Enzyme-Linked Immunosorbent Assay and Immunoblot Analysis of the Immunoglobulin G Response to Whole-Cell and Lipooligosaccharide Antigens of *Pasteurella pneumotropica* in Laboratory Mice with Latent Pasteurellosis

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The immunoglobulin G (IgG) response to whole-cell and lipooligosaccharide (LOS) antigens of *Pasteurella pneumotropica* was evaluated in mice with latent pasteurellosis by enzyme-linked immunosorbent assay (ELISA) and immunoblots. Antibodies to cell wall proteins of *P. pneumotropica* also reacted with several protein antigens from isolates of *Actinobacillus* spp. and other pasteurellae. Conversely, antibodies to LOS antigens of *P. pneumotropica* demonstrated no cross-reactivity with LOSs of other *Pasteurella* or *Actinobacillus* species. IgG to cell wall proteins was detected initially by ELISA 4 weeks after experimental oronasal inoculation of specific-pathogen-free mice; antibody to LOSs was first detected 7 weeks after infection and at that time exceeded titers to other cell wall antigens. Naturally infected conventional mice from a colony with endemic latent pasteurellosis had high IgG titers to *P. pneumotropica* antigens at 8 to 10 weeks of age, and, as in the experimentally infected mice, antibody to LOSs predominated. Thus, LOSs of *P. pneumotropica* can be used as an ELISA or immunoblot antigen to detect serospecific antibodies in laboratory mice with latent pasteurellosis.

Pasteurella pneumotropica is the major cause of pasteurellosis in laboratory rodents, especially mice, rats, and hamsters. Latent infections are common in both conventional and barrier-maintained colonies (2, 6, 11, 25). The organism is capable of causing primary disease, mainly local suppurative conditions (7, 18, 21, 27, 29, 30) and genital infections (1, 24, 28), but it is more often a secondary invader in viral and mycoplasmal pneumonias (3, 5, 9, 10, 13). Detection of latent infection by culture techniques is often unreliable, especially when only oropharyngeal samples are taken, because the organism also readily colonizes the mucosae of the genital and intestinal tracts (6, 19, 23, 32). A sensitive and specific serologic assay to detect antibodies to *P. pneumotropica* would facilitate detection of latent infections and would be especially valuable as a surveillance tool in large colonies of specific-pathogen-free rodents. An enzyme-linked immunosorbent assay (ELISA) for antibodies to *P. pneumotropica* whole-cell (WC) antigens to detect infections in mice has been described (31). However, antibodies detected by the assay appeared to lack specificity because cross-reactions were observed with WC antigens of several species of *Actinobacillus* and *Pasteurella*. This report describes studies using ELISAs and immunoblots to characterize the immunoglobulin G (IgG) antibody response to both WC and lipooligosaccharide (LOS) antigens in mice latently infected with *P. pneumotropica*. The cross-reactivity of *P. pneumotropica* antibodies to antigens shared by several species of *Pasteurella* and *Actinobacillus* was also studied by ELISA and immunoblot techniques.

MATERIALS AND METHODS

Bacterial isolates. Thirteen isolates of *P. pneumotropica* were obtained from tracheobronchial or oropharyngeal

washings of conventional mice. The 13 isolates were categorized into three groups on the basis of different electrophoretic patterns of LOSs in silver-stained polyacrylamide gels (see below). Three isolates representing each of these LOS patterns were used as a mixed bacterin in immunization and experimental infection studies. Other bacterial isolates from our laboratories used as sources of antigens to assess cross-reactivity of antibodies were *Pasteurella multocida* from a rabbit, *Pasteurella haemolytica* from a cat, *Actinobacillus suis* from a pig, *Actinobacillus equuli* from a horse, and *A. equuli* ATCC 19392. Isolates were stored at -80°C in otherwise sterile rabbit blood and were propagated on Trypticase soy agar with 3% sheep blood for 18 h at 37°C . Isolates were identified by standard microbiologic methods (4).

Preparation of bacterial antigens. WC antigens were prepared by harvesting confluent colonies from overnight cultures on nutrient agar with carbonate buffer (0.05 M sodium carbonate, pH 9.6, containing 0.02% sodium azide). The concentration was adjusted to 10^9 cells per ml by visual comparison with McFarland standards, and the cells were killed by heating at 100°C for 75 min. WC lysates (WC-L) were prepared from overnight cultures by harvesting colonies from three agar plates in 1 ml of carbonate buffer. After sonicating this suspension for 1 min at setting 30 (Fisher Scientific Co., Pittsburgh, Pa.), 3 ml of lysing buffer (2% sodium dodecyl sulfate [SDS], 4% 2-mercaptoethanol, 2% glycerol, 1 M Tris, pH 6.8) was added, and the mixture was heated at 100°C for 3 min and then passed through a 0.22- μm -pore-size filter. Some WC-L preparations were further treated with proteinase K to obtain samples rich in lipopolysaccharides (LPS) (8). Forty microliters of proteinase K (Boehringer GmbH, Mannheim, Federal Republic of Germany) (1 mg/ml in lysing buffer) was added to 4 ml of WC-L, and the sample was incubated for 2 h at 60°C . This treatment was repeated. These samples were diluted 1:100 in

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diluting buffer (0.02 M NaHPO₄, 0.15 M NaCl, 1% bovine serum albumin, 0.01% thimerosal, 0.03% gelatin) and labeled proteinase K lysates (PK-L). Some WC samples were subjected to periodate oxidation to destroy antigenic epitopes on oligosaccharides while leaving protein antigens intact by adding an equal volume of 0.04 M HIO₄ in 0.125 N H₂SO₄ to 3×10^9 WCs in carbonate buffer. The mixture was kept at room temperature for 30 min, dialyzed (3,500-molecular-weight cutoff) in several changes of distilled water for 4 h, and concentrated in polyethylene glycol. The samples were dissolved in lysing buffer, treated at 100°C for 3 min, and passed through a 0.22- μ m filter. Destruction of oligosaccharide epitopes was confirmed by parallel periodate treatment of *Salmonella minnesota* LPS (List Biologicals, Campbell, Calif.) and then by SDS-polyacrylamide gel electrophoresis (PAGE) and Western blots (immunoblots) treated with rabbit antiserum to *S. minnesota* LPS (14). Some periodate-oxidized samples were treated subsequently with proteinase K as described above (15).

Mouse studies. Serum specimens from mice with latent *P. pneumotropica* infection were obtained from young adult (10 to 12 weeks of age) Swiss mice (Biolab Corp., Centerville, Minn.), whereas serum specimens free of antibodies to *P. pneumotropica* were obtained from retired breeder C57BL/6J mice (Jackson Laboratory, Bar Harbor, Maine). For infection studies, *P. pneumotropica*-free female C57BL/6 mice (4 weeks of age; Simonson Laboratories, Gilroy, Calif.) were sedated with CO₂ and inoculated oronasally with a 50- μ l suspension of live *P. pneumotropica* containing 3×10^9 cells per ml in carbonate buffer. Control mice were given buffer. Mice were kept in groups of three to five animals in plastic cages fitted with fiberglass bonnets within a laminar air flow cabinet (Lab Products, Inc., Maywood, N.J.). Groups of mice were killed 2, 4, and 7 weeks after infection. Blood was obtained by severing the axillary arteries of mice anesthetized with CO₂. Serum was aspirated from the clotted blood, diluted 1:10 or 1:25 in diluting buffer, and stored at -20°C.

Electrophoresis and immunoblots. SDS-PAGE was performed with the Laemmli buffer system (12). The 5% stacking gel and 10 or 15% separating gel contained 0.2% SDS. Samples of bacterial lysates and LPS standards were heated at 100°C for 3 min and sonicated for 2 s (35% setting; Fisher Scientific) just prior to electrophoresis. Electrophoresis (Mini-Protean; Bio-Rad Laboratories, Richmond Calif.) was performed at room temperature at 20 mA for 1.5 h in Tris-glycine buffer, pH 8.0, with 0.1% SDS. Gels were silver stained for either proteins and LPS or for LPS, (Ag-LPS), as described by Hitchcock and Brown (8). After SDS-PAGE, some samples were electrotransferred (Western blot) from the gel onto nitrocellulose sheets (TransBlot; Bio-Rad) in the presence of 0.1 M glycine-0.01 M Tris, pH 8.0, in 20% methanol at 15 V for 30 min. Other steps in the immunoblot procedure were performed as described previously (16). Mouse serum was used at a dilution of either 1:10 or 1:25.

ELISA. Polystyrene wells were precoated with 0.3% gelatin overnight when PK-L was used as the antigen, whereas WC antigen was put into untreated wells. The ELISA procedures were otherwise performed exactly as described previously (16). Mouse serum was used at a dilution of 1:25 and protein A-conjugated horseradish peroxidase (Sigma Chemical Co., St. Louis, Mo.) was diluted 1:200. Control wells lacked either antigen or primary antibody, and all determinations were performed in duplicate or quadruplicate.

Statistical analysis. Differences in the mean optical density

FIG. 1. SDS-PAGE silver-stained gel. Lane A, LOS of Ra mutant of *S. minnesota*; lane B, combined WC-Ls of three mouse isolates of *P. pneumotropica*; lanes C through E, PK-Ls of each of the three mouse isolates of *P. pneumotropica*.

readings in the ELISAs were evaluated by Student's *t* test. A probability value of 5% or less was judged as statistically significant.

RESULTS

SDS-PAGE of *P. pneumotropica* antigens. Silver-stained profiles of WC-L and PK-L preparations from three mouse isolates of *P. pneumotropica* used in immunization and infection studies are shown in Fig. 1. WC-Ls had numerous distinct bands distributed over a molecular-size range of approximately 116 to 3.4 kilodaltons, while PK-Ls contained only one or two major bands located within a molecular-size range of approximately 14.3 to 3.4 kilodaltons. These two bands were shown to be predominantly LPS by the Ag-LPS staining technique, and they migrated slightly faster or slower than the Ra LOS of a semirough variant of *S. minnesota* which has a molecular weight of 4,311.

ELISA and immunoblots of serum specimens from naturally infected and uninfected mice. Serum specimens from 14 male Swiss mice from a commercial colony were evaluated by ELISA and immunoblots for antibodies to WC-L and PK-L antigens of *P. pneumotropica*. Chronic latent infection of these mice by *P. pneumotropica* has been repeatedly demonstrated by cultures in our laboratory, and *P. pneumotropica* was recovered from cultures of oropharyngeal or preputial washings just prior to euthanasia. The results (Table 1) indicate the presence of substantial serum antibody levels in the latently infected mice compared with mice free of *P. pneumotropica*. As expected from the ELISA results, immunoblots of serum specimens from uninfected mice revealed no detectable antibody to WC-L or PK-L antigens (Fig. 2A, lanes 1 through 4). Conversely, serum specimens from the infected mice contained antibodies to several WC-L antigens (Fig. 2B through D, lanes 1) including LOSs, which predominate at the end of the lane. The presence of LOS-specific antibody was confirmed by antibody distribution patterns against PK-Ls (Fig. 2B through D, lanes 2) and by lack of antibody reactivity in the LOS region of the blot after destruction of LOS antigenic epitopes by periodate oxidation of either WC-Ls or PK-Ls (Fig. 2B through D, lanes 3

TABLE 1. ELISA results on serum specimens from mice free of *P. pneumotropica* and from mice with chronic latent *P. pneumotropica* infection

No. of mice	Status	OD ₄₉₂ ^a	
		WC antigen	PK-L antigen
10	Uninfected	0.05 ± 0.01 ^b	0.05 ± 0.01 ^b
14	Infected	1.18 ± 0.12	1.57 ± 0.13

^a Mean ± SEM of serum specimens diluted 1:25. OD₄₉₂, Optical density at 492 nm.

^b Significantly lower than values from infected serum specimens (*P* < 0.005).

and 4). Immunoblots of PK-L preparations of *P. pneumotropica* also had up to five bands (1:10 antiserum dilution) with relative molecular masses between 38 and 84 kilodaltons (Fig. 2B through D, lanes 2). In SDS-PAGE, these bands were silver stained for proteins and LPS but were not stained by the Ag-LPS method, and periodate treatment of PK-Ls had a variable effect on immune serum reactivity to these bands (cf. Fig. 2B through D, lanes 4). These properties suggest that the bands are protease-resistant proteins or glycoproteins. The ELISA and immunoblot results indicate that serum specimens from mice free of *P. pneumotropica* had no demonstrable antibody to *P. pneumotropica* antigens, whereas mice with chronic latent *P. pneumotropica* infection had serum antibodies against a variety of cell surface antigens of this organism, including a major antibody response to serospecific LOS.

ELISA and immunoblots of serum specimens from experimentally infected mice. All of the experimentally infected mice remained clinically normal during the study, and *P. pneumotropica* was cultured from the oropharynges of mice sacrificed at 14 and 28 days after infection; owing to technical error, cultures taken at 48 days yielded no microbial growth. As measured by ELISA (Table 2), mice infected with *P. pneumotropica* for 2 weeks had no measurable antibody to WC-L antigens. At 4 weeks there was a modest but significant (*P* < 0.005) antibody response to WC antigens

TABLE 2. ELISA results on serum specimens from C57BL/6J mice with latent experimental *P. pneumotropica* infection^a

No. of mice	Status	OD ₄₉₂ ^b	
		WC antigen	PK-L antigen
10	Uninfected	0.05 ± 0.01	0.05 ± 0.01
10	Infected 14 days	0.06 ± 0.01	Not done
10	Infected 28 days	0.28 ± 0.09 ^c	0.02 ± 0.01
15	Infected 48 days	0.45 ± 0.08 ^c	0.66 ± 0.11 ^c

^a Mice were infected at 5 weeks of age by oronasal inoculation of 1.5×10^8 organisms in 50 µl.

^b Mean ± SEM of serum specimens diluted 1:25. OD₄₉₂, Optical density at 492 nm.

^c Significantly higher than preceding values in same column (*P* < 0.005).

but no measurable antibody to PK-Ls. After 7 weeks of infection, antibodies to both WC and PK-L antigens were significantly elevated relative to preceding values from infected mice and values from uninfected mice. Immunoblots of selected infected mice showed very faint antibody activity only to high-molecular-weight protein antigens at 28 days. At 48 days there was antibody reactivity to both protein and LOS antigens, but LOS antibody was restricted to serum specimens with the higher optical density values at 492 nm. At a serum dilution of 1:25, only mice with an optical density of >0.60 at 492 nm had detectable antibody to LOS on immunoblots. These results indicate that antibodies to cell envelope protein antigens emerged sooner than antibodies to LOSs, suggesting that WC antigens might be a more sensitive diagnostic antigen. However, the modest antibody response, especially 4 weeks after infection, plus the nonspecificity of antibodies to WC antigens (see below) reduces their utility as diagnostic antigens.

Antibody cross-reactivity. ELISAs and Western blots of sera from *P. pneumotropica*-free mice revealed no detectable antibodies against either WC-L or PK-L antigens of *P. multocida*, *P. haemolytica*, *A. equuli*, *A. equuli* ATCC 19392, and *A. suis* (data not shown). Conversely, serum specimens from mice with either natural or experimental *P. pneumotropica* infection had several cross-reactive antibodies to protein antigens in WC-Ls of these bacteria. Table 3 summarizes the results of ELISAs demonstrating strong reactivity of sera from *P. pneumotropica*-infected mice to WC-L protein antigens but not to PK-L antigens of *Actinobacillus* spp. Western blots confirmed these results by revealing several shared protein antigens (Fig. 3A and B), but in the case of PK-Ls, antibody reactivity was limited to *P. pneumotropica* antigens (Fig. 3C and D) and was especially prominent in the LOS region of the blot (Fig. 3C and D, lanes 1).

DISCUSSION

In these studies we explored the usefulness of somatic antigens or LPS of *P. pneumotropica* as an antigen for use in

FIG. 2. Immunoblots of serum specimens from mice free of *P. pneumotropica* (A) or with naturally occurring latent *P. pneumotropica* infection (B through D). Lanes 1 and 3 are WC-Ls and lanes 2 and 4 are PK-Ls of *P. pneumotropica*. Serum specimens from infected mice possess antibodies to several antigens of *P. pneumotropica*, including antibodies to LOSs which are located at the end of the lanes. Antibody reactivity to LOSs is abolished by periodate oxidation of either WC-L or PK-L preparations (lanes 3 and 4).

TABLE 3. Comparative ELISA values of serum specimens from 14 mice with chronic latent pasteurellosis to *P. pneumotropica* and *Actinobacillus* sp. antigens

Organism	OD ₄₉₂ ^a	
	Whole cell	Proteinase K lysate
<i>P. pneumotropica</i>	1.18 ± 0.12	1.57 ± 0.13
<i>A. equuli</i> ATCC 19392	0.83 ± 0.17	0.05 ± 0.01
<i>A. suis</i>	0.74 ± 0.09	0.08 ± 0.01

^a Mean ± SEM of serum specimens diluted 1:25. OD₄₉₂, Optical density at 492 nm.

FIG. 3. Immunoblots of serum specimens from mice with natural (A and C) or experimental (B and D) latent *P. pneumotropica* infection. Panels A and B demonstrate cross-reactivity of murine antibodies to *P. pneumotropica* with several protein antigens in WC-Ls from both *Actinobacillus* and *Pasteurella* species. Panels C and D demonstrate antibody reactivity to LOS antigens of *P. pneumotropica* (lane 1) and no antibody reactivity to LOSs of the other bacteria (lanes 2 through 4). Panels A and B are WC-Ls and panels C and D are PK-Ls of the following: lane 1, *P. pneumotropica*; lane 2, *A. equuli* 19392; lane 3, *A. suis*; lane 4, *P. multocida*.

the serodiagnosis by ELISA of latent pasteurellosis in laboratory mice. The results indicate that latently infected mice develop IgG antibodies to several cell wall antigens of *P. pneumotropica*. Many of these antibodies were not serospecific for this organism because they also reacted with several protein antigens from other bacteria. In contrast to the findings with protein antigens, antibodies to LOS antigens were serospecific and reacted only with LOS of *P. pneumotropica*.

The three *P. pneumotropica* isolates used in this research were chosen for infection and immunization studies because their silver-stained PK-L profiles in SDS-PAGE were representative of the slight variations observed in the LOS banding patterns in 13 isolates of this organism from mice housed in animal facilities at the University of Minnesota or from two commercial colonies. The number of LOS serotypes in *P. pneumotropica* is not known, but rabbit immune serum to a WC bacterin composed of a mixture of these three isolates reacted in immunoblots with LOS antigens of all 13 isolates, thereby suggesting that the LOS antigens in the bacterin represent common somatic serotypes of mouse isolates of *P. pneumotropica*.

The SDS-PAGE profiles of *P. pneumotropica* LOS in PK-Ls were similar to those of purified LPS from semirough variants of enterobacteria (8) and to those of purified somatic antigens from several other bacteria, including *P. multocida* (15), *Neisseria gonorrhoeae* (17), *Neisseria meningitidis* (26), and *Campylobacter jejuni* (22). LOSs of *P. multocida*, *Neisseria* spp., and *Campylobacter* sp. lack the several repeating oligosaccharide units characteristic of smooth strains of salmonellae and escherichiae, yet still possess serospecific antigens (15, 17, 22, 26).

The IgG response in experimentally infected mice was similar to but less pronounced than that observed in naturally infected mice. These differences most likely reflect environmental effects which differed for the two groups. The naturally infected mice were born and reared in conventional facilities. They were exposed to and probably contracted infection shortly after birth and were kept under conditions that ensure continual exposure to *P. pneumotropica*. The

serologic data from these mice were obtained from serum specimens from 10-week-old animals; by this age they had developed IgG antibodies to a variety of WC antigens, including high IgG titers to LOS antigens (Table 1; Fig. 2B through D). Preliminary results in studies with mice of various ages from the conventional colony indicate that LOS-specific antibody may be detectable before there is measurable antibody to WC-L protein antigens (unpublished observations). Although this observation appears to be at variance with results obtained from experimental infection studies, it may simply be a reflection of the more prolonged and intense antigenic stimulation that exists in a conventional colony.

The microbial and ambient environment of the experimentally infected mice was substantially different from that of the naturally infected mice. These studies were performed by using specific-pathogen-free mice kept in a clean environment since birth and maintained under barrier conditions while under study. Under these conditions, IgG to *P. pneumotropica* antigens was detected initially by ELISA 28 days after infection, when a modest IgG response was detected to WC-L protein antigens. LOS antibody was first detected 48 days after infection, but these antibodies probably began to emerge sometime earlier because by 7 weeks of infection, LOS-specific antibody predominated (Table 2).

Wullenweber-Schmidt et al. (31) reported that antibodies to a mouse isolate of *P. pneumotropica* cross-reacted with WC antigens of *A. equuli* and WC antigens of several members of the genus *Pasteurella*. Our experiments confirm these findings and extend them by demonstrating cross-reactivity to WC antigens of other actinobacilli and pasteurellae. It is notable that *P. pneumotropica* biotype Jawetz/Heyl from mouse lung has been shown by DNA hybridization to be unrelated to the pasteurellae and closely related to the *Actinobacillus* group (20). The extensive antibody cross-reactivity seen in our studies demonstrates clearly that antigenically similar protein epitopes in mouse isolates of *P. pneumotropica* are shared by other *Pasteurella* and *Actinobacillus* isolates. But our studies also show that despite the uncertainty as to the taxonomic status of some isolates of *P. pneumotropica*, their LOS antigens induce a strong and specific IgG response and these antibodies had no demonstrable activity against LOS antigens of several *Actinobacillus* and *Pasteurella* isolates tested.

In summary, mice latently infected with *P. pneumotropica* developed antibodies to several cell wall antigens, including proteins and LOSs. IgG antibodies to several of the proteins were not serospecific for *P. pneumotropica* antigens because they also reacted with cell wall proteins from other pasteurellae and actinobacilli. Conversely, antibodies to *P. pneumotropica* somatic antigens or LOSs were serospecific in that they reacted with LOSs from *P. pneumotropica* but did not react with the LOSs from any of the other isolates of *Pasteurella* or *Actinobacillus* spp. LOS-specific antibody was readily detected by ELISA in mice with either natural or experimental latent *P. pneumotropica* infection. These studies demonstrate the specificity of antibodies to *P. pneumotropica* LOSs and suggest the utility of LOS as an antigen for the serodiagnosis of latent *P. pneumotropica* infection in mice.

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