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NOTES

Vaccine Potential of *Haemophilus pleuropneumoniae* Oligosaccharide-Tetanus Toxoid Conjugates

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Oligosaccharides of smooth-type lipopolysaccharide (LPS) and oligosaccharides of rough-type LPS were isolated from *Haemophilus pleuropneumoniae* and conjugated to tetanus toxoid by reductive amination. The antigenic and imunogenic characteristics of the oligosaccharides, the oligosaccharide-tetanus toxoid conjugates, and the LPS of *H. pleuropneumoniae* were determined by passive hemagglutination, enzyme-linked immunosorbent assay, and inhibition enzyme-linked immunosorbent assay with antisera produced by immunization of rabbits and pigs. The findings were compared with the immunologic response induced by immunization of pigs with an *H. pleuropneumoniae* whole-cell vaccine and by infection of pigs with viable *H. pleuropneumoniae*. The results show that conjugation of isolated oligosaccharides of *H. pleuropneumoniae* to tetanus toxoid improves the immunogenicity of the oligosaccharides without significantly altering their antigenic character. These findings extend the understanding of the immunobiology of *H. pleuropneumoniae* infection in pigs and suggest the potential of purified oligosaccharides as vaccines to prevent porcine pleuropneumonia.

Haemophilus (*Actinobacillus*) *pleuropneumoniae* is the cause of a highly infectious pneumonia with pleuritis in pigs and is a significant problem in swine-producing areas of the United States and Canada (19, 21, 22). Infections in immunologically naive animals are commonly fatal, and surviving animals are stunted and frequently asymptomatic carriers of *H. pleuropneumoniae* (15–18). Presently used methods of controlling this economically important disease have been largely ineffective.

Preventing infections by immunization of pigs with *H. pleuropneumonia* bacterins has been only minimally successful. Bacterins provide some serotype-specific resistance to clinical disease but are frequently toxic (S. C. Henry and T. A. Marsteller, Abstr. Int. Pig Vet. Soc. Congr. 1982, p. 72). Immunization appears to reduce the mortality and the severity of the disease but routinely fails to prevent infection or the occurrence of carrier states (17; H. J. Riising, Abstr. Int. Pig Vet. Soc. Congr. 1984, p. 112). In contrast, swine that survive an infection with one serotype of *H. pleuropneumoniae* develop resistance to reinfection by any of the seven or more other serotypes of *H. pleuropneumoniae* (16, 17).

Previous studies have established the importance of heat-stable antigens in the serotyping of *H. pleuropneumoniae* isolates (3–5, 10, 11, 14, 21). The antigenic characteristics of Formalin-killed whole bacteria as compared with the immune response to autoclaved bacteria suggest that proteins are necessary for a significant humoral immune response to the heat-stable antigens of *H. pleuropneumoniae* (4, 11). Our previous observations showed that a significant immune response to the lipopolysaccharide (LPS) of *H. pleuropneumoniae* occurs as a result of infection but is not induced by immunization with bacterins and that serologic cross-reactive immunodeterminates are located in the LPS (1). The purpose of this study was to evaluate the vaccine potential of purified cell wall oligosaccharides isolated from the LPS of *H. pleuropneumoniae*.

H. pleuropneumoniae J45 (serotype 5) and 4047 (serotype 1) were grown in PPLO broth without crystal violet (Difco Laboratories, Detroit, Mich.) supplemented with 7% neutralized horse serum, 1 μg of NAD (Eastman Kodak Co., Rochester, N.Y.) per ml, and 4% yeast extract (GIBCO Laboratories, Salt Lake City, Utah). The LPS of *H. pleuropneumoniae* J45 was extracted by a modification of the procedure of Westphal and Jann (24). The majority of the LPS extracted was in the aqueous phase. This fraction was digested with RNase, DNase, and pronase and was concentrated and dialyzed versus saline and then water. The pelleted LPS was reconstituted in carbonate buffer (pH 8.5) and chromatographically purified. The fractions containing 2-keto-3-deoxyoctonate were pooled, concentrated,

TABLE 1. Geometric mean serum hemagglutination titers of immunized rabbits

Immunogens (200 μg/kg)	Serum titers[a] (n = 3) at the following days postimmunization:					
	0	5	10	15	20	30
HpS-OS[b]	0	0	5	10	16	42
HpS-OSTTC[c]	4	24	42	426	512	853
HpR-OS[d]	5	4	5	16	53	85
HpR-OSTTC[e]	16	42	64	426	597	1,024

[a] Homologous serum titers.
[b] Oligosaccharide core purified from smooth-type LPS of *H. pleuropneumoniae*.
[c] HpS-OS conjugated to tetanus toxoid.
[d] Oligosaccharide core purified from rough-type LPS of *H. pleuropneumoniae*.
[e] HpR-OS conjugated to tetanus toxoid.

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TABLE 2. Geometric mean serum immunoglobulin G (IgG) ELISA titers of immunized pigs

Immunogen (n = 3)	Mean serum IgG ELISA titer with the following antigens[a]:	
	HpS-LPS	HpR-LPS
HpS-OS	1,280	66
HpS-OSTTC	13,653	133
HpR-OS	93	855
HpR-OSTTC	426	34,133
Hp 1 (4047)[b]	4,266	3,413
Hp 5 (J45)[b]	8,533	4,266
Hp bacterin[c]	186	40
Control[d]	14	25

[a] Smooth type (HpS-LPS) and rough type (HpR-LPS) purified from *H. pleuropneumoniae* serotype 5, strain J45. Geometric mean titer of immunized swine (n = 3).

[b] Sera from experimentally infected convalescent pigs (3 weeks after last clinical signs of illness; n = 6). Hp, *H. pleuropneumoniae* serotype (strain).

[c] Bacterin containing *H. pleuropneumoniae* serotypes 1 and 5 (Pleuroguard; Norden Laboratories).

[d] Pigs unexposed to *H. pleuropneumoniae* (n = 9). See Table 1 for key.

redigested, dialyzed versus water, and lyophilized. The material was soluble in water and was labeled HpS-LPS.

The phenol-saturated bacteria remaining after the above procedure were subjected to phenol-chloroform-petroleum ether extraction by a modification of the methods of Galanos et al. (2). This fraction (HpR-LPS) was relatively insoluble in water and was therefore solubilized with 0.25% triethylamine (Sigma Chemical Co., St. Louis, Mo.) in 0.05 M carbonate buffer (pH 8.5), dialyzed against carbonate buffer, chromatographically purified, and stored at 4°C.

Because of the severe toxic effects of HpS-LPS and HpR-LPS when attempts were made to immunize pigs (1a), polysaccharide cores were isolated from each by acid hydrolysis and purified by gel filtration on a Sephadex G-25 column (Pharmacia, Inc. Piscataway, N.J.). The cores were dephosphorylated with 48% hydrogen fluoride and again purified on a Sephadex G-25 column as previously described (7) and lyophilized. The purified oligosaccharide cores (HpS-OS and HpR-OS) were not toxic when injected intramuscularly or subcutaneously into rabbits or pigs (Fenwick et al., in press).

In hopes of increasing the immunogenicity of HpS-OS and HpR-OS, samples of each were directly conjugated to tetanus toxoid by reductive amination through their reducing terminal, 2-keto-3-deoxyoctonate (23). Reaction mixtures of 275:1 (oligosaccharides to tetanus toxoid) after undergoing reductive amination for 10 days at pH 4.6 resulted in a conjugate ratio of 14:1 and 18:1 for HpS-OS and HpR-OS, respectively. The oligosaccharide from the rough type LPS (HpR-LPS) was coupled to tetanus toxoid slightly more efficiently than the larger and more complex oligosaccharide isolated from the smooth-type LPS (HpS-LPS). Relatively poor conjugation efficiencies have also been reported with smooth-type meningococcal polysaccharides (8, 9).

Antisera against HpS-LPS and HpR-LPS were produced in swine by the immunization methods of Moreno et al. (12). Antisera to HpS-OS and HpR-OS and their respective tetanus toxoid conjugates HpS-OSTTC and HpR-OSTTC were produced in rabbits and pigs by the same methods. Serum from pigs intramuscularly immunized three times at 2-week intervals with 2 ml of commercial *H. pleuropneumoniae* bacterin (Pleuroguard; Norden Laboratories, Lincoln, Nebr.) was collected 3 weeks after the last injection. Sera from convalescent pigs experimentally infected with *H. pleuropneumoniae* by intranasal inoculation with serotype 5 (strain J45) or serotype 1 (strain 4047) were collected 3 weeks after the last clinical signs of illness.

Serum immunoglobulin G titers against HpS-LPS and HpR-LPS were determined by enzyme-linked immunosorbent assay (ELISA) following a modification of the procedure described by Ito et al. (6). The results from each assay were corrected by an internal pooled negative serum and a bacterial-cell-wall fraction-specific hyperimmune positive control serum. The results are reported as titers, which were defined as the reciprocal of the lowest serum dilution with an optical density equal to at least three times the mean optical density of four wells containing pooled negative control sera.

Inhibition studies by ELISA were performed to evaluate the antigenic uniqueness of the various cell wall subunits. Hyperimmune, fraction-specific porcine antisera were diluted 10 times in saline and incubated for 2 h at room temperature with an equal volume (0.5 ml) of solutions containing graded concentrations of inhibitors. After the incubation, the antibody titer was redetermined by ELISA.

Hemagglutination assays were performed in 96-well microtiter plates with sheep erythrocytes as previously described (13). The sheep erythrocytes were sensitized with alkalinated HpS-LPS or HpR-LPS (0.25 N NaOH at 60°C for 90 min), purified oligosaccharides, or the oligosaccharide-tetanus toxoid conjugates and washed with phosphate-buffered saline (pH 7.2). Twofold dilutions of test sera were incubated for 2 h at room temperature in a 2% suspension of the sensitized sheep erythrocytes, and the titer was determined as the highest serum dilution causing agglutination.

Data in Table 1 clearly show that conjugation of the isolated oligosaccharide cores from *H. pleuropneumoniae* to tetanus toxoid significantly improved their immunogenicity. Mittal et al. (11) found that heat-stable antigens extracted from *H. pleuropneumoniae* were not capable of producing a type-specific antibody response but that antibodies specific for the heat-stable antigens were present in antisera produced against the whole organism. These findings suggested that autoclaved antigens (likely polysaccharides) require

TABLE 3. Geometric mean porcine serum hemagglutination titers

Antisera (n = 3)[a]	Mean serum hemagglutination titer with the following antigen:					
	HpS-LPS	HpS-OS	HpS-OSTTC	HpR-LPS	HpR-OS	HpR-OSTTC
HpS-OSTTC	10,922	8,192	16,384	170	8	213
HpR-OSTTC	85	16	341	21,845	16,384	54,613
Hp 1 (4047)	1,024	512	1,024	5,461	4,096	6,826
Hp 5 (J45)	16,384	10,922	21,845	8,192	8,192	16,384
Hp bacterin	32	8	10	4	4	5
Control	4	0	4	16	5	10

[a] See Table 1 for key.

TABLE 4. Fraction-specific inhibition of porcine antiserum

Inhibitor[a]	Inhibiting concn (μg/ml) of antisera[b]	
	HpS-LPS	HpR-LPS
HpS-LPS	>1	2,500
HpS-OS	>5	3,000
HpS-OSTTC	25	4,500
HpR-LPS	2,000	>1
HpR-OS	2,500	>5
HpR-OSTTC	4,000	15

[a] See Table 1 for key.
[b] Concentration of inhibitor giving 100% inhibition of ELISA titer.

heat-labile antigens (likely protein) as carriers in a haptenlike fashion to be fully immunogenic. Our findings with purified *H. pleuropneumoniae* oligosaccharides conjugated to tetanus toxoid confirm this notion. Studies involving other gram-negative polysaccharide-protein conjugates, including *Haemophilus influenzae*, have shown similar results, and their potential use as vaccines has been suggested (8, 9, 20).

The antibody titers to HpS-LPS and HpR-LPS of antisera produced in pigs against the purified oligosaccharides and the oligosaccharide-tetanus toxoid conjugates show that the polysaccharides prepared from the smooth-type and rough-type LPS were antigenically distinct (Tables 2 and 3). Serologic cross-reactivity between HpS-OS and HpS-OSTTC and between HpR-OS and HpR-OSTTC was found, and the tetanus toxoid conjugates induced greater titer than isolated oligosaccharide cores. Pigs immunized with the commercial *H. pleuropneumoniae* bacterin (containing both serotypes 1 and 5) had titers against HpS-LPS and HpR-LPS that were not significantly greater than the controls (Table 2), whereas sera from convalescent *H. pleuropneumoniae*-infected pigs had high titers against HpS-LPS and HpR-LPS. Furthermore, bacterin-immunized pigs did not have titers against either the oligosaccharide cores or their tetanus toxoid conjugates (Table 3). Many of the rabbits and pigs did have low titers against HpR-LPS before immunization which may reflect common antigenic determinants found within the LPS core of gram-negative bacterial cell walls. This suggests that the high titers induced by immunization with HpR-OSTTC may be, in part, due to an anamnestic immune response.

Quantitative adsorption studies also showed the antigenic specificity of HpS-LPS and HpR-LPS and their respective oligosaccharides (Table 4). The data also show that conjugation of tetanus toxoid to the oligosaccharides did not significantly alter their antigenic character. The minor serologic cross-reactions between the oligosaccharides isolated from HpS-LPS and HpR-LPS may be explained by a shared immunodeterminant, antibodies directed at the tetanus toxoid, or contamination. The reduced inhibiting ability of the oligosaccharide-tetanus toxoid conjugates with antisera may be due to a carrier effect or preexisting immunity to tetanus toxoid.

The findings of this study link cell wall structural subunits of *H. pleuropneumoniae* with specific immunologic characteristics and provide an improved understanding of the immunologic complexity associated with the infection in pigs. The results show that the polysaccharide portion of the cell wall of *H. pleuropneumoniae* plays a significant role in the porcine immune response after infection and that immunization with a whole-cell vaccine does not induce a significant immune response against these antigens. Furthermore, the immune response to purified oligosaccharides can be significantly improved by conjugation with a protein carrier (tetanus toxoid). The vaccine potential of the cell wall polysaccharide subunits is further suggested by the immunogenicity and antigenic characteristics of the nontoxic oligosaccharide-tetanus toxoid conjugates.

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LITERATURE CITED

1. **Fenwick, B. W., and B. I. Osburn.** 1986. Immune responses to the lipopolysaccharides and capsular polysaccharides of *Haemophilus pleuropneumoniae* in convalescent and immunized pigs. Infect. Immun. **54**:575–582.
1a. **Fenwick, B. W., B. I. Osburn, and H. J. Olander.** 1986. Isolation and biological characterization of two lipopolysaccharides and a capsular-enriched polysaccharide preparation from *Haemophilus pleuropneumoniae*. Am. J. Vet. Res. **47**:1433–1441.
2. **Galanos, C., O. Luderitz, and O. Westphal.** 1969. A new method for the extraction of R lipopolysaccharides. Eur. J. Biochem. **9**:245–249.
3. **Gunnarsson, A.** 1979. Evaluation of different antigens in the complement-fixation test for diagnosis of *Haemophilus pleuropneumoniae* (*parahaemolyticus*) infections in swine. Am. J. Vet. Res. **40**:1564–1567.
4. **Gunnarsson, A.** 1979. Serologic studies on porcine strains of *Haemophilus parahaemolyticus* (*pleuropneumoniae*): extraction of type specific antigens. Am. J. Vet. Res. **40**:469–472.
5. **Gunnarsson, A., B. Hurvell, and E. L. Biberstein.** 1978. Serological studies on porcine strains of *Haemophilus parahaemolyticus* (*pleuropneumoniae*): antigenic specificity and relationship between serotypes. Am. J. Vet. Res. **39**:1286–1292.
6. **Ito, J. I., A. C. Wunderlich, and J. L. Lyons.** 1980. The role of magnesium in the enzyme-linked immunosorbent assay for lipopolysaccharide of rough *Escherichia coli* strain J5 and *Neisseria gonorrhoeae*. J. Infect. Dis. **142**:532–537.
7. **Jennings, H. J., M. Bhattacharjee, L. Kenne, C. P. Kenny, and G. Calver.** 1980. The R-type lipopolysaccharide of *Neisseria meningitidis*. Can. J. Biochem. **58**:128–136.
8. **Jennings, H. J., and C. Lugowski.** 1981. Immunochemistry of group A, B, C meningococcal polysaccharide-tetanus toxoid conjugates. J. Immunol. **127**:1011–1018.
9. **Jennings, H. J., C. Lugowski, and F. E. Ashton.** 1984. Conjugation of meningococcal lipopolysaccharide R-type oligosaccharides to tetanus toxoid as route to a potential vaccine against group B *Neisseria meningitidis*. Infect. Immun. **43**:407–412.
10. **Mittal, K. R., R. Higgins, and S. Lariviere.** 1983. Detection of type-specific antigens in the lungs of *Haemophilus pleuropneumoniae*-infected pigs by a coagglutination test. J. Clin. Microbiol. **18**:1355–1357.
11. **Mittal, K. R., R. Higgins, and S. Lariviere.** 1983. Determination of antigenic specificity and relationship among *Haemophilus pleuropneumoniae* serotypes by an indirect hemagglutination test. J. Clin. Microbiol. **17**:787–790.
12. **Moreno, E., S. L. Speth, L. M. Jones, and D. T. Berman.** 1981. Immunochemical characterization of *Brucella* lipopolysaccharide and polysaccharides. Infect. Immun. **31**:214–222.
13. **Neter, E., L. F. Bertram, F. A. Zak, M. R. Murdock, and C. E. Arbesman.** 1952. Studies on hemagglutination and hemolysis by *Escherichia coli* antisera. J. Exp. Med. **96**:1–15.
14. **Nicolet, J.** 1971. Sur l'hemophilose du porc. III. Differenciation serologique de *Haemophilus parahaemolyticus*. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A **216**:487–495.

15. **Nielson, R.** 1970. *Haemophilus parahaemolyticus* as the cause of pleuropneumonia in swine. II. Studies on the identity and pathogenicity of the organisms isolated. Nord. Veterinaermed. **22**:246–255.
16. **Nielson, R.** 1979. *Haemophilus parahaemolyticus* serotypes pathogenicity and cross immunity. Nord. Veterinaermed. **31**:407–413.
17. **Nielson, R.** 1976. Pleuropneumonia in swine caused by *Haemophilus parahaemolyticus*. Studies on the protection obtained by vaccination. Nord. Veterinaermed. **28**:337–348.
18. **Nielson, R., A. D. Thomsen, and S. D. Vesterlund.** 1976. Pleuropneumonia caused by *Haemophilus parahaemolyticus*. An attempt to control the disease at two progeny testing stations by serological blood testing followed by removal of the seropositive animals and their litter mates. Nord. Veterinaermed. **28**:349–352.
19. **Rosendal, S., and W. R. Mitchell.** 1983. Epidemiology of *Haemophilus pleuropneumoniae* infection in pigs: a survey of Ontario pork producers. Can. J. Comp. Med. **47**:1–5.
20. **Schneerson, R., O. Barrera, A. Sutton, and J. B. Robbins.** 1980. Preparation, characterization, and immunogenicity of *Haemophilus influenzae* type b polysaccharide-protein conjugates. J. Exp. Med. **152**:361–364.
21. **Schultz, R. A., R. F. Ross, A. Gunnarsson, and R. Nielsen.** 1983. Serotyping 50 different isolates of *Haemophilus pleuropneumoniae* from swine pneumonia in Iowa and surrounding states. Vet. Med. Small Anim. Clin. **78**:1451–1453.
22. **Schultz, R. A., T. F. Young, R. F. Ross, and D. R. Jeske.** 1982. Prevalence of antibody to *Haemophilus pleuropneumoniae* in Iowa swine. Am. J. Vet. Res. **43**:1848–1851.
23. **Schwartz, B. A., and G. R. Grey.** 1977. Protein containing reductively aminated disaccharides. Arch. Biochem. Biophys. **181**:542–549.
24. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharides: extraction with phenol-water and further applications of the procedure. Methods Carbohydr. Chem. **5**:83–95.