INFECTION AND IMMUNITY, Sept. 1992, p. 3852–3860
0019-9567/92/093852-09$02.00/0

Vol. 60, No. 9

Inhibition of Bactericidal Activity of Anticapsular Antibody by Nonspecific Antibodies Reactive with Surface-Exposed Antigenic Determinants on *Actinobacillus pleuropneumoniae*

FRANK A. UDEZE AND SOLOMON KADIS†*

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

Received 28 March 1991/Accepted 1 July 1992

In an attempt to understand the mechanism of serum resistance in *Actinobacillus pleuropneumoniae*, in the present study we examined various interactions among the bacterial surface constituents, serum antibodies, and complement. Analysis of swine sera revealed the presence of anticapsular antibodies in convalescent-phase sera but not in preimmune sera. Both types of sera contained antibodies which reacted with each of 14 polypeptides present in saline extracts of the bacteria. Absorption of the preimmune sera with intact bacteria depleted antibodies to two of the polypeptides (27 and 32 kDa) and high-molecular-weight (>97.4,000) components which did not stain with Coomassie blue. Data derived from complement consumption and C3-binding experiments indicated that the organism was capable of initiating complement activation and binding C3 during incubation in preimmune and immune sera. Experiments designed to evaluate the bactericidal effectiveness of anticapsular antibody revealed that the purified antibody was bactericidal only when preimmune sera absorbed with intact bacteria were used as a source of complement. The bactericidal effects of anticapsular antibody and absorbed preimmune sera were inhibited in a dose-dependent manner by heat-inactivated preimmune sera and immunoglobulin G derived from the sera. The inhibitory activity of the preimmune sera was neutralized by preincubating the sera with column fractions of the saline extract which contained either the 27- or the 32-kDa polypeptide. These results indicate that serum resistance in *A. pleuropneumoniae* 4074 could be related to inhibition of the bactericidal action of anticapsular antibody by nonspecific antibodies which recognize surface-exposed epitopes on the polypeptides.

Swine pleuropneumonia is a severe and often fatal respiratory disease of pigs that is caused by the encapsulated gram-negative bacterium *Actinobacillus pleuropneumoniae* (24, 36, 37). Infected pigs may develop acute fibrinohemorrhagic necrotizing pleuropneumonia or localized chronic pulmonary necrosis with pleuritic adhesions (36, 37). Acute pleuropneumonia with a high rate of mortality is often observed with nonimmune animals after they are exposed to virulent strains. The chronic disease is observed with animals which survive the acute phase of disease and with herds with persistent infection (24, 36). Although *A. pleuropneumoniae* exhibits marked pathogenicity and pneumotropism for the swine respiratory tract (16, 38), septicemia and complications in the forms of meningitis, arthritis, endocarditis, abortion, and abscesses in various tissues have been observed previously (16, 24, 36). Attempts to control swine pleuropneumonia by vaccination with heat-killed or formalinized bacteria have not been successful, because these bacterins provide only minimal and serotype-specific protection against acute infection (9, 25, 32).

The failure of current vaccines to protect against *A. pleuropneumoniae* infections is reflected in part by the lack of basic understanding of the virulence mechanisms which enable the organism to evade host defense. One such host defense mechanism is the complement-mediated bactericidal activity of serum, which plays an important role in the prevention of certain gram-negative bacterial infections (7, 8, 31). Although most gram-negative bacteria are susceptible to killing by serum, the occurrence of serum-resistant bacteria has been noted previously (22, 29, 34, 44). Clinical findings on the relevance of serum resistance as a bacterial pathogenicity determinant have shown a strong correlation between serum resistance and the abilities of a variety of gram-negative bacteria to cause bacteremia, followed by shock and death (22, 27, 31, 44). Evidence from experimental infections of laboratory animals also supports the relationship between serum resistance and the ability of gram-negative bacteria to invade the blood system and produce disseminated infections (7). On the basis of these findings, the ability of *A. pleuropneumoniae* strains to survive in the bloodstream (2, 24) and subsequently cause extrapulmonary infections (16, 24, 36) may be related to their capacity to resist killing by preimmune and immune swine sera (10, 34).

In view of the crucial role of bacterial surface constituents in mediating resistance to humoral defense (4, 23, 30), it is implicitly apparent that a knowledge of *A. pleuropneumoniae* surface-exposed antigenic determinants and their interactions with serum components is fundamental to understanding the mechanism of *A. pleuropneumoniae* resistance to killing by serum. Although the capsule of *A. pleuropneumoniae* appears to constitute the outermost structure of the organism (11), the results of immunoabsorption studies have led some investigators to suggest that certain membrane proteins may possess epitopes which are surface exposed (28, 42). Thus, in order to understand the mechanism of *A. pleuropneumoniae* resistance to killing by serum, we have

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

examined the bacterial cell wall antigens for the presence of surface-exposed antigenic determinants whose interaction with serum components may be detrimental to bacterial killing by complement components.

MATERIALS AND METHODS

Bacteria and growth conditions. *A. pleuropneumoniae* 4074 (ATCC 27088), the reference strain of serotype 1, was obtained from the American Type Culture Collection and used in all experiments. The organism has been maintained as lyophilized cultures stored at 4°C.

Lyophilized cultures were routinely reconstituted in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.) supplemented with 0.01% NAD (Sigma Chemical Co., St. Louis, Mo.). The reconstituted culture was used to inoculate 20 ml of BHI-NAD in a 125-ml Erlenmeyer flask which was incubated overnight at 37°C without agitation. For complement consumption, C3-binding, and serum bactericidal assays, a 200-μl quantity of the overnight culture was transferred to 20 ml of fresh BHI-NAD, and then growth of the bacteria was continued at 37°C with slight agitation (100 rpm) until a Klett reading of 50 was reached (ca. 3 h), as determined by the use of a photoelectric colorimeter (no. 66 filter; Klett-Summerson, Trenton, N.J.).

Preparation of bacterial antigens. Surface constituents of *A. pleuropneumoniae* 4074 were extracted from mid-logarithmic-phase bacteria with a 0.15 M NaCl solution or a 0.15 M phosphate-buffered saline (PBS) solution, pH 7.0, at 56°C as described previously (33, 35). Capsular polysaccharide (CP) was purified from the PBS extracts by modification of a previously described method (1). Briefly, the bacteria were washed once in the 0.15 M NaCl solution and suspended in PBS. The suspension was agitated for 6 h with an electric shaker at 56°C and centrifuged at 10,000 × *g* for 10 min at 4°C. The supernatant fluid was filtered through a 0.45-μm-pore-size membrane filter, mixed with 3 volumes of cold acetone, and incubated overnight at −20°C. The precipitate formed was collected by centrifugation (17,000 × *g*, 20 min, 4°C) and dissolved in 20 mM Tris-HCl (pH 8.0) containing 2 mM $MgCl_2$. The solution was incubated with DNase (20 U/ml) and RNase (20 U/ml) at 37°C for 2 h, after which pronase was added to the solution at a final concentration of 200 μg/ml. After being incubated overnight at 37°C, the crude CP was precipitated with 3 volumes of 95% ethanol, dissolved in 10 mM Tris-HCl (pH 7.5), and extracted two times with 45% phenol at room temperature. The aqueous phase was collected, dialyzed extensively against 10 mM Tris-HCl (pH 7.5), and ultracentrifuged (105,000 × *g*, 4 h, 4°C) to remove residual lipopolysaccharide (LPS). The supernatant was concentrated, applied to a column (1.6 by 90 cm) of Sephadex G-75 (Pharmacia LKB Biotechnology, Piscataway, N.J.) which had been equilibrated in 0.15 M PBS (pH 7.5), and eluted with the same buffer. Carbohydrate-positive fractions were pooled, dialyzed against distilled water, and lyophilized.

Extracts obtained after a 1-h incubation of bacteria in the 0.15 M NaCl solution were used as the starting material for the chromatographic fractionation of extracted proteins. The saline extract was filter sterilized and incubated at 4°C for 1 h with the protease inhibitor phenylmethylsulfonyl fluoride at a final concentration of 1 mM. The solution was dialyzed overnight against 20 liters of 20 mM Tris-HCl (pH 7.8) at 4°C and applied to a column (2.6 by 10 cm) of DEAE-Sepharose CL-6B (Pharmacia) equilibrated with the Tris-HCl buffer. The column was washed with 2 column volumes of the same buffer, after which adsorbed material was eluted with a linear gradient (0 to 0.5 M) of NaCl in the same buffer. The eluate was collected in 5-ml fractions while the A_{280} was monitored. Fractions which eluted with the NaCl gradient were pooled, concentrated, and applied to a column (1.6 by 90 cm) of Sephacryl S-200HR (Pharmacia) which had been equilibrated with 50 mM Tris-HCl (pH 7.8) containing 0.15 M NaCl. Fractions corresponding to individual protein peaks were pooled, dialyzed against 20 mM Tris-HCl (pH 7.0), concentrated, and filter sterilized.

LPS was isolated and purified by using the method of Darveau and Hancock (5). The purified LPS was essentially free of contaminating protein, nucleic acids, cellular phospholipids, and CP, as reported previously (22).

Chemical analysis. Preparations of bacterial antigen were analyzed for protein, carbohydrate, endotoxin, and nucleic acid contents. Protein assays were performed by using the colorimetric method of Bradford (3), with bovine serum albumin as the standard. The carbohydrate content was determined by using the hot phenol-sulfuric acid method (6), with glucose as the standard. The concentration of the LPS endotoxin was measured by the colorimetric *Limulus* amoebocyte lysate assay with a kit obtained from Whittaker M. A. Bioproducts, Walkersville, Md. Purified *Escherichia coli* LPS was used as the standard. Nucleic acid contamination was determined from the ratio of A_{280} to A_{260}.

PAGE. Aliquots (100 μl) of concentrated saline extract and pools of protein peaks obtained from the columns were diluted in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (2% SDS, 4 mM dithiothreitol, 10% glycerol, 0.002% bromophenol blue in 0.065 M Tris-HCl [pH 6.8]). The samples were heated at 100°C for 5 min and subjected to electrophoresis on modified Laemmli gels (17): a 3% stacking gel and a 5-to-20% polyacrylamide gradient separating gel. Electrophoresis was carried out at a constant current of 15 mA until the dye front had migrated to the end of the gel. The gels were stained with silver stain (Bio-Rad Laboratories, Rockville Centre, N.Y.) and counterstained with 0.2% Coomassie brilliant blue in 40% methanol–5% acetic acid.

Preparation of swine sera. Nonimmune swine sera (NSS) were prepared from the blood of five clinically healthy pigs (4 to 5 months old) with no known history of *A. pleuropneumoniae* infection. The blood was allowed to clot for 1 h at room temperature and then centrifuged (2,000 × *g*, 20 min, 4°C). The sera were pooled, filter sterilized through a 0.22-μm-pore-size filter, and stored at −70°C in 1.0-ml aliquots. A 20-ml aliquot of the NSS was adsorbed with mid-logarithmic-growth-phase bacteria at 0°C in order to remove cross-reactive antibody. Approximately 10^{10} CFU/ml of sera was used in three sequential adsorptions for 30 min each. Bacteria were removed by centrifugation (10,000 × *g*, 10 min, 4°C) and filter sterilization. The adsorbed NSS, designated NSS-A, were stored at −70°C in 1.0-ml aliquots. Convalescent-phase sera (CS) were prepared from blood obtained from the same animals 28 days after intratracheal inoculation with 10^5 CFU of *A. pleuropneumoniae* 4074 in PBS solution, pH 7.2. The CS were heated at 56°C for 30 min, pooled, filter sterilized, and stored at −20°C in 1.0-ml aliquots.

Isolation of Ig. Antibodies to the CP were isolated from CS by affinity chromatography on a column (1.6 by 7 cm) of AH-Sepharose 4B (Pharmacia) to which cyanogen bromide (CNBr)-activated CP had been coupled. Briefly, CNBr was dissolved in a solution of CP (5 mg/ml) in 0.1 M $NaHCO_3$, pH 10.2, to a final concentration of 50 mg/mg of polysaccharide. The pH of the reaction mixture was maintained at 10.5

for 10 min while it was stirred, after which the reaction mixture was rapidly cooled to 4°C and the pH was adjusted to 8.5. The CNBr-activated CP was added to a suspension of AH-Sepharose 4B in coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl [pH 8.3]), and the mixture was tumbled gently for 2 h at room temperature. The gel was transferred to a solution of 0.2 M glycine (pH 8.0) to block the remaining active groups. The gel was packed in a column and washed sequentially with 10 column volumes of coupling buffer, 0.1 M acetate buffer containing 0.5 M NaCl (pH 4.0), and coupling buffer. The column was equilibrated with 50 mM Tris-HCl, pH 7.5, containing 0.1 M NaCl. Next, CS were diluted in the equilibration buffer and passed through the column. The column was washed with 10 column volumes of equilibration buffer, and anti-CP was eluted with 0.2 M glycine containing 0.5 M NaCl (pH 2.8) while the A_{280} was monitored. The fractions making up the immunoglobulin (Ig) peak were pooled, adjusted to a pH of 7.5, and dialyzed. The dialysate was concentrated and filter sterilized.

The IgG fraction of the anti-CP was separated from the affinity-purified anti-CP by anion-exchange chromatography on a column (2.6 by 10 cm) of DEAE-Sepharose CL-6B (Pharmacia) equilibrated with 20 mM Tris-HCl, pH 7.5. The nonbound protein fractions were pooled, concentrated, and applied to a Sepharose CL-6B (Pharmacia) gel filtration column (1.6 by 90 cm) equilibrated with 50 mM Tris-HCl (pH 7.5) and containing 0.1 M NaCl. Fractions of the eluate were assayed for IgG by an enzyme-linked immunosorbent assay (ELISA) with heavy-chain-specific, alkaline phosphatase-labelled goat anti-swine IgG. The fractions containing IgG were pooled, concentrated, and filter sterilized.

IgG was also isolated from NSS by anion-exchange chromatography on DEAE-Sepharose and gel filtration on Sepharose CL-6B essentially as described for the IgG fraction of anti-CP. The NSS were initially heated (56°C, 30 min) and subjected to ammonium sulfate (33% saturation) precipitation.

ELISA. Class-specific antibodies to the CP, LPS, and saline extract were measured by ELISA with microwell ELISA kits obtained from Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md. In order to ensure binding of the CP and LPS, microtiter wells were precoated with polylysine (50 mg/ml) overnight at 4°C and washed with PBS. Wells of microtiter plates (Flow Laboratories, Inc., McLean, Va.) were coated with 100 μl of CP, LPS, or saline extract in PBS for 2 h at room temperature. The wells were emptied, and residual liquid was removed by tapping the plates on a towel. Nonspecific binding sites in the wells were blocked with PBS containing 1% Ig-free bovine albumin (BA). Aliquots (100 μl) of serially diluted test sera were added, and the wells were incubated at room temperature for 2 h and washed four times in wash buffer (PBS, 0.02% Tween 20). Aliquots (100 μl) of alkaline phosphatase-conjugated goat antibody to swine IgM, IgA, or IgG were added, and the wells were incubated at room temperature for 1 h and washed four times in wash buffer. Wells were developed with 100 μl of *p*-nitrophenol phosphate disodium in 1.0 M diethanolamine–0.5 mM $MgCl_2$ (pH 9.8) for 30 min at room temperature. The reaction was stopped by the addition of 75 μl of 1.0 N NaOH, after which the A_{405} was measured with an ELISA reader (Flow Laboratories).

Immunoblots. Samples (25 μl) of the saline extract and pools of protein peaks obtained from column chromatography were fractionated by SDS-PAGE and transferred electrophoretically to 0.45-μm-pore-size nitrocellulose sheets (Bio-Rad) as described previously (43). The gels and nitrocellulose sheets were soaked in transfer buffer (25 mM Tris [pH 8.3] containing 192 mM glycine and 20% methanol) for 20 min prior to transfer. Electrophoretic transfer of separated proteins to nitrocellulose was accomplished by the use of a Transblot cell (Bio-Rad; 60 V for 5 h, 4°C). Nonspecific binding sites on the nitrocellulose sheets were blocked by incubating the sheets overnight in Tris-buffered saline (TBS) (10 mM Tris-HCl [pH 7.5], 150 mM NaCl) containing 3% BA. The blots were washed three times in TBS and incubated for 2 h at room temperature with either CS, NSS, or NSS-A diluted 1:50 in TBS-BA (0.5%). Following three 10-min washes in TBS, the blots were incubated for 2 h at room temperature with alkaline phosphatase-conjugated goat anti-swine IgG diluted 1:1,000 in TBS-BA (0.5%). After three 10-min washes in TBS, the blots were incubated in developing reagent (0.165 mg of 5-bromo-4-chloro-3-indolylphosphate and 0.33 mg of nitroblue tetrazolium per ml in 0.1 M Tris-HCl [pH 9.5] containing 0.1 M NaCl and 5 mM $MgCl_2$) for 30 min at room temperature in the dark. The reaction was stopped by rinsing the blots with 20 mM EDTA in TBS.

Complement consumption and C3-binding assays. Bacteria grown to the mid-logarithmic growth phase were harvested by centrifugation (10,000 × *g*, 15 min, 23°C), washed once in Veronal-buffered saline (VBS) solution, pH 7.4, and suspended in the same buffer. Aliquots (200 μl; 5×10^8 CFU/ml) of the bacterial suspension, followed by 200 μl of NSS or NSS-A as the complement source and 100 μl of serial dilutions of CS in VBS containing 0.5% BA (VBS-BA), were dispensed into polypropylene microcentrifuge tubes. VBS solution containing $MgCl_2$ (0.5 mM), $CaCl_2$ (0.15 mM), and BA (0.5%) was added to bring the reaction volume to 1.0 ml. Control tubes contained heat-inactivated serum and bacteria or unheated serum without bacteria. The tubes were incubated at 37°C for 60 min and then centrifuged (10,000 × *g*, 10 min, 4°C). The supernatant fluids were filter sterilized, and the total amount of hemolytic complement remaining in each supernatant fluid was titrated with optimally sensitized sheep erythrocytes (21). The consumption of complement in each reaction mixture was expressed as the percentage of complement activity lost relative to that in the control which contained unheated sera without bacteria.

For the measurement of C3 binding, reaction mixtures were set up as described above for the complement consumption assay. After 30 min of incubation at 37°C, bacteria were harvested and washed three times in PBS (pH 7.2). Nonspecific protein-binding sites were blocked by suspending the bacteria in 1.5 ml of PBS-BA (3%) and incubating the suspension for 30 min at room temperature. The bacteria were harvested by centrifugation (10,000 × *g*, 10 min, 23°C) and suspended in 500 μl of a 1:50 dilution of the IgG fraction of rabbit anti-swine C3 (Organon Teknika-Cappel, West Chester, Pa.). The suspension was incubated at room temperature for 1 h, after which the bacteria were harvested and washed three times in PBS containing 0.005% Tween 20. The cell pellet obtained from the final wash was suspended in 500 μl of the appropriately diluted peroxidase-conjugated IgG fraction of goat anti-rabbit IgG. After being incubated at room temperature for 1 h, bacteria were pelleted by centrifugation (10,000 × *g*, 10 min, 23°C), washed three times with PBS-Tween 20 (0.005%), and suspended in 1.0 ml of PBS. Samples (100 μl; 10^7 CFU), followed by 100 μl of a mixture of 2,2′-azino-di(3-ethylbenzthiazoline) sulfonate and hydrogen peroxide, were dispensed into wells of microtiter plates. The color was allowed to develop for 30 min, and then the

A_{405} was measured with an ELISA reader (Flow Laboratories). The relative C3 fixation was expressed as the A_{405}.

Serum bactericidal assay. The serum bactericidal assay used was a slight modification of the method described previously by Taylor (41). Mid-logarithmic-phase bacteria were harvested, washed once in VBS, and suspended in VBS at a concentration of 10^6 CFU/ml. Aliquots (50 μl; 5×10^4 CFU) of the bacterial suspension were mixed with 50 μl of VBS containing 2.5 mM $MgCl_2$ and 0.75 mM $CaCl_2$ (VBS-MC), 50 μl of NSS or NSS-A, 50 μl of the appropriate dilution of CS or anti-CP (IgG or whole fraction) in VBS-BA, and 50 μl of VBS-BA in sterile microcentrifuge tubes. Control reaction mixtures contained heated (56°C, 30 min) NSS-A or NSS instead of the unheated sera. The tubes were incubated in a 37°C shaking water bath. In order to determine the numbers of viable bacteria, samples (5 μl) were withdrawn from each tube after 0, 1, 2, or 3 h of incubation, diluted, and plated on BHI-NAD agar. Colonies were counted after being incubated overnight at 37°C, and the results were expressed as $\log_{10}$ CFU/ml. The extent of killing was expressed as $\log_{10}$ killing, which was calculated as $\log_{10}$ CFU/ml in heat-inactivated sera − $\log_{10}$ CFU/ml in unheated sera.

Blocking assay. The extent to which heated (56°C, 30 min) NSS or IgG derived from NSS could inhibit the bactericidal activity of anti-CP was determined by incubating 5×10^4 CFU with a mixture of 2 μg of anti-CP (IgG fraction), NSS-A (20%), VBS-MC, and dilutions of the heat-inactivated NSS or the IgG fraction derived from NSS. After an incubation period of 2 h at 37°C, samples (5 μl) were removed, diluted, and plated on BHI-NAD agar. The extent of killing was determined as described above. The controls for these experiments included reaction mixtures in which VBS-BA was substituted for heat-inactivated NSS or the IgG fraction.

Assay for neutralization of blocking activity. The extent to which fractions of bacterial surface antigens could neutralize the blocking activity of NSS was determined by preincubating 50 μl of NSS with 50 μl of serial dilutions of the CP, LPS, saline extract, and fractions derived from the chromatography columns. After 1 h of incubation at 4°C, 2 μg of anti-CP (IgG fraction), 5×10^4 CFU, and VBS-MC were added. The mixtures were incubated for an additional 2 h at 37°C, after which 5-μl samples were removed, diluted, and plated on BHI-NAD agar. The controls for these experiments included reaction mixtures in which NSS were preincubated with VBS-BA instead of the bacterial antigens.

Statistical analyses. Data obtained from the complement consumption, C3-binding, and serum sensitivity assays were analyzed by Student's *t* test.

RESULTS

Characterization of extracted bacterial antigens. Extraction of an *A. pleuropneumoniae* 4074 suspension in 0.15 M NaCl or PBS at 56°C resulted in the release of a number of cell wall antigens. Chemical analysis of the saline extracts revealed the presence of protein (8.0%), carbohydrate (14%), and LPS (5%). Nucleic acid contamination was estimated at less than 1.5% (A_{280}/A_{260} ratio, >1.25). The CP purified from the PBS extract was essentially free of LPS (0.01%), nucleic acids (0.01%), and protein (0.02%). The purified CP eluted from the Sephadex G-75 column as a sharp peak at the void volume, indicating that it had a high molecular weight. Fractionation of the saline extract on DEAE-Sepharose at pH 7.8 resulted in major (pool A) and minor (pool B) protein peaks which did not bind to the column as well as several protein peaks which bound to the column and eluted at NaCl concentrations of 0 to 0.5 M (Fig. 1). Further fractionation of the bound proteins (pool C) on Sephacryl S-200 resulted in four protein peaks, designated peaks 1, 2, 3, and 4 (Fig. 2). Chemical analysis of the pooled fractions showed that most of the carbohydrate and LPS present in the saline extract eluted from the Sephacryl S-200 column close to the void volume, along with protein peak 1. Analysis of the saline extract by SDS-PAGE revealed the presence of at least 14 bands and smears after staining with silver stain. Counterstaining with Coomassie blue diminished the intensity of the smear but not those of the bands, most of which were stained blue (Fig. 3). Fractionation of the saline extract by anion-

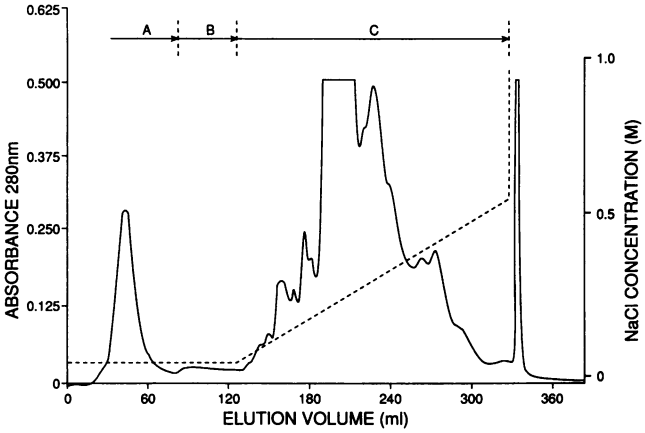

FIG. 1. Anion-exchange chromatography of saline extract obtained from *A. pleuropneumoniae* 4074. The saline extract (50 ml) was dialyzed extensively against 20 mM Tris-HCl (pH 7.8), applied to a column (2.6 by 10 cm) of DEAE-Sepharose CL-6B, and eluted with a linear gradient of 0 to 0.5 M NaCl (– – –) in the Tris-HCl buffer. The protein profile of the eluate was monitored at an optical density of 280 nm (——).

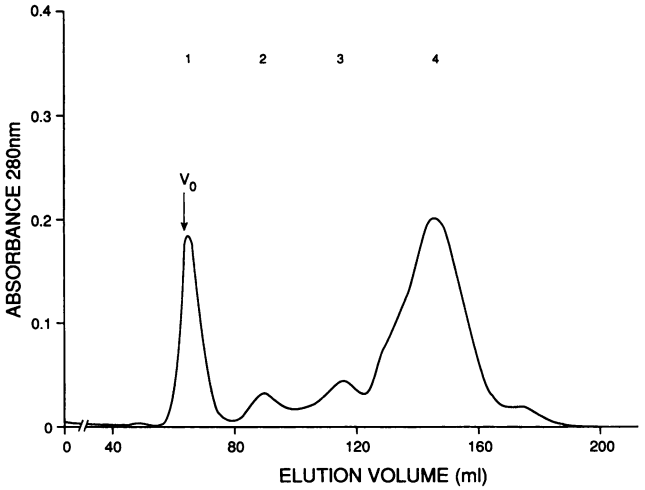

FIG. 2. Sephacryl S-200 chromatography of pool C derived from a DEAE-Sepharose column. Protein-containing fractions which eluted at 0 to 0.5 M NaCl were combined, concentrated, applied to a Sephacryl S-200 column (1.6 by 90 cm), and eluted with 50 mM Tris-HCl containing 0.15 M NaCl. The protein profile of the eluate was monitored by measuring the A_{280}. The void volume of the column was determined with dextran 2000.

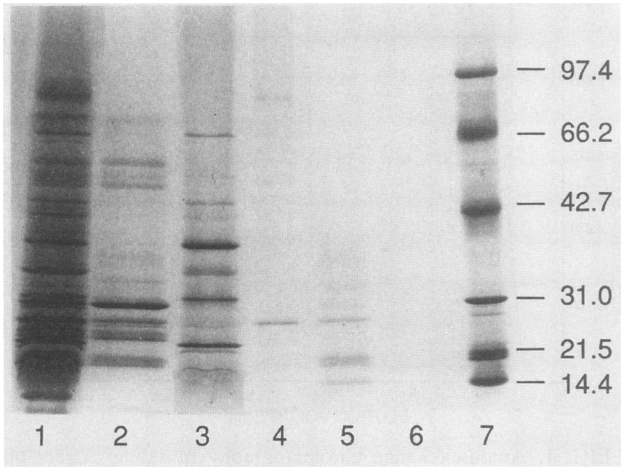

FIG. 3. SDS-PAGE analysis of saline extract and pooled fractions derived from chromatography columns. Samples were diluted in sample buffer, heated (100°C, 5 min), and fractionated on a 5-to-20% gradient polyacrylamide gel. The gel was stained with Coomassie blue. Lanes: 1, saline extract; 2, DEAE-Sepharose pool A; 3, Sephacryl S-200 peak 1; 4, Sephacryl S-200 peak 2; 5, Sephacryl S-200 peak 3; 6, Sephacryl S-200 peak 4; 7, molecular mass standards (in kilodaltons). Lane 6 (peak 4) contained a 38-kDa band which stains with silver stain but not with Coomassie blue.

exchange and gel filtration chromatography failed to separate the protein components into individual fractions, as evidenced by the profiles of the pooled column fractions. Samples of peak 4 which exhibited A_{280} contained a band at about 38 kDa after being stained with silver stain. This particular band destained during the process of staining with Coomassie blue. Because these observations are consistent with the properties of nucleic acid, it is likely that the peak 4 fraction represents nucleic acid contamination of the saline extract.

Characterization of sera and purified antibody. The pooled CS used in this study were obtained from five pigs 28 days after they were inoculated with *A. pleuropneumoniae* 4074. At this time, the animals showed no signs of ongoing clinical infection, although they remained chronically diseased, as evidenced by the residual lesions of chronic pneumonia observed at necropsy. The pooled preimmune sera obtained from the same animals prior to infection and the CS were examined for antibodies to the CP, LPS, and constituents of the saline extract by ELISA. Initial experiments showed that the preimmune sera reacted with the LPS and the saline extract. In order to quantitate the extent of these reactions, an aliquot (2 ml) of the NSS was adsorbed three times with a mixture of LPS (2 mg) and saline extract (2 mg) to remove the reactive antibodies. The adsorbed sera were used as the negative control sera for the ELISA. The antibody titer was determined as the reciprocal of the highest dilution at which the mean optical density at 405 nm of test sera exceeded the mean + 3 standard deviations of the negative control sera. As shown in Table 1, class-specific antibodies to the CP, LPS, and saline extract were well represented in the CS. In contrast to the CS, NSS lacked antibodies to the CP but contained antibodies which reacted with LPS and the saline extract. Absorption of the NSS with intact bacteria reduced the titer of antibodies to the saline extract but not those that were directed against the LPS. The ELISA results also showed that anti-CP isolated from CS was specific for the CP

TABLE 1. Characterization of antigen-specific Ig present in NSS-A, NSS, and CS by ELISA

Antigen and sera	Class-specific ELISA titer[a]		
	IgM	IgA	IgG
CP			
CS	724	362	406
NSS	<10	<10	<10
NSS-A	<10	<10	<10
LPS			
CS	5,560	3,502	2,206
NSS	<10	45	64
NSS-A	<10	45	64
Saline extract			
CS	1,280	806	2,032
NSS	<10	32	362
NSS-A	<10	<10	161

[a] Determined as the reciprocal of the highest dilution of serum at which the mean optical density at 405 nm exceeded the mean + 3 standard deviations of negative control serum. Data are the geometric means of three separate determinations. The geometric means were calculated by the equation $n\sqrt{x_1, x_2 \ldots \ldots x_n}$.

and contained IgM, IgA, and IgG isotypes in the same proportion as the CS (data not shown). Immunoblot analysis showed that both CS and NSS contained antibodies which reacted with most of the polypeptides present in the saline extract (Fig. 4). It also revealed that absorption of NSS with intact bacteria depleted antibodies to a 32-kDa polypeptide (Fig. 4D, lane 1), a 27-kDa polypeptide (Fig. 4D, lane 2), and high-molecular-weight bands (Fig. 4D, lanes 1, 2, and 3). The high-molecular-weight bands were not apparent in SDS-polyacrylamide gels of saline extracts stained with Coomassie blue. It is not clear, however, why the 32- and

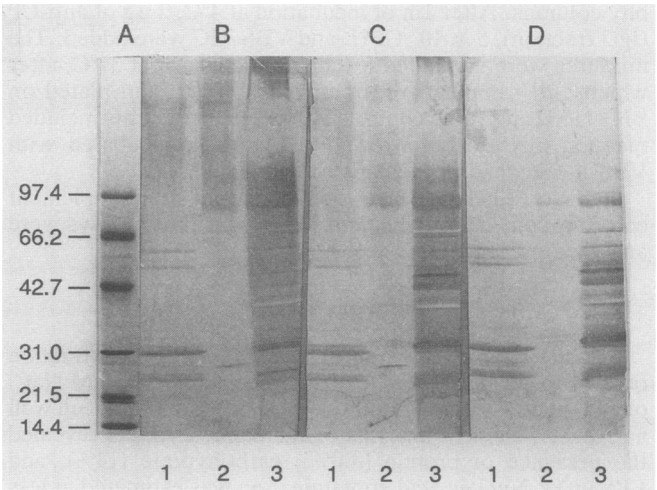

FIG. 4. Immunoblots of surface polypeptides extracted from *A. pleuropneumoniae* 4074. Samples were fractionated by SDS-PAGE, transferred to nitrocellulose sheets, and reacted with swine CS (B), preimmune sera (C), or preimmune sera that were absorbed three times with viable *A. pleuropneumoniae* 4074 isolates at 0°C (D). (A) The single lane contained molecular weight standards (in kilodaltons) stained with Coomassie blue. (B, C, and D) Lanes: 1, DEAE-Sepharose pool A; 2, Sephacryl S-200 protein peak 2; 3, saline extract. All sera were used at dilutions of 1:50.

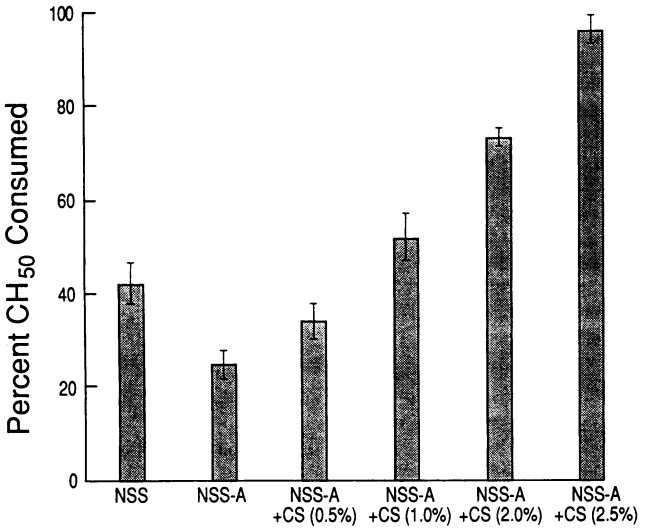

FIG. 5. Consumption of hemolytic complement by *A. pleuropneumoniae* 4074 in the absence and presence of specific antibodies. Bacteria (10^8 CFU) were incubated for 60 min at 37°C in NSS or NSS-A in the presence of different concentrations of CS. Percent CH_{50} consumed per milliliter is expressed relative to that of the control, which contained the appropriate serum without bacteria. Data presented are the means ± standard deviations of four experiments.

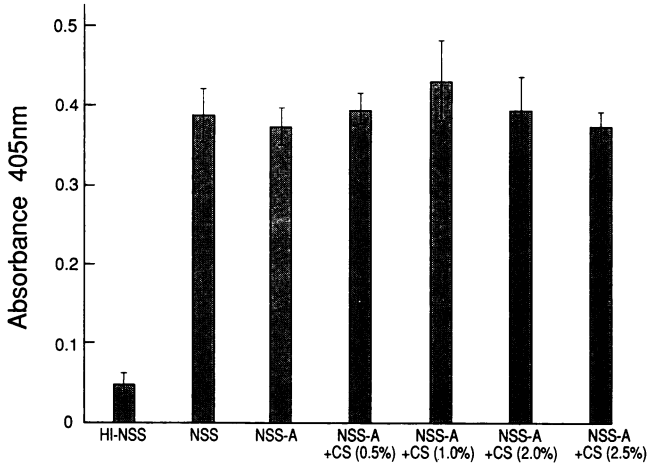

FIG. 6. The extent of C3 fixation to *A. pleuropneumoniae* 4074 in the absence or presence of specific antibodies. Bacteria (10^8 CFU) were incubated for 30 min at 37°C in NSS (20%), NSS-A (20%), or NSS-A (20%) supplemented with different concentrations of CS. The bacteria were washed and used as the antigen in an ELISA for C3, as described in the text. Data are the means ± standard deviations of three separate determinations, each performed in duplicate.

27-kDa polypeptides which stained intensely on SDS-polyacrylamide gels did not appear distinct on immunoblots of the crude saline extract developed with either CS or NSS. Further studies are required to determine whether antibody-binding sites on the polypeptides were occluded by comigrating components of the crude saline extract.

Complement consumption and C3 fixation. To determine whether the serum resistance of *A. pleuropneumoniae* is related to an inability of the organism to initiate complement activation, bacteria were incubated in NSS or NSS-A supplemented with CS for 1 h, after which residual complement was measured. Nonimmune swine sera incubated with buffer for 60 min exhibited residual complement hemolytic activity (CH_{50}) of 250 ± 8.0, whereas NSS-A possessed residual CH_{50} of 239 ± 10.2 per ml. Bacteria incubated in NSS and NSS-A consumed 42.1 and 24.6% of the CH_{50}, respectively (Fig. 5). The addition of increasing concentrations of CS to the NSS-A resulted in corresponding increases in the amount of CH_{50} consumed. The amount of CH_{50} consumed correlated with the concentration of CS added to the NSS-A and bacteria ($r = 0.99$).

To ascertain whether the activated C3 generated was stably fixed onto the bacterial surface, bacteria were incubated in heated or unheated sera for 30 min, after which C3 binding was measured. Bacteria incubated in buffer or in heat-inactivated sera did not bind C3, whereas bacteria suspended in NSS or NSS-A readily fixed C3 (Fig. 6). There was no significant difference between C3 binding to the bacteria incubated in NSS and those opsonized in NSS-A ($P > 0.05$). The addition of different concentrations of CS to the NSS-A did not significantly affect the amount of C3 bound to the bacteria ($P > 0.05$). Binding of C3 to the bacteria in the presence of purified anti-CP was also comparable to that observed when bacteria were incubated in NSS-A (data not shown).

Effects of anti-CP and nonspecific antibodies on complement-mediated killing of *A. pleuropneumoniae* 4074. Preliminary study indicated that the presence of anti-CP in immune sera was not synonymous with bacterial killing. In order to evaluate the bactericidal effectiveness of the anti-CP, the antibody was purified from CS and incubated with bacteria and complement. For these experiments, NSS or NSS-A were used as a source of complement in order to determine whether reactions between nonspecific antibodies and bacterial surface components are inhibitory to the bactericidal activity of the anti-CP. Serum sensitivity was defined as ≥1.0 $\log_{10}$ (90%) killing. The results of assays in which NSS-A were used as a source of complement are shown in Table 2. Both the IgG and whole fractions of anti-CP were bactericidal for *A. pleuropneumoniae* 4074 in a dose- and

TABLE 2. Effects of isolated anticapsular antibodies on serum sensitivity of *A. pleuropneumoniae*[a]

Anticapsular antibody (μg)	$\log_{10}$ killing[b] after the following h of incubation:		
	1	2	3
Whole fraction			
0.70	0.23 ± .05	0.30 ± .11	0.50 ± .05
2.80	0.56 ± .10	1.08 ± .06	1.20 ± .11
5.60	0.98 ± .06	2.10 ± .13	2.69 ± .15
11.20	1.74 ± .10	2.69 ± .02	2.69 ± .05
IgG fraction			
0.25	0.32 ± .05	0.55 ± .06	0.56 ± .09
1.0	0.65 ± .05	1.06 ± .12	1.30 ± .20
2.0	0.98 ± .09	2.69 ± .05	2.69 ± .12
4.0	1.72 ± .12	2.69 ± .02	2.69 ± .01
8.0	1.08 ± .04	2.69 ± .03	2.69 ± .13

[a] Preimmune sera that were absorbed with viable bacteria (NSS-A) were used as the complement source.

[b] The extent of killing ($\log_{10}$ killing) was determined after 1, 2, or 3 h of incubation. $\log_{10}$ killing was calculated from the formula $\log_{10}$ CFU in heat-inactivated NSS-A − $\log_{10}$ CFU in unheated sera. Data are the means ± standard deviations of five separate determinations. Serum sensitivity is defined as ≥1.0 $\log_{10}$ (90%) killing.

TABLE 3. Effects of heat-inactivated NSS and IgG prepared from NSS on the bactericidal activity of anticapsular antibody

Test condition[a]	Log_{10} killing[b]
Control	2.69 ± .12
Heat-inactivated NSS (%)	
0.16	1.72 ± .11
1.25	0.69 ± .09
5.0	0.26 ± .05
IgG prepared from NSS (μg)	
2.5	1.52 ± .15
10.0	0.76 ± .05
40.0	0.45 ± .11
100.0	0.25 ± .07

[a] Bacteria (5×10^4 CFU) were incubated at 37°C in a mixture of the IgG fraction of anti-CP (2.0 μg), NSS-A (20%), and the indicated amounts of heated (56°C, 30 min) NSS or IgG prepared from the NSS.

[b] The extent of killing (log_{10} killing) was determined after 2 h of incubation. Data are the means ± standard deviations of three separate determinations, each of which was performed in duplicate.

TABLE 4. Neutralization of blocking activity of NSS by protein fractions derived from column chromatography[a]

Fraction(s) preincubated with NSS	Protein concn (μg)	Log_{10} killing[b]
Control buffer		0.29 ± .08
DEAE-Sepharose		
Pool A	50	1.36 ± .13[c]
	10	1.17 ± .11[c]
Pool B	50	0.45 ± .16
Sephacryl S-200		
Peak 1	50	0.22 ± .05
	10	0.32 ± .02
Peak 2	50	2.69 ± .10[c]
	10	1.28 ± .13[c]
Peak 3	50	1.17 ± .07[c]
	10	0.97 ± .14[c]
Peak 4	50	0.50 ± .14
	10	0.48 ± .15

[a] Nonimmune swine sera (50 μl) were preincubated with the indicated amounts of protein fractions at 4°C for 1 h, after which bacteria (4×10^4 CFU), anti-CP (IgG; 2 μg), and buffer were added.

[b] The extent of killing (log_{10} killing) was determined after 2 h of incubation at 37°C. Data shown are the means ± standard deviations of four separate determinations, each performed in duplicate.

[c] $P < 0.001$ versus that of control.

time-dependent manner. In contrast to these results, the antibodies were not bactericidal for the bacteria when NSS were used as the source of complement (data not shown).

To determine whether NSS contained antibodies which inhibited the bactericidal action of anti-CP, the capacity of heat-inactivated NSS or IgG prepared from the NSS to inhibit the bactericidal action of anti-CP was examined. As shown in Table 3, increasing concentrations of the heated sera or the IgG fraction caused corresponding decreases in log_{10} killing.

In order to determine the specificity of blocking antibody, the extent to which the CP, LPS, saline extract, and protein fractions derived from the columns could neutralize the blocking activity of preimmune sera was assessed. Preincubation was performed at 4°C in order to prevent complement activation prior to the addition of bacteria. For controls in which NSS were preincubated with VBS-BA (0.5%), log_{10} killing was 0.29 ± 0.08 after 2 h of incubation at 37°C (Table 4). Preincubation of NSS with 10 to 500 μg of CP, LPS, or saline extract did not increase log_{10} killing over that observed for the control (data not shown). Similarly, samples of pool B derived from the DEAE-Sepharose column and peaks 1 and 4 obtained from the Sephacryl S-200 column did not neutralize the blocking activity of NSS, because log_{10} killing was not significantly higher than that of the control ($P > 0.01$) (Table 4). Significant increases in log_{10} killing were observed when NSS was preincubated with aliquots of pool A and protein peaks 2 and 3 ($P < 0.001$). The log_{10} killing in each instance was dependent on the concentration of protein in the sample preincubated with NSS.

DISCUSSION

The central question with regard to complement resistance is whether resistance is due to inefficient complement activation or involves subversion of the membrane attack process at some later step. Our data on complement consumption indicated that *A. pleuropneumoniae* 4074 is capable of initiating complement activation during incubation in preimmune sera and does so much more efficiently in the presence of specific antibodies present in immune sera. If serum resistance in *A. pleuropneumoniae* does not reflect a failure of the organism to initiate complement activation, it is certainly not due to a lack of binding of activated C3 to the bacterial surface. Although both specific and nonspecific antibodies present in CS and NSS, respectively, acted to enhance complement activation by *A. pleuropneumoniae*, these antibodies did not appear to increase C3 binding to the bacteria, because quantitatively equivalent C3 fixation occurred on bacteria that were incubated in NSS and NSS-A and in the presence of specific antibodies. Similar findings have been noted in studies of serum resistance in certain gram-negative bacteria (8, 12–14). These studies have demonstrated enhanced complement activation and stable and quantitatively comparable binding of C3 and late complement components to the surfaces of serum-sensitive and serum-resistant bacteria during incubation in serum in the presence and absence of specific antibodies. Whereas antibody has been essential for complement activation, the more important role of antibody has been to focus the complement attack at sensitive sites on the bacterial surface where successful insertion of the C5b-9 complex can be accomplished (8, 13–15). On the basis of these findings, it is clear that bactericidal effectiveness and presence are important in determining whether an antibody is bactericidal or nonbactericidal.

Experiments designed to determine the bactericidal effectiveness of anti-CP showed that the antibody was bactericidal upon separation from coexisting antibodies in immune sera and only when NSS-A were used as a source of complement. The implication of these findings is that NSS and possibly CS possess blocking activity which interfered with the bactericidal action of anti-CP. This view was supported by results of the blocking assays which showed that heat-inactivated NSS and IgG derived from the preimmune sera were capable of inhibiting the bactericidal effects of anti-CP and NSS-A in a dose-dependent manner. These results also implied that nonspecific antibodies of the IgG isotype could have contributed to the blocking activity of the preimmune sera. The blocking antibodies did not react with LPS and CP, because the purified antigens failed to neutralize the blocking effect of NSS. Furthermore, ELISA results

had shown that the preimmune sera lacked antibody to the CP. Consequently, it was reasonable to presume that the blocking antibodies were directed against any of the polypeptides present in the saline extract. The inability of the saline extract to neutralize the blocking activity of NSS can be explained by the presence of CP in the extract, which could bind anti-CP and prevent the antibody from binding to capsular determinants on the bacterial surface in order to exert a bactericidal effect.

In order to ascertain whether the blocking antibodies have specificities for the extracted polypeptides, it was necessary to fractionate the saline extract. The use of detergents and other rigorous conditions, which could denature the extracted proteins and disrupt secondary and higher-order antigenic determinants, was purposely avoided. Instead, potential differences in the charges and the sizes of the individual components of the saline extract were exploited in the fractionation protocols. Although these procedures failed to separate the individual components, most of the CP and LPS was effectively relegated to one of many fractions obtained. Analysis of data derived from neutralization experiments indicated that preincubation of NSS with samples of pool A (DEAE-Sepharose chromatography) and peaks 2 and 3 (Sephacryl S-200 chromatography) inhibited the blocking activity of the sera. Comparison of blots of pool A and peak 2 which were probed with NSS and NSS-A revealed that absorption of the preimmune sera with intact bacteria depleted antibodies to 32- and 27-kDa polypeptides, as well as high-molecular-weight bands. These findings imply that these cell wall antigens contain surface-exposed epitopes which bound and removed antibodies to the respective surface antigens during absorption of the NSS with intact bacteria. Because absorption of the preimmune sera with intact bacteria also removed the blocking activity of the sera, it is tempting to speculate that the nonspecific antibodies which recognized these surface antigens are responsible for the blocking activity of NSS. These results do not, however, preclude the existence of surface-exposed epitopes on the other polypeptides, since the capacities of immunoabsorption and immunoblots to identify antigens containing surface-exposed epitopes could depend on the proportion of antibodies to the individual antigen that is directed against the exposed determinants of the antigen (18, 40).

It was of interest that preimmune sera contained antibodies which reacted with most of the polypeptides extracted from *A. pleuropneumoniae* 4074. The origin of these antibodies is not clear, since there was no history of *A. pleuropneumoniae* infection in animals from which the pooled sera were obtained. Similar reactions between swine preimmune sera and *A. pleuropneumoniae* membrane proteins have been reported previously (26, 28). It seems likely that prior exposure of pigs to an organism(s) with antigenically similar membrane proteins could have induced these antibodies. This view is consistent with the report of MacInnes and Rosendal (19), who showed that *A. pleuropneumoniae* strains possess membrane proteins which contain epitopes that are antigenically similar to those present in other gram-negative bacteria.

In summary, data derived from this study have allowed us to infer that the resistance of *A. pleuropneumoniae* 4074 to killing by immune sera is related to the capacity of nonspecific antibodies to inhibit the bactericidal activity of anticapsular antibody. Resistance of the organism to killing by preimmune sera could be attributed to the absence of anti-CP and/or the presence of the nonspecific antibodies which may focus the complement attack to nonlethal sites on the bacterial surface.

ACKNOWLEDGMENTS

We thank Steven Mason for technical assistance.

This work was supported by grants from the National Pork Producers Council; the Veterinary Medical Experiment Station, University of Georgia; and the Georgia Department of Agriculture Swine Disease Eradication Program.

REFERENCES

1. **Altman, E., J. R. Brisson, and M. B. Perry.** 1986. Structural studies of the capsular polysaccharide from *Haemophilus pleuropneumoniae* serotype 1. Biochem. Cell Biol. **64**:707–716.
2. **Bertram, T. A.** 1985. Quantitative morphology of peracute pulmonary lesions in swine induced by *Haemophilus pleuropneumoniae*. Vet. Pathol. **22**:598–609.
3. **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.
4. **Cross, A. S.** 1990. The biologic significance of bacterial encapsulation. Curr. Top. Microbiol. Immunol. **150**:87–95.
5. **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.
6. **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.
7. **Durack, D. T., and P. B. Beeson.** 1977. Protective role of complement in experimental *Escherichia coli* endocarditis. Infect. Immun. **16**:213–217.
8. **Frank, M. M.** 1990. Complement in host defense against bacterial infections, p. 305–317. *In* E. M. Ayoub, G. H. Cassell, W. C. Branche, and T. J. Henry (ed.), Microbial determinants of virulence and host response. American Society for Microbiology, Washington, D.C.
9. **Higgins, R., S. Larriviere, K. R. Mittal, G. P. Mortineau, P. Rousseau, and J. Cameron.** 1985. Evaluation of a killed vaccine against porcine pleuropneumonia due to *Haemophilus pleuropneumoniae*. Can. Vet. J. **26**:86–89.
10. **Inzana, T. J., J. Ma, T. Workman, R. P. Gogolewski, and P. Anderson.** 1988. Virulence properties and protective efficacy of the capsular polymer of *Haemophilus* (*Actinobacillus*) *pleuropneumoniae* serotype 5. Infect. Immun. **56**:1880–1889.
11. **Jacques, M., B. Foiry, R. Higgins, and K. R. Mittal.** 1988. Electron microscopic examination of capsular material from various serotypes of *Actinobacillus pleuropneumoniae*. J. Bacteriol. **170**:3314–3318.
12. **Joiner, K. A., R. C. Goldman, C. H. Hammer, L. Leive, and M. M. Frank.** 1983. Studies of the mechanism of bacterial resistance to complement-mediated killing. V. IgG and F(ab)'2 mediate killing of *E. coli* 0111B4 by the alternative complement pathway without increasing C5b-9 deposition. J. Immunol. **131**:2563–2569.
13. **Joiner, K. A., R. Scales, K. A. Warren, M. M. Frank, and P. A. Rice.** 1985. Mechanism of action of blocking immunoglobulin G for *Neisseria gonorrhoeae*. J. Clin. Invest. **76**:1765–1772.
14. **Joiner, K. A., K. A. Warren, M. Tam, and M. M. Frank.** 1984. Bactericidal activity of monoclonal antibodies directed against protein I of *Neisseria gonorrhoeae*. Clin. Res. **32**:371A.
15. **Joiner, K. A., K. A. Warren, M. Tam, and M. M. Frank.** 1985. Monoclonal antibodies directed against gonococcal protein I vary in bactericidal activity. J. Immunol. **134**:3411–3419.
16. **Kilian, M., and E. L. Biberstein.** 1984. Genus II. *Haemophilus*, p. 558–569. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of determinative bacteriology, vol. 1. The Williams and Wilkins Co., Baltimore.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
18. **Loeb, M. R.** 1984. Immunoblot method for identifying surface

components, determining their cross-reactivity, and investigating cell topology: results with *Haemophilus influenzae* type b. Anal. Biochem. **143:**196–204.

19. **MacInnes, J. I., and S. Rosendal.** 1987. Analysis of major antigens of *Haemophilus* (*Actinobacillus*) *pleuropneumoniae* and related organisms. Infect. Immun. **55:**1626–1634.
20. **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.
21. **Mayer, N. N.** 1961. Complement and complement fixation, p. 133–149. *In* E. A. Kabat and M. N. Mayer (ed.), Experimental immunochemistry, 2nd ed. Charles C Thomas Publisher, Springfield, Ill.
22. **McCabe, W. R., B. Kaijser, S. Olling, M. Uwaydah, and L. A. Hanson.** 1978. *Escherichia coli* in bacteremia: K and O antigens and serum sensitivity of strains from adults and neonates. J. Infect. Dis. **138:**33–41.
23. **Moxon, E. R., and J. S. Kroll.** 1990. The role of bacterial polysaccharide capsules as virulence factors. Curr. Top. Microbiol. Immunol. **150:**65–85.
24. **Nicolet, J.** 1986. Haemophilus infections, p. 426–436. *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.
25. **Nielsen, R.** 1976. Pleuropneumonia of swine caused by *Haemophilus parahaemolyticus*. Studies on the protection obtained by vaccination. Nord. Vet. Med. **28:**337–348.
26. **Niven, D. F., J. Donga, and F. S. Archibald.** 1989. Responses of *Haemophilus pleuropneumoniae* to iron restriction: changes in the outer membrane protein profile and the removal of iron from porcine transferrin. Mol. Microbiol. **8:**1083–1089.
27. **Olling, S.** 1977. Sensitivity of gram-negative bacilli to the serum bactericidal activity: a marker of the host-parasite relationship in acute and persisting infections. Scand. J. Infect. Dis. **10**(Suppl.)**:**1–40.
28. **Rapp, V. J., and R. F. Ross.** 1986. Antibody response of swine to outer membrane components of *Haemophilus pleuropneumoniae* during infection. Infect. Immun. **54:**751–760.
29. **Rice, P. A., W. M. McCormack, and D. L. Kasper.** 1980. Natural serum bactericidal activity against *Neisseria gonorrhoeae* isolates from disseminated, locally invasive and uncomplicated disease. J. Immunol. **124:**2105–2109.
30. **Rice, P. A., H. E. Vayo, M. R. Tam, and M. S. Blake.** 1986. Immunoglobulin G antibodies directed against protein III block killing of serum-resistant *Neisseria gonorrhoeae* by immune serum. J. Exp. Med. **164:**1735–1748.
31. **Roantree, R. J., and L. A. Rantz.** 1960. A study of the relationship of the normal bactericidal activity of human serum to bacterial infection. J. Clin. Invest. **39:**72–81.
32. **Rosendal, S., D. S. Carpenter, W. R. Mitchell, and M. R. Wilson.** 1987. Vaccination against pleuropneumonia in pigs caused by *Haemophilus pleuropneumoniae*. Can. Vet. J. **22:**34–35.
33. **Rosendal, S., O. P. Miniats, and P. Sinclair.** 1986. Protective efficacy of capsular extracts of *Haemophilus pleuropneumoniae* in pigs and mice. Vet. Microbiol. **12:**229–240.
34. **Rycroft, A. N., and J. M. Cullen.** 1990. Complement resistance in *Actinobacillus* (*Haemophilus*) *pleuropneumoniae* infection of swine. Am. J. Vet. Res. **51:**1449–1453.
35. **Rycroft, A. N., and D. J. Taylor.** 1987. Preparation and characterization of envelope proteins from *Haemophilus pleuropneumoniae*. Vet. Microbiol. **15:**303–314.
36. **Sebunya, T. N. K., and J. R. Saunders.** 1983. *Haemophilus pleuropneumoniae* infection in swine: a review. J. Am. Vet. Med. Assoc. **182:**1331–1337.
37. **Shope, R. E.** 1964. Porcine contagious pleuropneumonia. I. Experimental transmission, etiology and pathology. J. Exp. Med. **119:**357–368.
38. **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.
39. **Smith, H.** 1977. Microbial surfaces in relation to pathogenicity. Bacteriol. Rev. **41:**475–500.
40. **Swanson, J., and O. Barrera.** 1983. Immunological characteristics of gonococcal outer membrane II assessed by immunoprecipitation, immunoblotting, and coagglutination. J. Exp. Med. **157:**1405–1420.
41. **Taylor, P. W.** 1985. Measurement of the bactericidal activity of serum, p. 445–455. *In* M. Sussman (ed.), Virulence of *Escherichia coli*: reviews and methods. Academic Press, New York.
42. **Thwaits, R. N., and S. Kadis.** 1991. Immunogenicity of *Actinobacillus pleuropneumoniae* outer membrane proteins and enhancement of phagocytosis by antibodies to the proteins. Infect. Immun. **59:**544–549.
43. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
44. **Vosti, K. L., and E. Randall.** 1970. Sensitivity of serologically classified strains of *Escherichia coli* of human origin to the serum bactericidal system. Am. J. Med. Sci. **259:**114–119.