Antibody Response of Swine to Outer Membrane Components of Haemophilus pleuropneumoniae during Infection

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Sera from pigs infected with Haemophilus (Actinobacillus) pleuropneumoniae were tested for antibodies to outer membrane proteins (OMPs) of the organism by immunoblotting. Convalescent sera were produced in naturally born, colostrum-fed pigs and in cesarean-derived, colostrum-deprived pigs given H. pleuropneumoniae serotype 5 intranasally twice at 5-week intervals. Sera, collected at weekly intervals, were reacted with Sarkosyl-insoluble, OMP-enriched preparations of H. pleuropneumoniae which had been separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose. Antibodies were detected to OMPs with an apparent molecular weight of 16,500 (16.5K OMP); to 29K, 38.5K, 43.5K, 45K, 49.5K, and 66.5K OMPs; and to several high-molecular-weight (≥94,000) OMPs, but not to the major 42K OMP. Antibodies to the heat-modifiable OMP (29K/43.5K) and the 38.5K OMP were detected in sera from noninfected pigs. Antibodies were also detected to two broad 54,000- and 95,000-molecular-weight bands which did not stain with Coomassie blue, stained with silver nitrate, resisted proteinase K digestion, and were eliminated by oxidation with sodium metaperiodate. This indicates that the 54,000- and 95,000-molecular-weight bands represent polysaccharide, possibly capsular or lipopolysaccharide immunogens. Adsorption of sera with cells from the homologous serotype 5 strain removed antibodies to the 45K, 49.5K, 66.5K, and ≥94K OMPs and to the two polysaccharide bands, indicating that these antibodies were directed primarily to surface-exposed epitopes. When tested with OMP preparations from other serotype 5 strains, heterogeneity was apparent, both in the reactions with OMPs and with the polysaccharide bands. Silver staining of proteinase K-treated, whole-cell lysates from serotype 5 strains also indicated variable expression of the polysaccharide bands. Sera also reacted with OMPs from H. pleuropneumoniae serotypes 1 and 7; however, several OMPs and the lipopolysaccharide or polysaccharide determinants of these serotypes appeared to be type specific.

Haemophilus (Actinobacillus) pleuropneumoniae is a leading cause of pleuropneumonia in swine throughout the world (36). Specific immunity to infection can be acquired following exposure to the organism (37) or vaccination (17, 24, 38, 51), and antibodies to H. pleuropneumoniae are routinely assayed by several techniques (31, 34, 35, 37). The complement fixation (CF) test, in particular, has proven to be useful for monitoring disease prevalence and for disease control (34, 52; R. Nielsen, Ph.D. thesis, Royal Veterinary and Agricultural University, Copenhagen, Denmark, 1982). Although antibody titers are an indication of past or current infection, they do not always correlate with immunity to disease (40).

Cellular components of H. pleuropneumoniae involved in induction of protective immunity have not been defined. The organism is encapsulated, and nine serotypes, presumably based on capsular antigens, have been recognized (12, 33, 43, 44, 50). Both CF antibodies following infection and immunity following vaccination are serotype specific (39, 41); thus, capsular antigens are believed to be important protective immunogens (Nielsen, Ph.D. thesis). Nielsen (42; Ph.D. thesis) reported that heat-extracted capsular preparations were protective for pigs. Roles for lipopolysaccharide (LPS) (42; D. W. Fenwick and H. J. Olander, Proc. 8th Int. Pig Vet. Soc. Congr., p. 94, 1984) and cytotoxin (R. Hesse, M. Stoll, J. Coon, and R. Simonson, Proc. 8th Int. Pig Vet. Soc. Congr., p. 111, 1984) in the induction of immunity have also been indicated.

Recently, we characterized the outer membrane proteins (OMPs) of H. pleuropneumoniae by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (47). A role for OMPs as protective immunogens has been established for several gram-negative pathogens, including Haemophilus influenzae type b (9), Neisseria meningitidis group B (6), Pseudomonas aeruginosa (8, 16), Salmonella typhimurium (25), and Bordetella bronchiseptica (32). Since immunogenicity of H. pleuropneumoniae OMPs has not been reported previously, we examined the immunogenicity of H. pleuropneumoniae OMPs in pigs experimentally exposed to H. pleuropneumoniae serotype 5. The subsequent antibody response to individual outer membrane (OM) components was determined by immunoblotting with OMP-enriched preparations from the homologous strain, heterologous serotype 5 strains, and serotype 1 and 7 strains. A determination of the individual OM components that elicit an antibody response in the infected host is an initial step in the elucidation of the H. pleuropneumoniae protective antigens.


MATERIALS AND METHODS

Bacterial strains. The acquisition, maintenance, biochemical, morphologic, and serologic characterization of the strains used in this investigation were reported previously (47, 48, 52), and the strains are summarized in Table 1.

Exposure of pigs to H. pleuropneumoniae. Convalescent sera were obtained from pigs exposed to H. pleuropneumoniae 4074 (serotype 1), WF83 (serotype 7), and 200
TABLE 1. *H. pleuropneumoniae* strains

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Serotype</th>
<th>Origin</th>
<th>Source*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A, 155, 163, 178, 200, 201</td>
<td>5</td>
<td>Swine lung, Iowa</td>
<td>IVDL</td>
</tr>
<tr>
<td>119,144</td>
<td>5</td>
<td>Swine lung, Illinois</td>
<td>ADL</td>
</tr>
<tr>
<td>M1</td>
<td>5</td>
<td>Swine lung, Iowa</td>
<td>VMRI</td>
</tr>
<tr>
<td>K17</td>
<td>5</td>
<td>Lamb joint, California</td>
<td>ATCC</td>
</tr>
<tr>
<td>4074</td>
<td>1</td>
<td>Swine lung, Argentina</td>
<td>ATCC</td>
</tr>
<tr>
<td>WF83</td>
<td>7</td>
<td>Swine lung, Ontario</td>
<td>OVC</td>
</tr>
</tbody>
</table>

*IVDL, L. Hoffman, Iowa Veterinary Diagnostic Laboratory, Ames, Iowa; ADL, D. Hoefling, Animal Disease Laboratory, Galesburg, Ill.; VMRI, R. Schultz, Veterinary Medical Research Institute, Ames, Iowa; ATCC, American Type Culture Collection, Rockville, Md.; OVC, S. Rosendal, Ontario Veterinary College, University of Guelph, Guelph, Ontario.*

(serotype 5) as described previously (52). Briefly, pigs from the Iowa State University Animal Resource Station (ARS) herd were inoculated intranasally (1 ml per nostril) with a 4- to 6-h-old culture grown in M96 mycoplasma broth (7) and containing $1.4 \times 10^5$ CFU/ml (strains 4074 and WF83), or $6 \times 10^4$ to $6 \times 10^5$ CFU/ml (strain 200). Sera were collected 28 days after inoculation. The ARS herd is maintained as a closed herd and is known to be free of porcine respiratory pathogens, including *Haemophilus* spp. Pigs were housed in isolation units and fed a 16% protein swine grower ration with no antibiotics or other growth promoters.

Convalescent sera to serotype 5 strain 200 were also produced in 6-week-old caesarean-derived, colostrum-deprived (CDCD) pigs. The Hampshire-cross CDCC pigs, obtained from Veterinary Associates, Manilla, Iowa, were maintained in isolation units and fed 18% protein swine starter ration without added antibiotics or other growth promoters. Three of the pigs were given 2 ml (1 ml per nostril) of *H. pleuropneumoniae* strain 200 that had been grown for 4 to 6 h in M96 mycoplasma broth and diluted to $3.5 \times 10^4$ CFU/ml in brain heart infusion broth (BHI; Difco Laboratories, Detroit, Mich.). *H. pleuropneumoniae* forms two colony types, designated adherent and smooth (47, 48).

The pigs were exposed to strain 200 cells of adherent colony morphology. Two CDCC pigs were held as nonexposed controls. Following inoculation, pigs were observed for signs of pleuropneumonia, and if clinical conditions indicated they were treated with penicillin-streptomycin (Combicid; Pfizer, Terre Haute, Ind.) to ensure survival. Thirty-five days following initial exposure, the three challenged pigs were inoculated with $7.5 \times 10^7$ CFU of strain 200 per ml prepared and administered as described above. All pigs were bled at weekly intervals, and the sera were collected for immunoblotting. Sera were also tested for CF antibodies to *H. pleuropneumoniae* antigen prepared from the homologous strain by previously described procedures (52).

**SDS-PAGE.** OMP-enriched fractions were prepared by selective solubilization with N-lauroyl sarcosinate ( Sarkosyl; Sigma Chemical Co., St. Louis, Mo.) as described previously (1, 47). OMP-enriched preparations for SDS-PAGE were routinely prepared from cultures with smooth colony morphology (47). Discontinuous SDS-PAGE was performed with a 3.8% stacking gel and a 10% separating gel (47). In addition to visualization of OMP bands by staining with Coomassie blue, selected gels were silver stained by a procedure which preferentially stains LPS (18, 57). A smooth LPS from *S. typhimurium* (Sigma) was used for comparison. To determine sensitivity to proteinase K, Sarkosyl-insoluble samples were solubilized in SDS-PAGE treatment buffer at 100°C for 5 min and then treated with 25 μg of proteinase K (Sigma) at 60°C for 60 min prior to SDS-PAGE (18). Sensitivity to periodate oxidation was determined, based on the procedure of Maeland (28). Briefly, samples were treated with 0.01 M sodium metaperiodate (Fisher Scientific Co., Fairlawn, N.J.) for 18 to 20 h at 4°C prior to solubilization in the SDS-PAGE treatment buffer. The SDS-PAGE profiles of whole-cell, proteinase K-treated lysates were determined by a modification of the procedure of Hitchcock and Brown (18). Cultures were grown overnight on modified BHI blood agar (48), harvested, and diluted in TS buffer containing 0.01 M Tris hydrochloride (Fisher) and 0.15 M NaCl (pH 7.4) to an optical density of 0.55 to 0.65 at 600 nm (Spectronic 20; Bausch & Lomb, Inc., Rochester, N.Y.). The preparation contained approximately $6 \times 10^8$ CFU/ml, as determined by viable count. A portion (0.5 ml) of this suspension was centrifuged (Eppendorf model 5412; Brinkmann Instruments, Inc., Westbury, N.Y.) for 5 min, and then cell pellets were solubilized in SDS-PAGE treatment buffer and treated with proteinase K as described above for the Sarkosyl-insoluble preparations.

**Immunoblotting.** Following separation by SDS-PAGE, proteins were electrophoretically transferred to nitrocellulose sheets (0.45 μm pore size; Bio-Rad Laboratories, Richmond, Calif.) by a modification of the procedure of Towbin et al. (56). The transfer buffer was 25 mM sodium phosphate (pH 7.4). Gels were washed for 1 h in transfer buffer, and then proteins were transferred overnight in a transblot cell (Bio-Rad) at a constant voltage of 16 V. In the morning the power was increased to 30 V for 1 h. The nitrocellulose was cut into strips, and subsequent procedures were performed at room temperature on a reciprocating shaker. Strips were first incubated for 1 h in TSM buffer containing 0.01 M Tris hydrochloride, 0.15 M NaCl (pH 7.5), and 5% nonfat dry milk (Carnation Co., Los Angeles, Calif.) as a blocking agent (22). Strips were then incubated for 2 h with the test sera diluted 1:100 in TSM buffer. Following a 30-min wash in TSM buffer, strips were incubated for 2 h with horseradish peroxidase-conjugated, rabbit anti-swine immunoglobulin G, heavy- and light-chain specific (Cappel Laboratories, Cochranville, Pa.) diluted 1:1,500 in TSM buffer. Following two 15-min washings in TSM buffer, bands were visualized by reacting for 15 to 20 min with 0.05% -chloroacetamide (Sigma) and 0.02% H2O2 in 20% methanol and TS buffer (15). The reaction was stopped by washing the strips with water. Each Sarkosyl-insoluble preparation tested was also reacted with gnotobiotic swine serum (supplied by G. Erickson, National Veterinary Services Laboratory, Ames, Iowa) as a negative control. The efficiency of transfer of the protein bands was determined by reacting one nitrocellulose strip for 4 to 6 h with Pelikan Fount India Ink (Pelikan AG, Hanover, Federal Republic of Germany) at a concentration of 1 μl/ml in TS buffer (13). For some gels, the efficiency of transfer was also monitored by Coomassie blue staining of the gel following electrophoretic transfer.

**Adsorption of sera.** Sera were adsorbed with the whole cells of the homologous *H. pleuropneumoniae* 200 by procedures based on those of Loeb (26). Following 5 h of growth in BHI broth containing NAD (47) cells were centrifuged for 20 min at 12,000 × g, suspended in 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; pH 7.4) buffer (Sigma) to a concentration of $6.8 \times 10^8$ CFU/ml, and 1-ml amounts were centrifuged (Eppendorf model 5412; Brinkmann) for 10 min. The cell button was suspended in 0.5 ml of HEPES buffer, and 0.5 ml of heat-inactivated (56°C for 30 min) serum was added to each tube. Following adsorption
for 90 min at 4°C, the serum samples were centrifuged twice, 15 min each, and stored at −20°C.

RESULTS

Immunoblotting with sera from naturally born, respiratory pathogen-free pigs infected with *H. pleuropneumoniae* serotype 5. The OMP profile of Sarkosyl-insoluble preparations of *H. pleuropneumoniae* serotype 5 strains has been reported previously (47) and is shown in Fig. 1 for strain 200. A major OMP with an apparent molecular weight of 16,500 (16.5K OMP), and 29K, 42K, 43.5K, and 66.5K OMPs were resolved in Coomassie blue-stained SDS-PAGE gels. The 29K and 43.5K OMPs represent the nondenatured and the heat-modified forms of a heat-modifiable OMP (29K/43.5K OMP). Bands of 20.5K, 32K, 38.5K, 45K, and 49.5K OMPs were less intensely stained, and numerous high-molecular-weight (≥67,000) bands were also apparent. The major OMPs were efficiently transferred to the nitrocellulose sheets as indicated by India ink staining, (Fig. 1, lane a).

Pigs from the ARS herd developed symptoms of acute pleuropneumonia following exposure to strain 200, and at necropsy on day 28 lesions of chronic pneumonia and pleuritis were evident. When reacted with sera from two pigs from the ARS herd, preexposure sera contained antibodies to the 29K, 32K, 38.5K, and 43.5K OMPs (Fig. 1, lanes b and d). The reactions with these OMPs were more intense in convalescent sera (Fig. 1, lanes c and e). In addition, antibodies to the 16.5K, 49.5K, and 66.5K OMPs and to two broad bands (approximate molecular weights of 54,000 and 95,000), were present in convalescent sera. The latter two bands were not apparent on Coomassie blue-stained SDS-PAGE gels. No antibodies to the major 42K OMP were detected. Gnotobiatic swine serum did not react with the OMP-enriched preparation (Fig. 1, lane f).

Exposure of CDCD pigs to *H. pleuropneumoniae* 200. Twelve hours after exposure to 7 × 10⁹ CFU of *H. pleuropneumoniae* 200, the three challenged CDCD pigs evidenced depression, dyspnea, and elevated temperatures ranging from 105.0 to 107.8°F (40.6 to 42.1°C). When clinical signs were evident for at least 6 h, each pig was treated with penicillin and streptomycin to ensure survival. After reexposure on day 35 to 1.5 × 10² CFU, two of the three challenged pigs evidenced no clinical signs of *H. pleuropneumoniae* infection. The third pig experienced a transient temperature of 106.0°F (41.1°C) but otherwise remained clinically normal. The two nonexposed pigs remained clinically normal throughout the observation period. At necropsy on day 49, no gross lesions indicative of *H. pleuropneumoniae* infection were apparent in either the exposed or nonexposed pigs, nor were *Haemophilus* spp. isolated from the lungs of the exposed pigs.

Preexposure sera from all pigs were negative (titer of <4) for CF antibodies to *H. pleuropneumoniae* serotype 5. Following exposure, the three exposed pigs developed CF antibody titers ranging from 32 to 64, while the two nonexposed pigs remained serologically negative (titer of <4).

Immunoblotting of sera from CDCD pigs with OMP-enriched preparations from serotype 5 strain 200. Sera collected at weekly intervals from two exposed (pigs 196 and 248) and one nonexposed (pig 199) CDCD pigs were reacted on immunoblots with a Sarkosyl-insoluble preparation of the homologous strain 200 (Fig. 2). Preexposure sera contained no antibodies which reacted with OMPs on immunoblots (Fig. 2, lanes b, f, and j). By test day 49, however, serum from the nonexposed pig reacted weakly with the 29K, 38.5K, and 43.5K OMPs (Fig. 2, lane e). Following initial exposure, sera from the two infected pigs reacted with the same OMPs and weakly with the 16.5K OMP (Fig. 2, lanes g, h, k, and l). In addition, sera from one of the pigs reacted with the 49.5K and 66.5K OMPs and with the two broad 54,000- and 95,000-molecular-weight bands (54K and 95K bands) (Fig. 2, lane 1). Reactions intensified following the

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FIG. 1. (A) Coomassie blue-stained SDS-PAGE profile of OMP-enriched, Sarkosyl-insoluble preparation from *H. pleuropneumoniae* 200 (serotype 5). Molecular weight (mw) markers are given in the left lane. (B) Immunoblot of OMP-enriched preparation from *H. pleuropneumoniae* 200 with pre- and postexposure sera from pigs from the ARS herd. Lane a, India ink stain; lane b, pig 232 preexposure; lane c, pig 232 postexposure; lane d, pig 233 preexposure; lane e, pig 233 postexposure; lane f, gnotobiatic swine serum. Sera were diluted 1:100. The positions of the molecular weight markers transferred to nitrocellulose and stained with India ink are indicated to the left of the immunoblot.

FIG. 2. Immunoblot of OMP-enriched preparation from *H. pleuropneumoniae* 200 (serotype 5) with sera from CDCD pigs exposed to the homologous strain and with sera from ARS pigs exposed to serotypes 1 (se1) and 7 (se7). Sera from CDCD pigs, taken preexposure, 21 and 35 days postexposure, and 14 days (day 49) following a second exposure to strain 200, were run in adjacent lanes (left to right, respectively): India ink stain (lane a); nonexposed (non) pig 199 (lanes b to e); exposed (inf) pig 196 (lanes f to i); exposed pig 248 (lanes j to m); gnotobiatic swine serum (lane n). Sera from ARS pigs were taken preexposure and 28 days postexposure: pig 3040 exposed to serotype 7 strain WF83 preexposure (lane 0) and postexposure (lane p); pig 1451 exposed to serotype 1 strain 4074 preexposure (lane q) and postexposure (lane r). Sera were diluted 1:100. Major OMPs are indicated to the left of the immunoblot.
The position of the organism (Fig. 2, lanes i and m), and were more intense with sera from pig 248 than with sera from pig 196. In blots in which the higher molecular weight OMPs were more efficiently transferred, antibodies to the 45K OMP and to several high-molecular-weight (≥94,000) OMPs were also apparent (Fig. 3, lanes b and d). Antibodies to the major 42K OMP were not detected in sera from either infected pig.

Treatment of the OMP-enriched preparation with proteinase K prior to SDS-PAGE and immunoblotting eliminated reactions with the Coomassie blue-stained OMPs but had no effect on the reactions with the two Coomassie blue-negative bands (Fig. 4). Conversely, treatment of the OMP-enriched preparation with sodium metaperiodate prior to SDS-PAGE eliminated antibody reactions with the 54K and 95K Coomassie blue-negative bands while it did not affect the reactions with the OMPs (data not shown).

Antibodies reacting with the 29K, 38.5K, or 43.5K OMPs were still detected in postexposure (day 49) sera which had been adsorbed with whole cells of the homologous strain 200 (Fig. 3). However, reactions of sera from the two exposed pigs with the 44.5K, 49.5K, and 66.5K OMPs; several high-molecular-weight OMPs; and the two broad 54K and 95K bands were eliminated or greatly reduced by adsorption of sera (Fig. 3, lanes c and e).

OMP-enriched preparations from strain 200 cells of both smooth and adherent colony morphology were reacted with pre- and postexposure sera from the CDCD pigs to determine if differences attributable to colony type could be detected on immunoblots. The reactions between convalescent sera and an OMP-enriched preparation from cells of adherent morphology were indistinguishable from those obtained with the preparations from cells of smooth colony morphology (data not shown). We have previously reported that Coomassie blue-stained OMP profiles of adherent and smooth clones from the same H. pleuropneumoniae strain are indistinguishable (47). Similarly, silver-stained SDS-PAGE profiles of proteinase K-treated, Sarkosyl-insoluble preparations of both colony types from strain 200 were identical (data not shown).

Immunoblotting of sera from CDCD pigs infected with serotype 5 strain 200 with OMPs from heterologous serotype 5 strains. Coomassie blue-stained, SDS-PAGE OMP profiles of the serotype 5 strains listed in Table 1 are indistinguishable, except for strain K17, for which the 42K and 43.5K OMPs are resolved only with difficulty (47). Sera from the CDCD pigs exposed to strain 200 were tested with OMP-enriched preparations from these nine heterologous serotype
5 strains (Fig. 5). Neither gnotobiotic swine serum nor preexposure sera from CDCD pigs reacted on immunoblots with OMP-enriched preparations from any of the strains examined (data not shown). Postexposure serum (day 49) from nonexposed pig 199 reacted weakly with the 29K, 38.5K, and 43.5K OMPs of all strains examined (data not shown). Antibodies in postexposure sera from the two CDCD pigs exposed to strain 200 reacted with the 29K, 38.5K, and 43.5K OMPs of all heterologous serotype 5 strains examined (Fig. 5). Reactions with the 38.5K OMPs of some strains (i.e., strains 119 and 178) were very weak, and resolution was lost with photographic reproduction. Reactions with the major 42K OMP of any of the strains were not detected. The high-molecular-weight bands (>94,000) were not always efficiently transferred to nitrocellulose, but when transfer was detected, sera from the exposed pigs recognized these OMPs from heterologous strains.

Other than these similarities, sera from the infected pigs varied both in intensity of reaction with and in recognition of OMPs from heterologous serotype 5 strains. For example, when tested with strain 119 (Fig. 5, lanes h and i) only weak reactions occurred with the 29K, 38.5K, and 43.5K OMPs. In contrast, when sera from pig 196 was tested with the reference strain K17, 11 bands were recognized (Fig. 5, lane c); with strain M1, reactions with at least 12 bands were distinguished (Fig. 5, lane c'). Recognition of the 45K, 49.5K, and 66.5K OMPs was variable; sera reacted with seven of nine 45K OMPs, six of nine 49.5K OMPs, and five of nine 66.5K OMPs from the heterologous strains. For four strains (K17, 1A, 155, and M1) some minor 35K to 40K OMPs reacted intensely with serum from pig 196 (Fig. 5, lanes c, f, o, and c'). This same serum sample reacted only weakly when tested with the homologous strain (Fig. 2, lane i). Reaction with a broad 54K band was detected when sera were tested with OMP preparations from strains K17, 1A, 155, 178, and M1. Diffuse reactions at this approximate position were also detected with OMP preparations from strains 144 and 163.

**SDS-PAGE of whole-cell, proteinase K-treated lysates from H. pleuropneumoniae strains.** To better elucidate the nature of the immunogens detected in immunoblots of sera with serotype 5 strains, whole-cell, proteinase K-treated lysates from the 10 strains were subjected to SDS-PAGE and then silver stained (Fig. 6). In preparations from all strains, a very intense broad doublet at the low-molecular-weight region of the gel was detected. In addition, diffuse 54K and 95K bands were apparent in preparations from strains 200, 144, and 178. Similar bands were detected for strain 163, although the bands migrated at a lower apparent molecular weight than did the corresponding bands of the former three strains. Less intense bands with an apparent molecular weight slightly lower than 54,000 were present for strains 155 and 201, and a very broad diffuse band was detected in preparations from strain K17. For strain M1, only a very faint band was detected at the 54,000-molecular-weight region of the gel, while no corresponding bands were detected for strains 1A or 119. LPS extracted from strain 200 by the hot aqueous phenol method (supplied by A. Jensen, National Animal Disease Center, Ames, Iowa) was subjected to SDS-PAGE. When loaded at 10- to 30-μg levels, the 54K band and the low-molecular-weight bands were apparent in silver-stained profiles of the purified LPS. The 95K band, however, was not apparent (data not shown).

**Effect of proteinase K and periodate treatment on silver-stained SDS-PAGE profiles.** Representative results obtained when silver-stained immunogens in the OMP-enriched preparations from H. pleuropneumoniae serotype 5 strains were treated with proteinase K and sodium metaperiodate are shown in Fig. 7. Proteinase K treatment did not affect the broad, silver-stained, low-molecular-weight or the 54K bands of any of the 10 strains examined. However, the 95K silver-stained bands and a higher molecular weight band of strain 178 were reduced by proteinase K treatment. Treatment with periodate eliminated the high-molecular-weight, silver-stained bands and significantly reduced the intensity of the low-molecular-weight doublet. Concomitantly, a new band appeared at the low-molecular-weight end of the gel.

**Immunoblotting of sera from CDCD pigs infected with**
serotype 5 with OMP-enriched preparations from heterologous H. pleuropneumoniae serotypes. Postexposure (day 49) sera from CDCD pigs exposed to serotype 5 was tested with OMP-enriched preparations from serotype 7 (strain WF83) and serotype 1 (strain 4074) reference strains (Fig. 8). Pre- and postexposure sera from ARS pigs exposed to serotype 7 or to serotype 1 were also tested with the homologous antigen preparations (Fig. 8).

In preparations from serotype 7 strains, 16K, 29K, 38.5K, 42K, 43.5K, and 66.5K OMPs and several bands of apparent higher molecular weight are resolved on Coomassie blue-stained, SDS-PAGE gels (47); and India ink staining indicated efficient transfer of these OMPs to nitrocellulose (Fig. 8, lane a). Preexposure sera from the ARS pig reacted with the 29K and 43.5K OMPs (Fig. 8, lane b). Following exposure to the homologous serotype 7 strain, the reactions with these bands intensified, and additional reactions with the 38.5K and three high-molecular-weight OMPs were apparent (Fig. 8, lane c). The 42K and 43.5K OMPs were not well resolved; thus, it was difficult to discern which band reacted with the serum. In addition, antibodies to numerous bands, arranged in a step ladder array at the top half of the gel, were apparent in postexposure serum. These bands did not stain with Coomassie blue, were resistant to proteinase K treatment, and stained with silver (Fig. 6, lane m). Serum (day 49) from the nonexposed CDCD pigs reacted weakly with the 29K and 43.5K OMPs (Fig. 8, lane d), while sera from the CDCD pigs exposed to serotype 5 reacted with these two OMPs and with the 38.5K OMP (Fig. 8, lanes e and f). Gnotobiotic swine sera (Fig. 8, lane g) did not react with the serotype 7 preparation.

In preparations from serotype 1 strains, 16K, 29K, 38.5K, 39K, 42.5K, 50K, and 66.5K OMPs and several bands of apparent higher molecular weight are resolved on Coomassie blue-stained SDS-PAGE gels (47). India ink staining indicated transfer of these OMPs to nitrocellulose (Fig. 8, lane h). Preexposure serum from the ARS pig reacted with the 29K, 38.5K, and 42.5K OMPs (Fig. 8, lane i). This reaction was intensified in serum taken 28 days postexposure (Fig. 8, lane j). In addition, antibodies to the 66.5K OMP and a diffuse, broad reaction at the top of the gel were evident. No antibodies to the major 39K OMP were detected. The diffuse band in the high-molecular-weight region of the gel did not stain with Coomassie blue, was resistant to proteinase K treatment, and stained with silver nitrate (Fig. 6, lane l). Sera from the nonexposed CDCD pig reacted with the 29K, 38.5K, and 42.5K OMPs from the serotype 1 preparation (Fig. 8, lane k). Sera from the two CDCD pigs infected with serotype 5 reacted more intensely with the same OMPs recognized by the serum sample from the noninfected pig (Fig. 8, lane l and m), while gnotobiotic swine serum did not react with the serotype 1 preparation (Fig. 8, lane n).

Sera from the ARS pigs infected with serotype 1 or serotype 7 were also tested with OMP-enriched preparations from serotype 5 strain 200 to examine the reciprocal cross-reactions (Fig. 2, lanes o through r). Preexposure sera reacted with the serotype 5 29K and 43.5K OMPs. These reactions intensified in sera from ARS pigs exposed to both heterologous serotypes. In addition, antibodies to the 16.5K, 38.5K, and 66.5K serotypes 5 OMPs were evident in postexposure sera.

DISCUSSION

We identified several OM components which elicit a specific antibody response in pigs infected with H. pleuropneumoniae serotype 5. These include the 45K, 49.5K, and 66.5K OMPs; several high-molecular-weight OMPs (≥94,000), and two polysaccharide-containing immunogens which migrated as broad 54K and 95K bands. The fact that antibodies to these components were eliminated by whole-cell adsorption of sera suggests that they were directed primarily to surface-exposed epitopes (26).

The challenged CDCD pigs developed clinical symptoms indicative of acute pleuropneumonia following initial exposure to strain 200. Although antibiotic treatment may have limited the infection and the subsequent immune response, the pigs resisted the second exposure to a higher dose which would cause acute disease and death in a majority of pigs (unpublished data). The CF antibody response and an absence of gross lesions of pneumonia at necropsy also indicate that the pigs were infected and recovered following the initial challenge exposure. Thus, in addition to being directed to surface-exposed epitopes, the antibodies we detected to the OM components developed concomitantly with protective immunity in the pigs. These are important factors for consideration when identifying potential protective immunogens.

Detection of antibodies in sera from noninfected pigs that reacted with several OMPs was not entirely unexpected. Similar findings have been reported when normal, preexposure, or acute-phase human sera have been examined for antibodies to OMPs of H. influenzae type b (10, 27, 59), nontypable H. influenzae (14), Campylobacter jejuni (3, 30), and N. meningitidis (53, 60). It is believed that this recognition is due to either previous undetected exposure to the organism or to exposure to an organism with serologically cross-reacting OMPs. The respiratory pathogen-free ARS herd from which we initially obtained pigs is free of Haemophilus spp., as indicated by periodic bacteriologic, serologic,
and necropsy examination of pigs from the herd. Thus, it seems likely that exposure of these pigs to an organism with cross-reacting OMPs was responsible for the antibodies which reacted with \textit{H. pleuropneumoniae} OMPs on immunoblots. The strict isolation in which the CDCD pigs were reared apparently limited their exposure to potentially cross-reacting organisms, since sera from these pigs did not react with \textit{H. pleuropneumoniae} OMPs prior to challenge exposure. By test day 49, however, sera from the nonexposed pigs had antibodies to several OMPs, indicating that as they matured in our isolation facilities it is likely that the pigs were exposed to cross-reacting organisms. Cross-reactions among OMPs of members of the family \textit{Enterobacteriaceae} (2, 19) and between \textit{Enterobacteriaceae} spp. and \textit{H. influenzae} (58) have been reported. Proteins are denatured during SDS-PAGE, and the unfolding likely exposes conserved internal epitopes which are potentially cross-reactive among gram-negative organisms (54). In particular, heat-modifiable OMPs of gram-negative organisms are highly conserved (2). The 29K and 43.5K bands represent the nondenatured and heat-modified forms of a heat-modifiable OMP of \textit{H. pleuropneumoniae} serotype 5 (47), and it was to these bands that antibodies in nonexposed swine sera were primarily directed. Whole-cell adsorption of sera did not eliminate these antibodies, concurring with the observations of others that such cross-reactive antibodies are primarily directed to internal epitopes (10, 54).

We found India ink staining of nitrocellulose to be essential for identification of immunogenic OMPs, inasmuch as some OMPs migrated at similar apparent molecular weights or were detected only as minor bands in Coomassie blue-stained gels. Indeed, ink staining of nitrocellulose indicated that the \textit{H. pleuropneumoniae} OMPs were effectively transferred to nitrocellulose; however, the efficiency (especially for high-molecular-weight bands) and intensity varied to some extent with each transfer. Loeb (26) also noted such variability in replicate testing, especially when reactions with minor proteins were detected. Loeb (26) suggested that in addition to antibody concentration, efficiency of transfer and binding to nitrocellulose are considerations when attempting to quantitatively interpret data from immunoblots; false-negative reactions are possible. Thus, it is difficult to make definitive comparisons of immunoblots of sera from pigs infected with strain 200 with OMP preparations of heterologous serotype 5 strains. The variable reactions with OMPs may reflect variable intensity of transfer or binding, variable expression of the OMPs on the whole cell or in the Sarkosyl-insoluble preparations, or actual antigenic differences among the OMPs. Recognition of the 45K, 49.5K, and 66.5K OMPs was variable when India ink stains indicated comparable transfer, possibly indicating that OMPs of the same apparent molecular weight differ antigenically among serotype 5 strains. The strong reactions with the minor OMPs of some strains, not detected when sera were tested with the homologous OMP preparation, likely reflect a difference in expression of the OMPs in whole cells or Sarkosyl-insoluble preparations.

Antibodies to the 42K OMPs of the serotype 5 strains were not detected, in spite of the fact that this is a major OMP in Coomassie blue-stained gels (47) and that it is effectively transferred to nitrocellulose. Also, no antibodies to the 42K OMP of strain 200 were apparent in immunoblots with hyperimmune rabbit sera (V. Rapp, unpublished data). Similar observations have been made with \textit{H. influenzae} type b, in which antibodies to a major OMP were not detected in convalescent human sera (10, 27, 59), or in hyperimmune rabbit sera (4, 26). Antibodies to the major \textit{Bordetella pertussis} OMP were not detected in sera from immunized infants or mice (49). The porin protein of \textit{Serratia marcescens} strains reacted weakly in immunoblots with rabbit antisera (21). This lack of detection may be due to the denaturing of antigenic epitopes during SDS-PAGE (21, 26, 46), and it is possible that antibodies to the \textit{H. pleuropneumoniae} 42K OMP were present but not detected by immunoblot analysis. Immunologic competition has been shown to have a profound effect on the antibody response to \textit{Escherichia coli} proteins (55) and may be a factor in the response observed to \textit{H. pleuropneumoniae}. In spite of the fact that antibodies to the major 42K OMP were not detected, the pigs proved to be resistant to reinfection, and it is possible that antibodies to the 42K OMP may not be an essential factor in immunity following infection with \textit{H. pleuropneumoniae} serotype 5.

During in vitro passage, stable, smooth colony variants of \textit{H. pleuropneumoniae} may spontaneously appear (48; J. Nicolet, Ph.D. Thesis, University of Bern, Bern, Switzerland, 1971). Although it has been reported that this phenomenon may reflect the loss of capsule (Nicolet, Ph.D. thesis), our findings indicate that both the smooth and the original adherent colony types are encapsulated (47, 48). Clones of both colony types from the same strain have identical OMP profiles (47). Results with strain 200 indicate that preparations from cells of both colony types also have indistinguishable silver-stained profiles, and sera from pigs exposed to cells of the adherent colony type reacted identically in immunoblots with OMP-enriched preparations of both colony types. Thus, the factors responsible for the distinctive colony morphologies of \textit{H. pleuropneumoniae} remain unknown. A chemical change in the presumed polysaccharide capsule may be involved. Biochemical and morphological characterization of two serotype 5 strains indicated that structural differences in capsule may be associated with virulence (20), but it was not reported whether these structural differences were also correlated with differences in colony morphology.

Immunoblotting detected proteinase K-resistant bands in OMP-enriched preparations from strain 200 and from several of the other serotype 5 strains which were not apparent in Coomassie blue-stained SDS-PAGE gels. These bands were visualized by a silver nitrate procedure believed to stain carbohydrate components of LPS (18, 57). However, it has recently been reported that the lipid component of LPS may be stained by this procedure (23). The serotype 5 proteinase K-resistant bands were eliminated by oxidation with periodate, indicating that they are polysaccharide-containing immunogens. Expression of these polysaccharide bands was variable among serotype 5 strains. They were not detected in whole-cell lysates from 2 of the 10 strains examined, and bands migrating at apparent molecular weights different from those observed with strain 200 were detected with 5 strains. In addition to variable expression, variable recognition of the bands with sera from pigs infected with strain 200 was also evident. For example, although only a faint band of approximately 54,000 molecular weight was detected in silver-stained preparations of strains 155 and M1 and no band was detected with strain 1A, an intense reaction was detected at this approximate region when convalescent sera to strain 200 were tested with OMP preparations from these strains. Conversely, the intense band detected in silver-stained preparations from strain 163 reacted relatively weakly with convalescent sera to strain 200. The silver-stained profiles of whole-cell lysates and Sarkosyl-insoluble
preparations from the same strain were qualitatively similar, indicating that the presence of the bands was not influenced by the Sarkosyl procedure.

The heavily stained doublet apparent at the low-molecular-weight region of the gel was suggestive of a rough LPS. The higher molecular weight proteinase K-resistant bands most likely represent LPS, inasmuch as a 54K band was also present in silver-stained SDS-PAGE profiles of phenol-extracted LPS from strain 200. The 95K band, however, was not apparent in profiles of purified LPS. It may represent aggregated LPS or LPS which comigrates with the OMPs in this region. The partial loss of the 95K band with proteinase K treatment is consistent with the latter hypothesis. Alternatively, the bands could represent capsular material present in either whole-cell or Sarkosyl-insoluble preparations. However, with other organisms, capsular polysaccharide appears as a diffuse smear at the very top of the gel (45, 46, 59). The fact that antibodies to these components were eliminated by whole-cell adsorption of convalescent sera suggests that they are surface exposed and potentially significant immunogens.

Immunoblotting of convalescent sera and OMP preparations from heterologous *H. pleuropneumoniae* serotypes revealed cross-reactions between the heat-modifiable 29K and 55K OMPs and the 38.5K CFP of serotype 5 and the comparable OMPs of serotypes 1 and 7. These may not be specific reactions, inasmuch as antibodies cross-reacting with these proteins were found in sera from noninfected pigs. The silver-stained immunogen detected at the top of the gel in preparations from serotype 1 strain 4074 and in immunoblots with convalescent sera from a pig infected with the homologous strain likely represents capsular polysaccharide. For serotype 7 strain WF83, the silver-stained profile is suggestive of a smooth LPS, and blotting with convalescent sera from a pig infected with the homologous strain indicated that the bands were immunogenic. No cross-reactions between the polysaccharide immunogens of serotypes 1, 5, and 7 were detected, suggesting that they may contribute to the type specificity of these serotypes. In immunoblots of OMP-enriched preparations with hyperimmune rabbit serum, the reference strains (Rapp, unpublished data), serotype 9 antiserum reacted with the high-molecular-weight polysaccharide immunogen of serotype 1. Similarly, serotype 4 antiserum reacted with the presumed LPS bands of serotype 7. Serological cross-reactions between serotypes 1 and 9 (43) and serotypes 4 and 7 (48) have been reported. It has been suggested that type specificity for *H. pleuropneumoniae* may involve both capsular and LPS determinants (11, 42, 44), and preliminary data from immunoblots support these observations.

Recently, there have been several reports on purification and characterization of *H. pleuropneumoniae* LPS. Maudsley et al. (29) purified the LPS of the serotype 2 reference strain and reported that it is smooth with a relatively high content of heptose and glucose. We also found the silver-stained profile of the serotype 2 reference strain to reflect a smooth LPS, as did the profiles of the reference strains for serotypes 3, 4, 6, and 8 (Rapp, unpublished data). The profile of the reference strain of serotype 9 was similar to that of serotype 1 (Rapp, unpublished data). Fenwick et al. (5) reported the isolation of smooth- and rough-type LPSs from a serotype 5 strain by the use of the aqueous-phenol and phenol-chloroform-petroleum ether techniques, respectively. Biologic assays indicated that the rough-type LPS was more toxic than the smooth LPS preparation. Our SDS-PAGE analysis of whole-cell, proteinase K-treated preparations from 10 serotype 5 strains, as well as aqueous-phenol-purified LPS from strain 200, indicated a rough-type LPS for all strains. Jensen and Bertram (20) characterized the LPS and capsular material from two serotype 5 strains, one virulent and one avirulent, and found them to differ biochemically and serologically. The virulent strain that they characterized was strain 200, and the avirulent strain B8 is believed to have originated from strain M1 (T. Bertram, personal communication). Our observations, based on SDS-PAGE profiles and immunoblotting, corroborates those of Jensen and Bertram (20) regarding the heterogeneity of surface polysaccharides of serotype 5 strains.

Serotype 5 is the most prevalent isolate from swine in the midwestern United States (48). Although several vaccines are available, they consist of inactivated, adjuvanted, whole-cell bacterins which do not apparently protect against chronic infection (17, 24, 38), and their use may be associated with local or systemic untoward reactions (38, 51). The OMP and polysaccharide immunogens we identified for *H. pleuropneumoniae* serotype 5 are considered candidates for protective antigens based on the fact that antibodies to surface-exposed epitopes of these immunogens were detected in sera of pigs concomitantly with the development of immunity following infection. The variable expression of the polysaccharide immunogens and the apparent antigenic heterogeneity of the OMP and polysaccharide immunogens among serotype 5 strains are factors that should be considered in the development of vaccines against *H. pleuropneumoniae*.

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LITERATURE CITED

SWINE ANTIBODY RESPONSE TO OM OF *H. PLEUROPNEUMONIAE* 759


