. G., and N. S. M. Macleod.** 1977. The isolation of *Haemophilus somnus* following sudden deaths in suckler calves in Scotland. Vet. Rec. **100**:126–127.

40. **Saxen, H., M. Nurminen, N. Kuusi, S. B. Svenson, and P. H. Mäkelä.** 1986. Evidence for the importance of O antigen specific antibodies in mouse-protective Salmonella outer membrane protein (porin) antisera. Microb. Pathogen. **1**:433–441.

41. **Stephens, L. R., P. B. Little, B. N. Wilkie, and D. A. Barnum.** 1984. Isolation of *Haemophilus somnus* antigens and their use as vaccines for prevention of bovine thromboembolic meningoencephalitis. Am. J. Vet. Res. **45**:234–239.

42. **Svenson, S. B., M. Nurminen, and A. A. Lindberg.** 1979. Artificial *Salmonella* vaccines: O-antigenic oligosaccharide-protein conjugates induce protection against infection with *Salmonella typhimurium*. Infect. Immun. **25**:863–872.

43. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose. Proc. Natl. Acad. Sci. USA **76**:4350–4354.

44. **von Corboz, L., and J. Nicolet.** 1975. Infektionen mit sogenannten *Haemophilus somnus* beim Rind: Isolierung und Charakterisierung von Stämmen aus Respirations- und Geschlechtsorganen. Schweiz. Arch. Tierheilk. **117**:493–502.

45. **Watson, D. L.** 1981. Immunologically-specific resistance to infection with particular reference to staphylococcal mastitis. Adv. Exp. Med. Biol. **137**:579–590.

46. **Watson, D. L.** 1984. Evaluation of attenuated, live staphylococcal mastitis vaccine in lactating heifers. J. Dairy Sci. **67**:2608–2613.

47. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharides. Extraction with phenol-water and further applications of the procedure. Methods Carbohydr. Chem. **5**:83–91.

48. **Widders, P. R., L. G. Paisley, R. P. Gogolewski, J. F. Evermann, J. W. Smith, and L. B. Corbeil.** 1986. Experimental abortion and the systemic immune response in cattle to *Haemophilus somnus*. Infect. Immun. **54**:555–560.

49. **Wikse, S. F.** 1985. Feedlot cattle pneumonias. Vet. Clin. N. Am. Large Anim. Pract. **1**:289–310.