INFECTION AND IMMUNITY, Aug. 1992, p. 3042–3051
0019-9567/92/083042-10$02.00/0

Vol. 60, No. 8

# Preparation, Characterization, and Immunogenicity of Conjugate Vaccines Directed against *Actinobacillus pleuropneumoniae* Virulence Determinants

WYATT BYRD AND SOLOMON KADIS†*

*Department of Medical Microbiology, College of Veterinary Medicine, University of Georgia, Athens, Georgia 30602*

Received 30 January 1992/Accepted 27 April 1992

**Conjugate vaccines were prepared in an attempt to protect pigs against swine pleuropneumonia induced by *Actinobacillus pleuropneumoniae* (SPAP). Two subunit conjugates were prepared by coupling the *A. pleuropneumoniae* 4074 serotype 1 capsular polysaccharide (CP) to the hemolysin protein (HP) and the lipopolysaccharide (LPS) to the HP. Adipic acid dihydrazide was used as a spacer to facilitate the conjugation in a carbodiimide-mediated reaction. The CP and the LPS were found to be covalently coupled to the HP in the conjugates as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and detergent gel chromatography analyses. Following a booster vaccination, pigs exhibited significantly high ($P < 0.05$) immunoglobulin G antibodies against CP, LPS, and HP. The anti-CP and anti-LPS immunoglobulin G antibodies were found to function as opsonins in the phagocytosis of *A. pleuropneumoniae* by polymorphonuclear leukocytes, whereas antibodies to the HP neutralized the cytotoxic effect of the HP on polymorphonuclear leukocytes. No killing of *A. pleuropneumoniae* was observed when the effects of the antibodies were tested in the presence of complement. Thus, polysaccharide-protein *A. pleuropneumoniae* conjugates elicit significant antibody responses against each component of each conjugate, which could be instrumental in protecting swine against SPAP.**

Swine pleuropneumonia induced by encapsulated strains of the gram-negative bacterial organism *Actinobacillus pleuropneumoniae* (SPAP) is a highly contagious and often fatal disease of swine that is currently considered to be one of the most economically important diseases of swine worldwide (34, 46). This disease can occur in a peracute, acute, subacute, or chronic form depending on the virulence of the infectious agent (43), the infective dose (43, 49), the immune status of the infected animal (36), and stress caused by adverse environmental conditions (34, 45). The lesions observed in pigs suffering from the peracute form of SPAP are indicative of an intense inflammatory response evidenced by hemorrhage, edema, and fibrin exudation stemming from increased vascular permeability and neutrophil infiltration of the lung tissue (3, 28). The acute form of SPAP is characterized by fibrinous pleuritis and hemorrhagic necrotizing pneumonia (29, 45). Animals that survive the acute stage of the disease can exhibit manifestations of the chronic form of SPAP, become carriers of the organism, and infect nonimmune pigs (34, 37).

Despite extensive information concerning the clinical and histopathological manifestations of SPAP, the pathogenesis of the disease is incompletely understood. Evidence has implicated the capsular polysaccharide (CP) (22, 23), lipopolysaccharide (LPS) endotoxin (17, 52), and outer membrane proteins (7, 8) in the pathogenesis of this disease. However, antibodies directed only against these cell wall components do not appear to protect swine against SPAP, because heat-killed and formalin-treated bacterins, which induce antibodies to these bacterial components, provide only limited protection (16, 21, 36). Thus, it appears that a component(s) elaborated by viable *A. pleuropneumoniae* cells is required for the complete disease spectrum to be manifested. A likely candidate is the extracellular hemolysin protein (HP). The HP from an *A. pleuropneumoniae* serotype 1 strain was shown to be the predominant antigenic protein when convalescent-phase field serum or serum samples from experimentally infected pigs were used to develop immunoblots (10). Serum from pigs experimentally infected with strains representative of each of the 12 known *A. pleuropneumoniae* serotypes strongly reacted with the HP from the *A. pleuropneumoniae* serotype 1 strain (11). In addition, the HP from this *A. pleuropneumoniae* serotype 1 strain has been shown to be cytotoxic for porcine neutrophils, alveolar macrophages, and lymphocytes (51).

Although SPAP is one of the most economically important diseases affecting the swine industry, no effective ways of protecting animals have been developed. Our approach has been to prepare two vaccines directed against the aforementioned virulence factors, CP, LPS, and HP, which, when used together, would induce an immune response. On the basis of the reported effectiveness of various polysaccharide-protein conjugate vaccines in protecting against a number of different diseases (13, 24, 40), we prepared two subunit conjugate vaccines by (i) conjugating *A. pleuropneumoniae* CP to the HP and (ii) conjugating the LPS to the HP. The levels of antibody titers raised to each component of each vaccine and the functions of these antibodies were determined.

## MATERIALS AND METHODS

**Cultivation of bacteria.** *A. pleuropneumoniae* 4074, serotype 1, has been maintained in our laboratory, lyophilized in skim milk, and stored at 4°C. For the isolation of CP and LPS, the bacteria were cultured at 37°C in Trypticase soy

* Corresponding author.
† Present address: 7 Deauville Court, Apartment 2A, Pikesville, MD 21208.

broth containing 0.6% yeast extract and 0.01% NAD. For HP isolation, a chemically defined medium (29) was used for the bacterial cultures to avoid contamination of the isolated HP with other proteins and components of enriched media.

**LPS assay.** The concentration of LPS in the purified CP, LPS, and HP preparations was determined by the colorimetric *Limulus* amebocyte lysate assay, using the kit provided by Whittaker Bioproducts, Walkersville, Md., for the rapid chromogenic quantitation of bacterial endotoxin.

**SDS-PAGE.** The purity of the hemolysin preparation was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (27). The separating gel consisted of a 5 to 20% gradient of acrylamide and bisacrylamide in 0.37 M Tris-HCl (pH 8.8)–0.1% (wt/vol) SDS. The stacking gel consisted of 5% (wt/vol) acrylamide and bisacrylamide in 0.125 M Tris-HCl (pH 6.8)–0.1% (wt/vol) SDS. The hemolysin preparation was diluted into sample buffer (65 mM Tris-HCl [pH 6.8] containing 10% [vol/vol] glycerol, 2% [wt/vol] SDS, and 5 mM dithiothreitol), heated at 95°C for 5 min, and subjected to electrophoresis at a constant current of 15 mA. The gel was stained with 0.5% Coomassie blue in 40% (vol/vol) methanol–10% (vol/vol) acetic acid.

**Isolation and purification of CP.** A modification of a published procedure (1) was used to isolate and purify CP from *A. pleuropneumoniae*. The organism was cultured for 6 h at 37°C in Trypticase soy broth containing yeast extract and NAD, harvested, and resuspended in 0.2 M phosphate-buffered saline solution (PBS) (pH 7.0). The CP was released from the surface of the bacteria by vigorous agitation, and the supernatant fluid containing the CP was filtered through a membrane filter (pore size, 0.45-μm). Three volumes of cold (−20°C) acetone were mixed with the supernatant fluid and allowed to remain overnight at −20°C. The mixture was then centrifuged (13,300 × *g* for 15 min), and the pellet was suspended in 10 mM Tris-HCl (pH 8.0) containing 2 mM $MgCl_2$. Both DNase (200 μg/ml; Sigma Chemical Co., St. Louis, Mo.) and RNase (50 μg/ml; Sigma) were added, and the mixture was incubated for 2 h at 37°C. Next, proteinase (Sigma) (200 μg/ml) was added, and the mixture was incubated overnight at 37°C. Three volumes of cold (−20°C) acetone were again added, the mixture was centrifuged, and the pellet was dissolved in 10% saturated sodium acetate–77% (wt/vol) phenol. The resultant phenol phase was extracted three times with fresh 10% saturated sodium acetate. The aqueous phase was dialyzed against 0.1 M $CaCl_2$ for 24 h and centrifuged at 105,000 × *g* for 3 h to remove LPS. Three volumes of 95% ethanol containing 1% (vol/vol) saturated sodium acetate were added to the supernatant, and the mixture was centrifuged (13,300 × *g* for 1 h). The ethanol-purified CP was air dried, dissolved in pyrogen-free, sterile distilled water, and lyophilized. The purity of the CP was measured by three methods. First, measurements of $A_{260}$ and $A_{280}$ were performed to test for nucleic acid and protein contamination, respectively. Second, the extent of protein contamination was determined by the use of the Bradford procedure (4) with a kit provided by Bio-Rad Laboratories, Rockville Center, New York, N.Y. Third, LPS contamination was determined by the use of the colorimetric *Limulus* amebocyte lysate assay as indicated above. The CP preparation was found to be essentially free of nucleic acid and protein (both less than 0.1% by weight) and LPS (less than 0.001% by weight). The CP preparation contained less than 10 ng of endotoxin per mg of CP, as determined by the *Limulus* amebocyte lysate assay.

**Isolation and purification of LPS.** The procedure of Darveau and Hancock (6) was used to isolate and purify LPS from *A. pleuropneumoniae*. Disrupted *A. pleuropneumoniae* cells were treated with DNase, RNase, proteinase, and SDS, and the LPS extract was subjected to ethanol-magnesium chloride precipitation and high-speed centrifugation. The LPS preparation was essentially free (less than 0.1% by weight) of contaminating nucleic acid, protein, and CP when analyzed by procedures previously described (30).

**Isolation and purification of HP.** *A. pleuropneumoniae* 4074, serotype 1, was cultured in a chemically defined medium (29) at 37°C for 4 to 6 h (i.e., until the middle to the end of the logarithmic phase of growth). The culture was centrifuged (27,200 × *g* for 20 min at 4°C) and then filter sterilized with a low-protein-binding 0.2-μm filter to remove bacterial cells. The pH of the cell-free supernatant fluid was adjusted to pH 6.2, ammonium sulfate (85% saturation) was added, and the suspension was stirred at 4°C for 12 h. The precipitate that formed was removed by centrifugation (27,200 × *g* for 30 min at 4°C). The pellet was resuspended at 0 to 4°C in 3 to 4 ml of 10 mM Tris-HCl (pH 7.2) containing 50 mM NaCl and 0.5 mM $CaCl_2$, and the suspension was applied to a Sephacryl S-200HR (Pharmacia Fine Chemicals, Piscataway, N.J.) gel filtration column (1.6 by 96 cm). The HP was eluted from the chromatographic column at a flow rate of 24 ml/h with an eluant of 10 mM Tris-HCl (pH 7.2) containing 50 mM NaCl and 0.5 mM $CaCl_2$. The $A_{280}$ was used to monitor the elution of protein from the column. The eluant volume containing protein was dialyzed against 0.05 M PBS (pH 7.2) for 48 h at 4°C and lyophilized. Approximately $4 \times 10^3$ endotoxin units of LPS per mg of isolated HP was measured in our purified HP preparations.

**Conjugation of CP and HP.** A modification of a published (15) procedure for coupling polysaccharide to protein was used for conjugation of CP and HP. The purified *A. pleuropneumoniae* CP was partially depolymerized by heating a solution of the CP in 0.1 M glycine-NaOH buffer (pH 10), containing 0.1 M $CaCl_2$, at 80°C for 4 h (15). The CP was converted to the tetra-*n*-butylammonium salt via ion-exchange chromatography with Dowex resin in the $(N\text{-}Bu)_4N^+$ form. The CP was dissolved in dimethyl sulfoxide followed by the addition of adipic acid dihydrazide (ADH) to a final concentration of 0.125 M and then 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) to a final concentration of 0.125 M. CP was coupled to ADH at room temperature for the first 1 to 2 h and then at 4°C for the remainder of the 24-h period, both with end-over-end tumbling. The coupling reaction was maintained at pH 4.5 to 5.0 during the initial 1 to 2 h of the coupling reaction. After the coupling reaction was complete, the reaction mixture was dialyzed extensively against distilled water for 24 h at 4°C. The ADH derivative of CP was subjected to ion-exchange chromatography at pH 7.0 by using the $Na^+$ form of a Dowex resin to remove electrostatically attached ADH from the CP.

To the ADH derivative of CP was added HP and then EDAC at a final concentration of 0.125 M. The ADH-CP derivative was coupled to the HP in an EDAC-mediated coupling reaction as described above, with distilled water as the reaction medium. On completion of coupling, the reaction mixture was extensively dialyzed against distilled water and lyophilized. The CP-ADH-HP conjugate was dissolved in 0.05 M PBS (pH 7.2) and passed through a Sepharose CL-4B (Pharmacia) gel chromatography column (2.6 by 100 cm) at 4°C with 0.05 M PBS (pH 7.2) as the eluant. A flow rate of 48 ml/h was used, and the conjugate-containing fractions were determined by using a UV monitor at 280 nm and the phenol-sulfuric acid assay (14) for protein and carbohydrate determination, respectively. The conjugate-

containing fractions were pooled, lyophilized, resuspended in distilled water, dialyzed against distilled water for 24 h at room temperature, and lyophilized again. The concentrations of protein and carbohydrate present in the CP-AH-HP conjugate were measured by the Bradford procedure (4), using a kit provided by Bio-Rad and the phenol-sulfuric acid method (14), respectively. The ADH content was determined by the TNBS (2,4,6-trinitrobenzenesulfonic acid) assay (15).

**Conjugation of LPS and HP.** A modification of a published (44) procedure for coupling polysaccharide to protein was used for conjugation of LPS and HP. The purified *A. pleuropneumoniae* LPS was detoxified by alkaline hydrolysis with 0.1 N NaOH in 99% ethanol at 65°C for 1 h (44, 48). The *Limulus* amebocyte lysate gelation assay (Whittaker) was used to measure the extent of LPS detoxification by alkaline hydrolysis. After the deacylated LPS was dissolved in ethylene glycol, ADH and EDAC were added, both at a final concentration of 0.125 M. LPS was coupled to ADH at room temperature for the first 1 to 2 h and then at 4°C for the remainder of the 24-h period, both with end-over-end tumbling. The coupling reaction was maintained at pH 4.5 to 5.0 during the initial 1 to 2 h of the coupling reaction. After the coupling reaction was complete, the reaction mixture was dialyzed extensively against distilled water for 24 h at 4°C. The ADH-derivatized LPS was subjected to ion-exchange chromatography at pH 7.0 with the $Na^+$ form of a Dowex resin to remove electrostatically attached ADH from the CP.

The hemolysin protein was treated and conjugated to the ADH-LPS derivative as described for the CP-HP conjugation. The concentrations of protein, carbohydrate, and ADH present in the LPS-AH-HP conjugate were measured as described above.

**Evaluation of covalent coupling of the CP-HP and LPS-HP conjugates.** Analyses involving SDS-PAGE and sodium deoxycholate detergent column chromatography were performed to determine whether the CP was covalently coupled to the HP and whether the LPS was covalently coupled to the HP in the CP-HP and LPS-HP conjugates, respectively (44). Both conjugates, as well as their CP and LPS components, were subjected to SDS-PAGE. The procedure involves the use of the Laemmli (27) buffer system and stacking and separating gels of 4% and 12% acrylamide-bisacrylamide, respectively. Gels were stained by the alcian blue-silver stain procedure (31). In addition, both conjugates, as well as their CP, LPS, and HP components, were subjected to column chromatography in a sodium deoxycholate detergent medium. A Sephadex G-100 (Pharmacia) column (1.5 by 5.5 cm) was equilibrated with 0.05 M sodium phosphate buffer (pH 8.8) containing 0.2% sodium deoxycholate (44). The fractions in which the protein and the carbohydrates eluted from the column were determined by the use of a UV monitor at 280 nm and the phenol-sulfuric acid assay (14), respectively.

**Preparation of porcine serum.** Serum samples were obtained from healthy 10- to 14-week-old pigs free of SPAP. All pigs were tested for exposure to *A. pleuropneumoniae* by a highly sensitive enzyme-linked immunosorbent assay (ELISA) (35). The pigs were randomly assigned to one of three groups. In group 1 ($n = 12$), each pig was vaccinated intramuscularly with both conjugates simultaneously in a 1-ml volume and, 10 days after booster immunization, challenged intratracheally with $5 \times 10^7$ cells of *A. pleuropneumoniae* 4074, serotype 1, suspended in 10 ml of 0.05 M PBS (pH 7.2). In group 2 ($n = 17$), each pig was injected intramuscularly with 1 ml of adjuvant-containing pyrogen-free sterile distilled water and then 10 days later challenged intratracheally with 10 ml containing $5 \times 10^7$ bacterial cells. In group 3 ($n = 2$), each pig was vaccinated intramuscularly with the conjugate vaccines simultaneously in a 1-ml volume and was not challenged but, 10 days after the booster immunization, administered 10 ml of sterile PBS intratracheally.

The conjugate vaccines were dissolved in pyrogen-free sterile distilled water, emulsified with an equal amount of either Freund's complete adjuvant or Freund's incomplete adjuvant, and administered intramuscularly simultaneously. In the initial vaccination, the conjugates were emulsified with Freund's complete adjuvant. In the booster vaccination, administered 2 weeks after the initial vaccination, the conjugates were emulsified with Freund's incomplete adjuvant. The amounts of polysaccharide (CP and LPS) and protein (HP) in each conjugate inoculated per pig were 27.8 μg of CP and 2.0 μg of HP for the CP-HP conjugate and 25.8 μg of LPS and 2.0 μg of HP for the LPS-HP conjugate. A total of 4 μg of HP was inoculated per pig, with both conjugates being administered simultaneously.

**Antibody measurement.** The CP, LPS, and HP antibody titers in pigs administered the conjugate vaccines were measured by the use of a previously described ELISA procedure (32). $A_{405}$ readings were measured on an ELISA reader (Titertek Multiscan; Flow Laboratories, Inc., McLean, Va.). The procedure involved the use of goat anti-swine immunoglobulin G (IgG)(γ), IgM(μ), and IgA(α) antibody alkaline phosphatase conjugates (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.). Blood samples for serological studies were collected from each animal 14 days after initial vaccination, 10 days after booster vaccination, and 7 days after challenge. Antibody titers to IgG(γ), IgM(μ), and IgA(α) heavy chains were determined. The titers were defined as the reciprocal of the highest dilution of test immune serum at which the $A_{405}$ was at least twice that of the nonimmune serum.

**Antibody function(s).** Antisera were obtained from 10- to 14-week-old pigs immunized simultaneously with the CP-HP and LPS-HP conjugate vaccines as already indicated. The pigs were bled 10 days after the last injection. The antisera obtained from the pigs were pooled, heat inactivated (at 56°C for 1 h), and subjected to repeated ammonium sulfate (33% saturation) precipitation and extensive dialysis against 0.05 M PBS (pH 7.2). Separation and purification of monospecific antibodies against the CP, LPS, and HP were performed with the aid of immunosorbent columns to which appropriate antigens had been coupled. Specific anti-CP and anti-LPS antibodies were prepared by passing the antibody suspension through equilibrated (0.05 M PBS, 0.15 M NaCl [pH 7.4]) epoxy-activated Sepharose 6B (Pharmacia) columns (1.6 by 20 cm) to which either the CP or LPS had been coupled by standard procedures (38). Specific anti-HP antibodies were prepared by passing the antibody suspension through an equilibrated (0.05 M PBS, 0.15 M NaCl [pH 7.4]) EAH-Sepharose 4B (Pharmacia) column (1.6 by 20 cm) to which the HP had been coupled by standard procedures (38). Since the HP preparation contains LPS, the antibody suspension was absorbed against LPS to remove anti-LPS antibodies (25) before the antibody suspension was passed through the column. The monospecific antibodies were eluted initially with 2 M and finally with 3 M sodium thiocyanate. Eluted proteins were detected by measurement at 280 nm with a UV monitor and verified by the Bradford assay (4), using a Bio-Rad kit. The antibody-containing samples were dialyzed extensively by using dialysis tubing

treated to be low protein binding and then concentrated by ultrafiltration with a YM30 membrane filter (Amicon Division, Beverly, Mass.) until the original volume of immune serum was obtained. Aliquots of the purified antibodies were stored frozen at −20°C.

**Phagocytosis assay.** Polymorphonuclear leukocytes (PMN) were isolated from heparinized blood drawn from clinically healthy pigs. The blood, diluted 1:1 with PBS, was overlaid onto Histopaque 1077 (Sigma) and centrifuged (700 × *g* for 30 min). Erythrocytes were removed by hypotonic lysis with distilled water as previously described (19). The PMN were suspended in RPMI 1640 (Sigma) supplemented with 5% fetal bovine serum (pH 7.2; Biofluid, Inc., Rockville, Md.) heat inactivated at 56°C for 1 h. Only the suspensions containing at least 98% PMN and exhibiting 98% viability, as determined by Wright staining and trypan blue exclusion, respectively, were used in the assay.

A modification of the method of Vray et al. (54) was used to determine, by the use of flow cytometry, the ability of antibodies to the CP, LPS, or HP to act as opsonins to enhance the uptake of bacteria by PMN. These bacteria were heat killed (at 60°C for 1 h) and treated with fluorescein isothiocyanate (20). The fluorescein isothiocyanate-treated bacterial cells (FB) were opsonized with affinity-purified antibodies to CP, LPS, or HP at concentrations of 0.15, 0.3, 0.6, and 1.2 μg/ml for 15 min at 37°C with gentle shaking. The FB were mixed with freshly isolated PMN at an FB:PMN ratio of 10:1 and were incubated for 20 min at 37°C with gentle shaking. Phagocytosis was halted by rapid cooling to 0 to 4°C in an ice-water bath, and cells were centrifuged (6,630 × *g* for 5 min). After lysozyme (Sigma) (0.1 mg/ml) was added, the suspension was incubated for 15 min at 37°C to lyse all extracellular bacteria. The PMN were washed twice and resuspended in RPMI 1640 containing 5% heat-inactivated fetal bovine serum (pH 7.2). Flow cytometry (Coulter EPIC 753; Coulter Corp., Hialeah, Fla.) was performed to measure the percentage of PMN that contain internalized FB.

**Bactericidal assay.** To determine whether antibody to CP, LPS, or HP could interact with complement to kill the bacteria, we used a bactericidal assay based on a modification of previously described methods (5, 26, 42, 50). Early-logarithmic-phase broth culture of *A. pleuropneumoniae* 4074 was harvested by centrifugation (17,400 × *g* for 15 min), washed once in Veronal-buffered saline solution (pH 7.2) containing 0.15 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.1% gelatin ($GVBS^{2+}$), and suspended in $GVBS^{2+}$ to a final concentration of $5 \times 10^7$ bacteria per ml. Normal pig serum was absorbed three times against *A. pleuropneumoniae* 4074 for 30 min each at 4°C with gentle shaking. This was done to remove any antibodies that might have been present in the normal porcine serum and thus might adversely affect the bactericidal assay by acting as either cross-reacting or blocking antibodies. The absorbed normal pig serum was passed through a 0.45-μm filter to eliminate any residual bacteria. A part of the absorbed normal pig serum was heat inactivated (at 56°C for 30 min). *A. pleuropneumoniae* cells ($2.5 \times 10^6$) were incubated with affinity-purified antibodies to CP, LPS, or HP at concentrations of 0.12, 0.3, 0.6, or 1.2 μg/ml and with either absorbed normal pig serum or heat-inactivated absorbed normal pig serum (20%), for 0 to 3 h at 37°C in a shaking water bath. Samples (100 μl) were removed from each assay mixture at 0, 1, 2, and 3 h and plated out in triplicate on Trypticase soy agar. The number of viable cells per milliliter was determined. Killing was accorded to those reactions in which at least a 50% reduction in the number of viable cells was observed after the specific incubation period compared with the number of organisms present in the same tubes at the onset of the reaction.

**Toxin neutralization assay.** Since HP has been shown to be cytotoxic for porcine PMN (51), an attempt was made to determine whether affinity-purified antibodies to the HP were capable of neutralizing the biological activity of the HP. The MTT [3-(4,5-dimethylthiazoyl-2-yl)-2,5-diphenyltetrazolium bromide] hemolysin neutralization assay (53) was used to measure the leukocidal activity of the HP for porcine PMN and thus to determine the HP neutralization titer of antibodies to the HP. Porcine PMN were isolated as described above, except that RPMI 1640 was used without the addition of fetal bovine serum and 100-μl aliquots were placed into wells of a microtiter plate at a concentration of $2.5 \times 10^5$ cells per 100 μl. The PMN were incubated for 1 h at 37°C under a 5% $CO_2$–humidified-air atmosphere to allow for adherence of the porcine PMN. The HP was prepared by ammonium sulfate precipitation from cell-free supernatant fluid as described above. The precipitate was dissolved in 2 to 3 ml of 0.05 M PBS (pH 7.2) and dialyzed against PBS at 4°C. The HP preparation was then diluted in RPMI 1640 to 1 hemolytic unit/ml. The antibodies to the HP were serially diluted in RPMI 1640 (pH 7.4), and each 100-μl sample of the antibody preparation was incubated with 100 μl of HP for 10 min at 37°C. The mixture was then added to the PMN and incubated for 45 min at 37°C under a 5% $CO_2$–humidified-air atmosphere. The unreacted MTT dye and medium were removed by aspiration. Then 100 μl of acid-isopropanol (0.04 N HCl in isopropanol) and 100 μl of 3% SDS were added, and the amount of formazan in each well was measured at 570 nm with an ELISA reader.

**Statistical analysis.** The Tukey multiple-range test was used to calculate *P* values for antibody responses at a 0.05 level of significance.

## RESULTS

**Preparation of CP-HP and LPS-HP conjugates.** The composition of each of the two conjugate vaccines was analyzed. It was determined that the ADH-to-carbohydrate ratios in terms of percentages (wt/wt) were 2.0 and 2.5% for the derivatized CP and LPS, respectively. The protein-to-carbohydrate ratios in terms of percentages (wt/wt) were 6.7 and 7.2% for the CP-HP and LPS-HP conjugates, respectively. Figures 1A and B depict the elution profiles of the CP-HP and LPS-HP conjugates, respectively. Both conjugates eluted in the void volume.

The CP was partially depolymerized by alkaline hydrolysis, which generated phosphate monoester termini at C-1 of galactose, and it is through the phosphate monoester termini that the CP is considered to be derivatized with ADH in an EDAC-mediated reaction. The purified LPS preparation contained approximately $10^7$ EU/mg as determined by the *Limulus* amebocyte assay. The LPS was detoxified by treatment with 0.1 N NaOH in 99% ethanol for 1 h; this caused the removal of the ester-linked fatty acids but left intact the amide-linked fatty acids (44, 48). This treatment detoxified the LPS 1,000- to 10,000-fold compared with the untreated LPS, as determined by the *Limulus* amebocyte lysate gelation assay. Via a EDAC-mediated reaction, the LPS is considered to be derivatized with ADH through the C-8 carboxyl of 2-keto-3-deoxyoctulosonic acid present in the inner core region of this LPS.

The HP was conjugated to the ADH 6-carbon spacer of both the CP-ADH and LPS-ADH derivatives via another

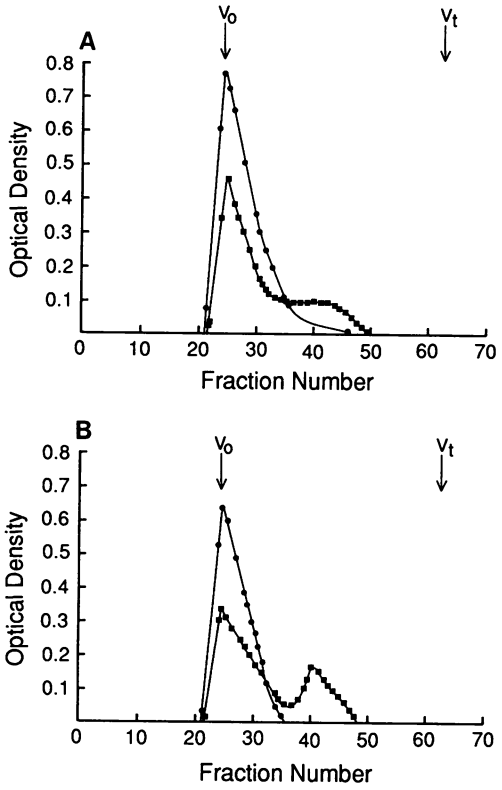


FIG. 1. Gel filtration of conjugates through a CL-4B Sepharose column. (A) CP-HP; (B) LPS-HP. The optical density at 280 nm was used to measure protein (■), and the phenol-sulfuric acid assay (optical density at 480 nm) (14) was used to measure carbohydrate (●). Fractions having both protein and carbohydrate and eluting in the void volume, 23 to 33 for the CP-HP conjugate and 23 to 32 for the LPS-HP conjugate, were pooled and subsequently used in vaccine trials. Abbreviations: $V_0$, void volume; $V_t$, total volume.

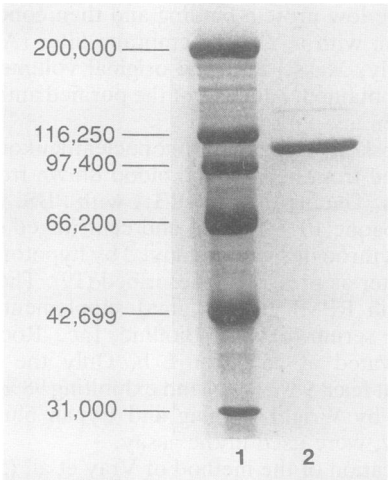


FIG. 2. Coomassie blue-stained SDS-polyacrylamide gel of *A. pleuropneumoniae* hemolysin preparation. The hemolysin preparation (lane 2) sample (25 μl; 8 μg of protein) was applied to the 5 to 20% polyacrylamide gradient gel. A strongly stained protein band was detected with a molecular weight of 107,000 (lane 2). In addition, a very faint band appears on the gel with an estimated molecular weight of approximately 120,000 (lane 2). Lane 1, molecular weight standards.

EDAC-mediated reaction through available C-terminal carboxyl groups and carboxyl groups on the acidic amino acids, aspartic acid and glutamic acid. Figure 2 depicts the SDS-PAGE gel of the hemolysin preparation and reveals the presence of a strongly stained protein band with an estimated molecular mass of 107 kDa. In addition, one other very faint band with an estimated molecular mass of approximately 120 kDa appears on the gel.

**Evaluation of covalent coupling of the CP-HP and LPS-HP conjugates.** To determine whether the CP and the LPS were each covalently coupled to the HP in the conjugate vaccines, we performed analyses involving SDS-PAGE and sodium deoxycholate column chromatography. The conjugates, as well as their individual components, were subjected to SDS-PAGE. The CP and LPS could be detected in the gel when unconjugated to HP, but when the conjugates were formed, they were too large to enter into the separating gel and neither stained CP nor LPS was detected in the lanes loaded with the CP-HP and LPS-HP conjugates (Fig. 3). Likewise, determination of CP-HP and LPS-HP conjugate covalent coupling was confirmed by detergent gel chromatography. Figures 4A and C depict the elution profiles of the CP-HP and LPS-HP conjugates, respectively, following gel filtration with sodium deoxycholate detergent medium, and Fig. 4B and D depict the elution profiles of the CP and HP components and LPS and HP components, respectively, following gel filtration under conditions identical to those for the conjugates. When carbohydrate and protein were monitored, detergent gel chromatography revealed that the CP-HP and LPS-HP conjugates eluted in the void volume ($V_0$) whereas the CP, LPS, and HP components eluted from the column after the $V_0$. Since the sodium deoxycholate detergent exerts an effect on electrostatic interactions but does not disrupt covalent bonds, this indicated that the

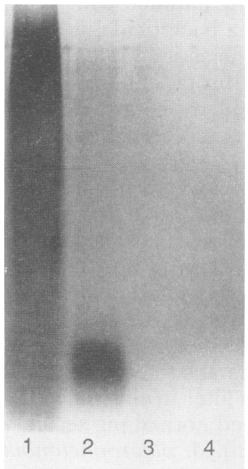


FIG. 3. Alcian blue–silver-stained SDS-polyacrylamide gel of the CP and LPS of *A. pleuropneumoniae* and the conjugates, CP-HP and LPS-HP. The CP (lane 1), LPS (lane 2), CP-HP conjugate (lane 3), and LPS-HP conjugate (lane 4) samples (20 μl; 20 μg of carbohydrate) were applied to the polyacrylamide gel. The CP and LPS could be detected in the gel when not conjugated to the HP, but when the conjugates were applied to the gel neither the CP nor the LPS was detected in the lanes loaded with the CP-HP and LPS-HP conjugates.

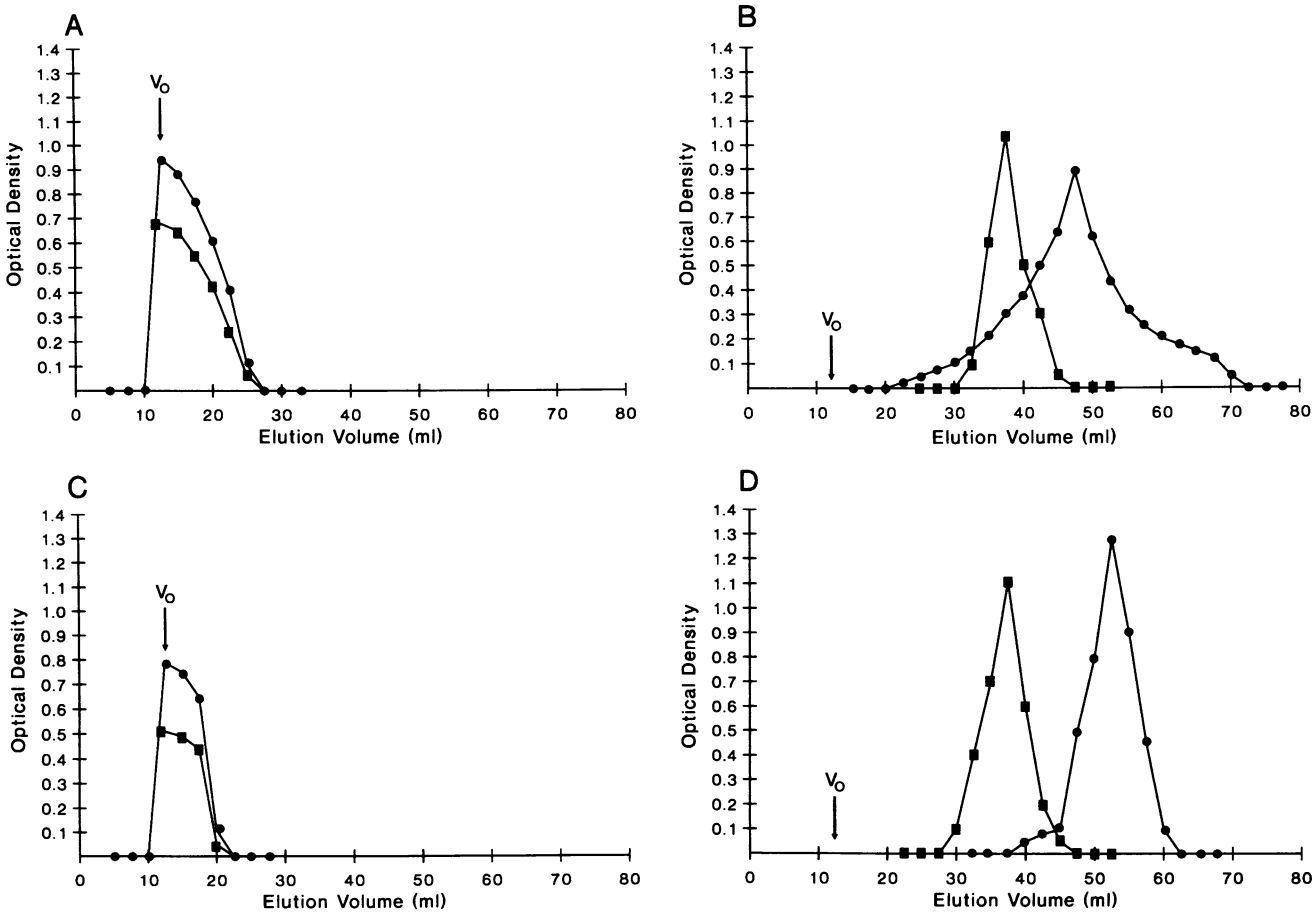


FIG. 4. Elution profiles of (A) the CP-HP conjugate and (C) the LPS-HP conjugate following gel filtration with sodium deoxycholate detergent medium, and elution profiles of (B) the CP and HP components and (D) the LPS and HP components, following gel filtration under conditions identical to those for the conjugates. Protein (■) was determined by measuring optical density at 280 nm, and carbohydrate (●) was measured by the phenol-sulfuric acid assay (optical density at 480 nm) (14). Detergent gel chromatography revealed that the CP-HP and LPS-HP conjugates eluted in the void volume ($V_0$) whereas the CP, LPS, and HP components eluted from the column after the void volume.

conjugates were held together by covalent bonds and not by electrostatic interactions.

**Antibody generated against each component of the conjugate vaccines.** The antibody titers for the CP, LPS, and HP components of the CP-HP and LPS-HP conjugate vaccines are depicted in Table 1. No IgA antibodies could be detected following either the initial or booster vaccinations. IgM antibodies were measured after both the initial and booster immunizations, but in both cases the titers were very low, i.e., less than twice the titers of the preimmune serum. The antibody titers presented are of the IgG class. After the initial vaccination with the conjugates, IgG antibodies were detected at low titers. Following the booster vaccination, antibodies against the CP, LPS, and HP could be detected at significantly higher titers in the vaccinated challenged and vaccinated unchallenged groups, with the HP antibody titers being the highest ($P < 0.05$). Seven days after challenge, high IgG antibody titers were detected for the CP, LPS, and

TABLE 1. IgG antibody titers generated against components of conjugate vaccines in pigs

| Pig[a] | IgG antibody titer[b] after[c]: | | | | | |
|---|---|---|---|---|---|---|
| | Booster | | | Challenge | | |
| | CP | LPS | HP | CP | LPS | HP |
| VC ($n$ = 10)[d] | 2.2*(1.8–2.7) | 2.4*(1.8–3.0) | 2.7*(1.2–3.3) | 2.6**(1.8–3.0) | 2.7(2.1–3.0) | 3.5**(3.0–3.9) |
| UC ($n$ = 8)[d] | 0 | 0 | 0 | 2.1(1.8–2.7) | 2.2(1.2–2.7) | 3.1(2.4–3.9) |
| VU ($n$ = 2) | 2.0*(1.8–2.1) | 2.4* | 2.9*(2.7–3.0) | 2.0(1.8–2.1) | 2.3(2.1–2.4) | 2.6(2.4–2.7) |

[a] VC, vaccinated challenged; UC, unvaccinated challenged; VU, vaccinated unchallenged.
[b] IgG antibody titers are expressed as the mean of $\log_{10}$ values. The values in parentheses represent the range of the antibody titers. Symbols: *, $P < 0.05$ when compared with initial vaccination levels; **, $P < 0.05$ when compared with booster vaccination levels.
[c] Serum samples were collected 10 days after booster immunization and 7 days after challenge with *A. pleuropneumoniae*.
[d] Since two pigs in group VC and nine pigs in group UC died within 18 h of challenge, $n$ = 10 and $n$ = 8, respectively, are used.

TABLE 2. Effect of antibodies to conjugate vaccine components on phagocytosis of *A. pleuropneumoniae*

| Antibody concn (μg/ml) | % PMN containing phagocytized bacteria[a] in presence of: | | |
|---|---|---|---|
| | Anti-CP | Anti-LPS | Anti-HP |
| 0.00 | 14.10 ± 4.54 | 14.10 ± 4.54 | 14.10 ± 4.54 |
| 0.15 | 20.86 ± 4.21 | 31.63 ± 6.91 | 17.25 ± 1.79 |
| 0.30 | 37.92 ± 4.05 | 38.50 ± 2.56 | 15.10 ± 4.08 |
| 0.60 | 44.29 ± 6.18 | 41.23 ± 3.10 | 18.90 ± 3.23 |
| 1.20 | 51.42 ± 5.02 | 48.10 ± 3.75 | 21.20 ± 4.18 |

[a] Values are means ± standard deviations of three experiments. The three experiments were performed with the same lot of antibodies fractionated from antisera pooled from three pigs.

TABLE 3. Neutralization of HP by anti-HP antibodies

| Anti-HP antibody concn (μg/ml) | HP neutralization (%)[a] |
|---|---|
| $3.0 \times 10^{-1}$ | 96.2 ± 5.3 |
| $1.5 \times 10^{-1}$ | 86.4 ± 6.0 |
| $7.5 \times 10^{-2}$ | 75.8 ± 5.2 |
| $3.8 \times 10^{-2}$ | 70.5 ± 4.9 |
| $1.9 \times 10^{-2}$ | 53.8 ± 7.4 |
| $9.4 \times 10^{-3}$ | 22.5 ± 2.1 |
| $4.7 \times 10^{-3}$ | 8.3 ± 7.2 |
| $2.3 \times 10^{-3}$ | 0.0 |

[a] Values are means ± standard deviations of three experiments. The three experiments were performed with the same lot of antibodies fractionated from antisera pooled from three pigs.

HP in the unvaccinated challenged group. After challenge the antibody titers in the vaccinated challenged group increased and were higher than those in the unvaccinated challenged group. The vaccinated unchallenged group exhibited a decrease in antibody titers for the LPS and the HP during the experimental period.

**Phagocytosis assay.** The percentage of PMN that contained phagocytized *A. pleuropneumoniae* 4074 bacteria, as measured by flow cytometry, is shown in Table 2. As the CP and LPS antibody concentrations increased to 1.2 μg/ml, there was an accompanying increase in the percentage of PMN that contained phagocytized bacteria. At CP or LPS antibody concentrations greater than 1.2 μg/ml, the bacteria tended to agglutinate and could not be resuspended in solution. Therefore the highest antibody concentration we used was 1.2 μg/ml. When PMN and *A. pleuropneumoniae* bacteria were incubated without the addition of antibodies, 14.1% of the PMN contained phagocytized bacteria. This percentage was the control for PMN with phagocytized bacteria in the absence of antibodies to function as opsonins. Initial experiments demonstrated that when the *A. pleuropneumoniae* bacteria were subjected to the lysozyme treatment in the absence of PMN, all the bacteria were lysed and were not included in the spectrofluorimetric count.

At a CP antibody concentration of 0.6 μg/ml, 44.3% of the PMN contained phagocytized bacteria, a value 3.2 times the control value. At the same LPS antibody concentration, 41.2% of the PMN contained phagocytized bacteria (2.9 times the control). With anti-HP antibodies at an identical concentration, 18.9% of the PMN had engulfed bacteria (1.4 times the control).

**Bactericidal assay.** To assess whether the anti-CP, anti-LPS, or anti-HP antibodies exhibited any bactericidal activity, we tested the effects of each of these antibodies together with normal pig serum absorbed with *A. pleuropneumoniae* 4074 or heat-treated absorbed normal pig serum. When the serum was heat treated, the complement was inactivated. Thus, no killing of the *A. pleuropneumoniae* bacteria would be expected and none was observed. There was also no observed killing of the *A. pleuropneumoniae* bacteria when the effects of the antibodies were tested in the presence of normal porcine serum having active complement.

*Escherichia coli* K-12 was used as a positive control to ensure that the normal porcine serum complement was active and that the heat-treated normal porcine serum had been inactivated. The *E. coli* K-12 bacteria were completely killed by the normal pig serum within 1 h of incubation, but these bacteria were unaffected and grew rapidly when incubated with the heat-treated normal porcine serum. Likewise, when unheated or heat-treated normal pig serum was incubated with sheep erythrocytes and rabbit anti-sheep erythrocyte antibodies, the erythrocytes were lysed in the presence of the unheated pig serum and sufficient titers of the erythrocyte antibodies but were not lysed by the heat-treated serum at any antibody titer.

**Toxin neutralization assay.** The ability of anti-HP antibodies to neutralize the cytotoxic effect of the HP on PMN is depicted in Table 3. At the lowest dilutions of antibodies to the HP that were used, there was almost complete neutralization of the cytotoxic activity of the HP against PMN.

As the anti-HP antibody concentration decreased, a subsequent increase was noted in the killing of the PMN by the HP. The highest dilution of antibodies to the HP able to cause some neutralization of the HP was 1/128, at which a 8.3% neutralization was measured. The neutralization titer of the affinity-purified antibodies to the HP was measured at a 1/32 dilution, at which 53.8% neutralization of the HP was observed. These results indicate that antibodies to the HP neutralize the ability of the HP to kill PMN.

## DISCUSSION

The rationale for preparing two conjugate vaccines by which the CP of *A. pleuropneumoniae* is coupled to the HP and the LPS is coupled to the HP is that these vaccines could induce an immune response against three important virulence factors of *A. pleuropneumoniae*. In addition, since bacterial polysaccharides are T-cell-independent antigens (12, 41), we anticipated that conjugation of CP and LPS to the HP would produce T-cell-dependent polysaccharide-protein conjugates which could lead to both anti-polysaccharide and anti-protein antibody production, as well as a cellular immune response.

The LPS of *A. pleuropneumoniae*, like the LPS of most gram-negative bacteria, is a toxic and highly immunogenic molecule (17, 18, 52). However, it can be rendered nontoxic while retaining its antigenic determinants by modification of the lipid A portion of the molecule (44, 48). Alkaline treatment of the LPS removes 95% of the ester-linked fatty acids but leaves intact the amide-linked fatty acids; this results in a nontoxic LPS molecule (44). This treatment greatly reduced the toxicity of *A. pleuropneumoniae* LPS as measured by the *Limulus* amebocyte lysate gelation assay. Unfortunately, this treatment also reduces the immunogenicity of the LPS compared with that of the untreated LPS (44). However, it has been shown that the immunogenicity of the alkali-detoxified LPS can be restored and enhanced by covalent coupling to a protein carrier (44).

We used the HP as the protein carrier because it appears to be a critical virulence factor elaborated by *A. pleuropneu-*

*moniae*. Evidence indicates that *A. pleuropneumoniae* releases HP in vivo and that the HP is considered to be responsible for the necrotic changes associated with *A. pleuropneumoniae*-induced pneumonic lesions (9, 10). The HP is toxic for porcine phagocytes and lymphocytes, and this would enhance the ability of the *A. pleuropneumoniae* to evade nonspecific host defense mechanisms (2, 39). This cytotoxicity would greatly enhance the ability of *A. pleuropneumoniae* to evade clearance from the lungs of a pig during the course of an infection and thus would allow the bacteria to proliferate extensively within the lungs. Investigations indicate that the minimal protective effectiveness of bacterins is due to the absence of the HP in such preparations (9). The HP is normally secreted by viable bacterial cells but is not present in the bacterins because the bacteria are killed by the treatment used to prepare the bacterin and are no longer able to synthesize and secrete the HP.

With respect to antibodies generated against components of the conjugate vaccines, only antibodies of the IgG subclass were detected in significant titers in the serum of pigs after vaccination with the conjugate vaccines. Both IgG and IgM antibodies were detected at low and essentially equal titers after the initial vaccination. However, after the booster vaccination, the IgM titers were about the same as those in the initial vaccination whereas the IgG titers increase significantly over those of the initial vaccination. This was seen for all the virulence determinants used to prepare the conjugates. The booster response to the polysaccharides, CP and LPS, suggests that in the conjugates they were no longer acting as T-cell-independent antigens but as T-cell-dependent antigens. Booster response is accompanied by class switching, i.e., IgM to IgG, which is indicative of a T-cell-dependent antibody response.

To obtain some indication of how antibodies against each of the conjugate vaccine components might be acting in protecting pigs against SPAP, we investigated specific functions of antibodies to the three conjugate vaccine components. Since phagocytosis plays a major role in the nonspecific and specific immunity of the mammalian host (33, 47), a study was directed at determining which of the antibodies could act as effective opsonins to enhance the uptake of bacteria by PMN. Antibodies to both the CP and LPS exhibited pronounced opsonic activity, whereas antibodies to the HP enhanced uptake only marginally.

To evaluate phagocytosis, the *A. pleuropneumoniae* cells were heat killed prior to uptake by the PMN because these viable bacteria synthesize and secrete an HP which inactivates and kills PMN and thus inhibits phagocytosis. To measure the ability of antibodies to act as opsonins, it was necessary to eliminate the ability of the bacteria to elaborate HP. This underlines the extreme importance of the ability of HP antibodies to neutralize the cytolytic effects of the HP and allow the phagocytic cells to take up the bacteria. Therefore, even when antibodies to surface structures, such as CP, LPS, and outer membrane proteins, are present, if the HP is not neutralized by anti-HP antibodies, the PMN and macrophages will be unable to survive and participate in their phagocytizing functions.

Whereas antibodies to the CP and LPS acted as opsonins, antibodies to the HP acted as opsonins only in a marginal capacity. It appears that the surface of the *A. pleuropneumoniae* cell may contain some HP, to which the antibodies to the HP are able to bind and enhance uptake by the PMN. As the HP is synthesized within the bacterial cell and must traverse the cell wall in the process of transport to the exterior, some of this HP might be trapped in or on the surface of the bacterial cell wall. This might be the HP to which the anti-HP antibodies are binding.

We also examined the ability of these antibodies to be bactericidal for *A. pleuropneumoniae* 4074 in the presence of porcine complement. Studies in our laboratory have shown that *A. pleuropneumoniae* 4074 is resistant to the bactericidal activity of serum. Likewise, when antibodies to the CP, LPS, and HP were incubated with normal porcine serum as the source of complement, no bacteriolysis of the *A. pleuropneumoniae* 4074 cells was observed. It appears that antibodies to the CP, LPS, and HP do not interact with complement to cause killing of the bacteria.

Evidence indicates that the nonimmune swine cannot eliminate the *A. pleuropneumoniae* 4074 by either complement-mediated lysis or phagocytosis. Therefore, for the pig's immune system to eliminate the agent, specific antibodies to permit phagocytosis of these encapsulated bacteria by phagocytic cells must be produced.

## ACKNOWLEDGMENTS

We thank Frank A. Udeze for helpful discussions and critical analysis of the manuscript, Richard N. Thwaits for his skillful performance of the pig experiments, John Brown for assistance in analyzing the data statistically, and Julie Golden for technical assistance with the flow cytometry experiments.

This work was supported by grants from U.S. Department of Agriculture Animal Health; the Veterinary Medical Experiment Station, University of Georgia; and the Georgia Department of Agriculture Swine Disease Eradication Program.

## REFERENCES

1. **Adlam, C., J. M. Knights, A. Mugridge, J. C. Lindon, P. R. W. Baker, J. E. Beesley, B. Spacey, G. R. Craig, and L. K. Nagy.** 1984. Purification, characterization and immunological properties of the serotype-specific capsular polysaccharide of *Pasteurella haemolytica* (serotype A1) organisms. J. Gen. Microbiol. **130**:2415–2426.
2. **Bendixen, P. H., P. E. Shewen, S. Rosendal, and B. N. Wilkie.** 1981. Toxicity of *Haemophilus pleuropneumoniae* for porcine lung macrophages, peripheral blood monocytes, and testicular cells. Infect. Immun. **33**:673–676.
3. **Bertram, T. A.** 1985. Quantitative morphology of peracute pulmonary lesions in swine induced by *Haemophilus pleuropneumoniae*. Vet. Pathol. **22**:598–609.
4. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
5. **Corbiel, L. B., K. Blau, T. J. Inzana, K. H. Nielsen, R. H. Jacobson, R. R. Corbeil, and A. J. Winter.** 1988. Killing of *Brucella abortus* by bovine serum. Infect. Immun. **56**:3251–3261.
6. **Darveau, R. P., and R. E. W. Hancock.** 1983. Procedure for isolation of bacterial lipopolysaccharides from both smooth and rough *Pseudomonas aeruginosa* and *Salmonella typhimurium* strains. J. Bacteriol. **155**:831–838.
7. **Deneer, H. G., and A. A. Potter.** 1989. Identification of a maltose-inducible major outer membrane protein in *Actinobacillus* (*Haemophilus*) *pleuropneumoniae*. Microb. Pathog. **6**:425–432.
8. **Deneer, H. G., and A. A. Potter.** 1989. Effect of iron restriction on the outer membrane proteins of *Actinobacillus* (*Haemophilus*) *pleuropneumoniae*. Infect. Immun. **57**:798–804.
9. **Devenish, J., S. Rosendal, and J. T. Bosse.** 1990. Humoral antibody response and protective immunity in swine following immunization with the 104-kilodalton hemolysin of *Actinobacillus pleuropneumoniae*. Infect. Immun. **58**:3829–3832.
10. **Devenish, J., S. Rosendal, J. T. Bosse, B. N. Wilkie, and R.**

**Johnson.** 1990. Prevalence of seroreactors to the 104-kilodalton hemolysin of *Actinobacillus pleuropneumoniae* in swine herds. J. Clin. Microbiol. **28:**789–791.
11. **Devenish, J., S. Rosendal, R. Johnson, and S. Hubler.** 1989. Immunoserological comparison of 104-kilodalton proteins associated with hemolysis and cytolysis in *Actinobacillus pleuropneumoniae*, *Actinobacillus suis*, *Pasteurella haemolytica*, and *Escherichia coli*. Infect. Immun. **57:**3210–3213.
12. **Dick, W. E., Jr., and M. Beurret.** 1989. Glycoconjugates of bacterial carbohydrate antigens. A survey and consideration of design and preparation factors. Contrib. Microbiol. Immunol. **10:**48–114.
13. **Donnelly, J. J., R. R. Deck, and M. A. Liu.** 1990. Immunogenicity of a *Haemophilus influenzae* polysaccharide-*Neisseria meningitidis* outer membrane protein complex conjugate vaccine. J. Immunol. **145:**3071–3079.
14. **Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28:**350–356.
15. **Egan, W., R. Schneerson, K. E. Werner, and G. Zon.** 1982. Structural studies and chemistry of bacterial capsular polysaccharides. Investigations of phosphodiester-linked capsular polysaccharides isolated from *Haemophilus influenzae* types a, b, c, and f: NMR spectroscopic identification and chemical modification of end groups and the nature of base-catalyzed hydrolytic depolymerization. J. Am. Chem. Soc. **104:**2898–2910.
16. **Fedorka-Cray, P. J., M. J. Huether, D. L. Stine, and G. A. Anderson.** 1990. Efficacy of a cell extract from *Actinobacillus (Haemophilus) pleuropneumoniae* serotype 1 against disease in swine. Infect. Immun. **58:**358–365.
17. **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.
18. **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.
19. **Field, T. R., M. R. Williams, and K. J. Bunch.** 1985. An improved method for the isolation of a neutrophil-rich fraction from porcine blood. Br. Vet. J. **141:**355–361.
20. **Gelfand, J. A., A. S. Fauci, I. Green, and M. M. Frank.** 1976. A simple method for the determination of complement receptor-bearing mononuclear cells. J. Immunol. **116:**595–599.
21. **Higgins, R., S. Lariviere, K. R. Mittal, G. P. Martineau, P. Rousseau, and J. Cameron.** 1985. Evaluation of a killed vaccine against porcine pleuropneumonia due to *Haemophilus pleuropneumoniae*. Can. Vet. J. **26:**86–89.
22. **Inzana, T. J.** 1987. Purification and partial characterization of the capsular polymer of *Haemophilus pleuropneumoniae* serotype 5. Infect. Immun. **55:**1573–1579.
23. **Inzana, T. J., and B. Mathison.** 1987. Serotype specificity and immunogenicity of the capsular polymer of *Haemophilus pleuropneumoniae* serotype 5. Infect. Immun. **55:**1580–1587.
24. **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.
25. **Kabir, S.** 1983. The serological properties of the cell surface proteins of *Vibrio cholerae*. J. Gen. Microbiol. **129:**2199–2206.
26. **Kimura, A., and E. J. Hansen.** 1986. Antigenic and phenotypic variations of *Haemophilus influenzae* type b lipopolysaccharide and their relationship to virulence. Infect. Immun. **51:**69–79.
27. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
28. **Liggett, A. D., L. R. Harrison, and R. L. Farrell.** 1987. Sequential study of lesion development in experimental haemophilus pleuropneumonia. Res. Vet. Sci. **42:**204–221.
29. **Maudsley, J. R., and S. Kadis.** 1986. Growth and hemolysin production by *Haemophilus pleuropneumoniae* cultivated in a chemically defined medium. Can. J. Microbiol. **32:**801–805.
30. **Maudsley, J. R., S. Kadis, and W. R. Mayberry.** 1986. Isolation, purification, and partial characterization of a lipopolysaccharide from *Haemophilus pleuropneumoniae*. Infect. Immun. **51:**501–506.
31. **Min, H., and M. K. Cowman.** 1986. Combined alcian blue and silver staining of glycosaminoglycans in polyacrylamide gels: application to electrophoretic analysis of molecular weight distribution. Anal. Biochem. **155:**275–285.
32. **Morris, D. D., and R. H. Whitlock.** 1987. Therapy of suspected septicemia in neonatal foals using plasma-containing antibodies to core lipopolysaccharide (LPS). J. Vet. Intern. Med. **1:**175–182.
33. **Neveu, P. J.** 1986. The mononuclear phagocyte system. Bull. Inst. Pasteur **84:**23–66.
34. **Nicolet, J.** 1986. *Haemophilus* infections, p. 426–439. *In* A. D. Leman, B. Straw, R. D. Glock, W. L. Mengeling, R. H. C. Penny, and E. Scholl (ed.), Diseases of swine, 6th ed. Iowa State University Press, Ames.
35. **Nicolet, J., P. Paroz, M. Krawinkler, and A. Baumgartner.** 1981. An enzyme-linked immunosorbent assay, using an EDTA-extracted antigen for the serology of *Haemophilus pleuropneumoniae*. Am. J. Vet. Res. **42:**2139–2142.
36. **Nielsen, R.** 1976. Pleuropneumonia of swine caused by *Haemophilus parahaemolyticus*. Studies on the protection obtained by vaccination. Nord. Vet. Med. **28:**337–348.
37. **Nielsen, R., and M. Mandrup.** 1977. Pleuropneumonia in swine caused by *Haemophilus parahaemolyticus*. A study of the epidemiology of the infection. Nord. Vet. Med. **29:**465–473.
38. **Pharmacia.** 1986. Affinity chromatography: principles and methods, p. 18–32. Pharmacia, Laboratory Separation Division. Ljungforetagen AB, Sweden.
39. **Pijoan, C.** 1986. Effect of *Pasteurella multocida* and *Haemophilus pleuropneumoniae* toxins on swine alveolar macrophages. Vet. Immunol. Immunopathol. **13:**141–149.
40. **Robbins, J. B., and R. Schneerson.** 1990. Polysaccharide-protein conjugates: a new generation of vaccines. J. Infect. Dis. **161:**821–832.
41. **Robbins, J. B., R. Schneerson, W. B. Egan, W. Vann, and D. T. Liu.** 1980. Virulence properties of bacterial capsular polysaccharides—unanswered questions, p. 115–132. *In* H. Smith, J. J. Skehel, and M. J. Turner (ed.), The molecular basis of microbial pathogenicity. Verlag Chemie GmbH, Weinheim, Germany.
42. **Romano, P. J.** 1980. Cytolytic methods, p. 29–34. *In* N. R. Rose and H. Friedman (ed.), Manual of clinical immunology, 2nd ed. American Society of Microbiology, Washington, D.C.
43. **Rosendal, S., D. A. Boyd, and K. A. Gilbride.** 1985. Comparative virulence of porcine *Haemophilus* bacteria. Can. J. Comp. Med. **49:**68–74.
44. **Sadoff, J. C., S. L. Futrovsky, H. F. Sidberry, B. H. Iglewski, and R. C. Seid, Jr.** 1982. Detoxified lipopolysaccharide-protein conjugates. Semin. Infect. Dis. **4:**346–354.
45. **Sanford, S. E., and G. K. A. Josephson.** 1981. Porcine *Haemophilus pleuropneumoniae* epizootic in southwestern Ontario: clinical, microbiological, pathological and some epidemiological findings. Can. J. Comp. Med. **45:**2–7.
46. **Sebunya, T. N. K., and J. R. Saunders.** 1983. *Haemophilus pleuropneumoniae* infection in swine: a review. J. Am. Vet. Med. Assoc. **182:**1331–1337.
47. **Segal, A. W.** 1981. The antimicrobial role of the neutrophil leukocyte. J. Infect. **3:**3–17.
48. **Seid, R. C., Jr., and J. C. Sadoff.** 1981. Preparation and characterization of detoxified lipopolysaccharide-protein conjugates. J. Biol. Chem. **256:**7305–7310.
49. **Shope, R. E., D. C. White, and G. Leidy.** 1964. Porcine contagious pleuropneumonia. II. Studies of the pathogenicity of the etiological agent, *Haemophilus pleuropneumoniae*. J. Exp. Med. **119:**369–375.
50. **Taylor, P. W.** 1985. Measurement of the bactericidal activity of serum, p. 445–456. *In* M. Sussman (ed.), The virulence of *Escherichia coli*. Reviews and methods. Academic Press, Inc., New York.
51. **Udeze, F. A., and S. Kadis.** 1988. Effect of *Actinobacillus pleuropneumoniae* hemolysin on viability and function of por-

cine phagocytosis. Proc. Int. Pig Vet. Soc. **10**:64.
52. **Udeze, F. A., K. S. Latimer, and S. Kadis.** 1987. Role of *Haemophilus pleuropneumoniae* lipopolysaccharide endotoxin in the pathogenesis of porcine *Haemophilus* pleuropneumonia. Am. J. Vet. Res. **48**:768–773.
53. **Vega, M. V., S. K. Maheswaran, J. R. Leininger, and T. R. Ames.** 1987. Adaptation of a colorimetric microtitration assay for quantifying *Pasteurella haemolytica* Al leukotoxin and antileukotoxin. Am. J. Vet. Res. **48**:1559–1564.
54. **Vray, B., J. Hoebeke, M. Saint-Guillain, R. Leloup, and A. D. Strosberg.** 1980. A new quantitative fluorimetric assay for phagocytosis of bacteria. Scand. J. Immunol. **11**:147–153.