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Humoral Antibody Response and Protective Immunity in Swine following Immunization with the 104-Kilodalton Hemolysin of *Actinobacillus pleuropneumoniae*

J. DEVENISH,* S. ROSENDAL, AND J. T. BOSSÉ

Department of Veterinary Microbiology and Immunology, University of Guelph, Guelph, Ontario N1G 2W1, Canada

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Five cesarean-derived, colostrum-deprived pigs were given three adjuvant-supplemented subcutaneous and one intravenous injection of the purified 104-kDa hemolysin from serotype 1 *Actinobacillus pleuropneumoniae* CM-5. Six control animals received phosphate-buffered saline only. Five of six control pigs died within 24 h after challenge. The sixth control pig was moribund and euthanized after 48 h. All six pigs had pleuropneumonia, and *A. pleuropneumoniae* was isolated from all six lungs. None of the vaccinated pigs died as a result of challenge. After being euthanized, two pigs in this group had no lung lesions, but three had chronic pleuropneumonia involving 10, 20, and 40% of the lung tissue. *A. pleuropneumoniae* was isolated from lung lesions of these three animals but not from the two pigs without lesions. The prechallenge hemolysin-neutralizing antibody titers in the vaccinated pigs were 1:10,900, 1:10,600, 1:4,800, 1:3,900, and 1:3,000, in order of increasing lung involvement. None of the control pigs had neutralizing antibodies. Enzyme-linked immunosorbent assay (ELISA) antibodies to capsule, lipopolysaccharide, and hemolysin were not detected in serum samples collected from the control pigs. In the vaccinated group, prechallenge sera did not contain ELISA antibodies to capsule or lipopolysaccharide. ELISA antibodies to the hemolysin were detected only in the prechallenge and postchallenge serum samples. These results indicate that pigs immunized with the 104-kDa hemolysin of serotype 1 *A. pleuropneumoniae* are protected against challenge with virulent bacteria. The association between neutralizing antibodies and protection indicates indirectly that the hemolysin is an important virulence factor.

Actinobacillus pleuropneumoniae is the causative agent of fibrinohemorrhagic pleuropneumonia of swine. The capsule and endotoxin have been shown to be important for disease production (8, 11, 13, 17, 21, 24), but the rapid onset of acute disease, with increases in vascular permeability resulting in hemorrhage and fibrin exudation, suggests that potent toxins other than endotoxin contribute to disease (14, 20, 22). Of the 12 known serotypes of *A. pleuropneumoniae*, strains of serotype 1 have been shown to be highly virulent in pigs (18), which may be reflected in the greater zone of hemolysis observed on blood agar plates with serotype 1 strains (J. Nicolet, Ph.D. thesis, University of Berne, Bern, Switzerland, 1970). The hemolysin of serotype 1 *A. pleuropneumoniae* is a protein with a molecular mass of 104 kDa (4, 10) and is related immunoserologically and genetically to the RTX group of cytolysins (6, 15). It has toxic activity for neutrophils, and pigs convalescent from pleuropneumonia have neutralizing antibodies to this hemolysin (20). Thus, the extensive interest in this cytotoxic factor is due to its putative role in the pathogenicity of disease and its potential use in a vaccine to protect against pleuropneumonia. Fedorka-Cray et al. (7) have shown that a crude concentrated extracellular extract of serotype 1 *A. pleuropneumoniae* protected against challenge with virulent bacteria. However, it could not be determined whether this protection was due to the immune response against the hemolysin, capsular material, lipopolysaccharide (LPS), or possibly other unknown factors present in the crude extract. The purpose of the present study was to determine the humoral response and immunoprotection in pigs immunized with the purified 104-kDa hemolysin of serotype 1 *A. pleuropneumoniae* CM-5 and challenged with virulent bacteria.

MATERIALS AND METHODS

Production and purification of the 104-kDa hemolysin. Serotype 1 *A. pleuropneumoniae* CM-5 was grown in Roux flasks containing 150 ml of tryptic soy agar supplemented with 10 mM $CaCl_2$ and 0.01% NAD for 18 h at 37°C. The bacterial lawn was removed by washing each Roux flask with 75 ml of RPMI 1640. Following incubation at 37°C for 90 min, the culture was centrifuged, and the supernatant was filtered with polycarbonate filters. The hemolysin was precipitated by addition of ammonium sulfate to 80% saturation, centrifuged, and redissolved in a volume of phosphate-buffered saline, pH 7.4, corresponding to approximately 1/30 the original volume of RPMI 1640 culture. The precipitate was stored at −20°C in 2-ml quantities until required for electrophoresis. The redissolved precipitates had an average hemolytic activity of 9×10^3 hemolytic units (defined as the dilution of the precipitate which caused 50% hemolysis of a 1% washed bovine erythrocyte suspension [6, 20]) per ml and a protein concentration of 300 μg/ml. To purify the 104-kDa hemolysin, the fractions were denatured and subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis by using a 4% stacking gel and an 11% separating gel. The gel was stained by Coomassie blue, and the 104-kDa protein band was excised and minced with a glass rod. To this was added two times the volume of electroelution buffer (9 mM Tris-borate buffer with 0.005% SDS, pH 8.0), and the 104-kDa protein was electroeluted from the gel and stored at −20°C until required for injection.

Injection of the 104-kDa protein. A litter of 11 cesarean-derived, colostrum-deprived 5-week-old piglets were placed

* Corresponding author.

in a control group and a test group of six and five animals, respectively. The pigs were randomly selected for each group and numbered by ear tag; the control group was assigned numbers 301, 302, 303, 305, 306, 314, and the test group was assigned numbers 307, 308, 309, 311, 312. The animals remained together throughout the trial. The five test pigs were injected subcutaneously with 50 μg of the electroeluted 104-kDa protein in 1.5 ml of buffer with 1 mg of Quil A as adjuvant (Cedarlane Laboratories, Hornby, Ontario, Canada). The injection was repeated 2 weeks later with 110 μg of protein and Quil A. After another 2 weeks, each pig was injected intravenously with 230 μg of protein without adjuvant. Because hemolysin-neutralizing antibody titers 1 week after the intravenous injection were considered to be low, 3 weeks later the pigs were given a final subcutaneous injection of 170 μg of the electroeluted 104-kDa hemolysin with Quil A. The control pigs received the same schedule of injections except that phosphate-buffered saline, pH 7.4, replaced the 104-kDa protein solution. The pigs were exposed 10 days later to an aerosol suspension of virulent CM-5 containing 2×10^7 CFU/ml in phosphate-buffered saline, pH 7.4, as described previously (23). The pigs were necropsied immediately after death or euthanasia. The percentage of the total lung volume with pleuropneumonia was estimated. Samples of lungs and tonsils were cultured on chocolate blood agar supplemented with 0.01% NAD and incubated in CO_2 at 37°C for 24 h. Typical colonies were subcultured and confirmed as *A. pleuropneumoniae* as described elsewhere (16). Serum samples were taken from each pig before the first subcutaneous injection (preimmune), 1 week after the last injection but before challenge (prechallenge), and after challenge (postchallenge). For the test group animals, the postchallenge serum samples were collected 10 days after challenge when these animals were euthanized.

Determination of ELISA and hemolysin-neutralizing antibody titers. All antigens for enzyme-linked immunosorbent assay (ELISA) tests were prepared with strain CM-5. The capsular polysaccharide (CPS) antigen was prepared as described previously (2), ultracentrifuged for 16 h at 100,000 × *g* to remove LPS, and used in the ELISA at a concentration containing 0.15 μg of galactose per ml. The LPS antigen was prepared by the method of Darveau and Hancock (3) and used at a concentration of 0.625 μg/ml. The hemolysin antigen was captured out of crude culture supernatant containing 100 hemolytic units per ml onto wells of polystyrene plates coated with a rabbit antiserum specific for the 104-kDa hemolysin as described previously (5). Test sera were diluted twofold in duplicate, commencing at 1:100. Attachment of peroxidase-labeled rabbit anti-swine immunoglobulin G (heavy- plus light-chain activity) (Miles Scientific, Naperville, Ill.) to specific antibodies bound in the ELISA wells was detected by the hydrolysis of H_2O_2 in the presence of 2,2′-azino bis-(3-ethylbenzthiazoline sulfonic acid)-diammonium salt (Boehringer Mannheim, Dorval, Quebec, Canada) at a wavelength of 405 nm. Positive (from pigs experimentally infected with *A. pleuropneumoniae* CM-5) and negative (from cesarean-derived, colostrum-deprived pigs fed *Escherichia coli*, fecal *Streptococcus* spp., and *Lactobacillus* spp.) control sera were also tested on each microdilution plate. Mean optical densities at each dilution were calculated, and titers were expressed as the highest dilution at which the mean optical density of each test serum sample exceeded the mean plus 3 standard deviations of the negative control sera. Titers of hemolysin-neutralizing antibodies of all 11 preimmune and prechallenge serum samples were determined as described previously (6).

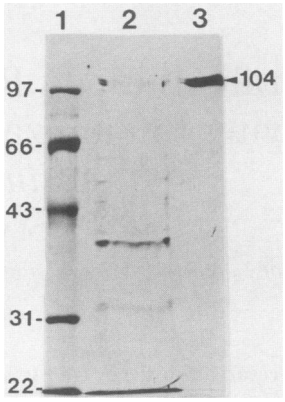

FIG. 1. Coomassie blue-stained SDS-polyacrylamide gel of Bio-Rad low-molecular-weight protein standards (lane 1), crude hemolysin (5 μg) concentrated from serotype 1 *A. pleuropneumoniae* CM-5 RPMI 1640 culture supernatant (lane 2), and the purified 104-kDa hemolysin protein (1 μg) used to inject animals and obtained after electroelution of the same protein from the crude hemolysin in lane 2 (lane 3). The molecular masses (in kilodaltons) of the protein standards are shown on the left; the arrowhead on the right indicates the position of the 104-kDa hemolysin in lanes 2 and 3.

RESULTS

The purity of the 104-kDa protein injected into pigs in this study is demonstrated in the Coomassie blue-stained SDS-polyacrylamide gel shown in Fig. 1. The health status of all 11 animals after challenge and the neutralizing antibody titers to the active 104-kDa hemolysin in preimmune and prechallenge sera are shown in Table 1. The six control animals were dyspneic, anorexic, and prostrated 18 h after challenge. Within 24 h, one animal in this group had died and four were euthanized in extremis for humane reasons. At necropsy, the entire lung tissue of these pigs had typical acute pleuropneumonia with extensive hemorrhage and serofibrinous exudation. The sixth animal was euthanized 48 h postchallenge and had typical fibrinohemorrhagic pleuropneumonia involving 80% of the lungs. *A. pleuropneumoniae* was isolated in pure culture and large numbers from all six lungs. Antibodies to CPS, LPS, and hemolysin were not detected by ELISA in any of the serum samples from this group (Table 2). Hemolysin-neutralizing antibodies were also absent in the preimmune and prechallenge sera of these animals (Table 1).

There was no mortality in the test group. The most seriously affected animal (no. 309) showed signs of dyspnea and anorexia, and preferred to lie down for a period of 5 to 6 days after challenge. At necropsy, the lungs had typical chronic pleuropneumonia, including edema, pulmonary necrosis, sequestra, abscesses, scars, and fibrous adhesions in the pleural cavity. The lesions were estimated to involve 40% of the lung tissue. A second animal (no. 312) was anorexic for a few days and had chronic pleuropneumonia in 20% of the lung tissue. Pig no. 308 appeared clinically normal following challenge but had chronic pleuropneumonia in 10% of the lung tissue upon necropsy. *A. pleuropneumoniae* was isolated in pure culture from the lungs of all three pigs with lesions but from the tonsil of no. 312 only. The remaining two pigs (no. 307 and 311) in the test group showed no

TABLE 1. Health status of control and test pigs following challenge with serotype 1 *A. pleuropneumoniae* CM-5 and neutralizing antibody titers to the 104-kDa hemolysin in preimmune and prechallenge sera

Pig no.	Postchallenge signs[a]	Mortality	% Lung involvement[b]	CM-5 isolation		Neutralizing titer[c]	
				Lung	Tonsil	1	2
Control							
301	sd; a; p	+	100	+	ND[d]	47	29
302	sd; a; p	+[e]	100	+	ND	39	40
303	sd; a; p	+[e]	100	+	ND	41	34
305	sd; a; p	+[e]	100	+	ND	61	41
306	sd; a; p	−	80	+	ND	49	40
314	sd; a; p	+[e]	100	+	ND	85	40
Test							
307	None	−	0	−	−	43	10,600
308	None	−	10	+	−	59	4,800
309	md; a; p	−	40	+	−	45	3,000
311	None	−	0	−	−	36	10,900
312	a	−	20	+	+	52	3,900

[a] s, Severe; m, mild; d, dyspnea; a, anorexia; p, prostrate.
[b] Percentage of total lung volume with pleuropneumonia.
[c] Determined as the reciprocal of the dilution of antiserum that neutralized 50% hemolysis of a 1% bovine erythrocyte suspension exposed to 10 hemolytic units of CM-5 RPMI 1640 culture supernatant. 1, Preimmune serum; 2, prechallenge serum.
[d] ND, Not done.
[e] Euthanized in extremis.

clinical signs of disease postchallenge and had no visible lesions of pleuropneumonia in lungs at necropsy, and *A. pleuropneumoniae* was not cultured from lungs or tonsils. Antibodies to CPS and LPS antigens of CM-5 were detected by ELISA in the postchallenge, but not the preimmune or prechallenge, serum samples of these five animals. Antibodies to the 104-kDa hemolysin were detected in the pre- and postchallenge, but not preimmune, serum samples (Table 2). Hemolysin-neutralizing antibody titers were detected only in the prechallenge, not preimmune, sera of these pigs. The neutralizing titers ranged from 1:3,000 to 1:10,900 and correlated inversely to the percentage of lung tissue with chronic pleuropneumonia that developed after challenge (Table 1).

TABLE 2. ELISA titers of preimmune, prechallenge, and postchallenge sera from control and test pigs against CPS, LPS, and hemolysin antigen of serotype 1 *A. pleuropneumoniae*

Pig no.	ELISA titer[a]		Hemolysin	
	CPS (postchallenge serum)	LPS (postchallenge serum)	Prechallenge serum	Postchallenge serum
Control				
301	NA	NA	<100	NA
302	NA	NA	<100	NA
303	NA	NA	<100	NA
305	NA	NA	<100	NA
306	NA	NA	<100	NA
314	NA	NA	<100	NA
Test				
307	400	800	6,400	12,800
308	800	3,200	3,200	51,200
309	1,600	6,400	3,200	51,200
311	800	800	12,800	12,800
312	800	800	6,400	51,200

[a] Determined as the reciprocal of the highest dilution at which the mean optical density at 405 nm of each test serum exceeded the mean + 3 standard deviations of the negative control sera. For all pigs, titers of preimmune and prechallenge sera against CPS and LPS and titers of preimmune sera against hemolysin were <100. NA, Not applicable, since the animal died or was euthanized within 48 h after challenge.

DISCUSSION

Whole-cell bacterins decrease mortality but not morbidity when used for protection against disease caused by *A. pleuropneumoniae* in swine (19, 22). This finding indicates that these vaccines lack or have insufficient quantities of important bacterial antigens that would drive a protective immune response. Because the current vaccines do not alleviate the severe economic losses suffered by pork producers, there have been attempts to define more specifically and accurately the immune response to important virulence factors of *A. pleuropneumoniae*. It has been demonstrated that antibodies to LPS and CPS of *A. pleuropneumoniae* protect pigs only partially (8, 9, 11, 12). Other disadvantages include (i) the undesirability of using LPS in a vaccine, since it has been shown that pigs are highly sensitive to its toxic effects (9), and (ii) the fact that CPS alone is poorly immunogenic and protects only against the homologous serotype of *A. pleuropneumoniae* (11, 12). Vaccination of animals with whole-cell bacterins has been shown to produce little or no antibody response to the hemolysin of *A. pleuropneumoniae*, whereas animals convalescing from disease were found to have high neutralizing antibody levels to this molecule (20). This information indicated that the insufficient protective efficacy of bacterins was due to the absence of the secreted cytotoxin antigen. One recent study has attempted to evaluate the contribution of secreted antigens to protective immunity conferred by *A. pleuropneumoniae* vaccination (7). Pigs were vaccinated with a crude cellular extract of *A. pleuropneumoniae* serotype 1 which was shown to contain LPS, CPS, and hemolysin. Protection was only partial, and the authors stated that the identity of any single primary protective immunogen could not be determined because of the impurity of the material used to vaccinate.

The development, in this study, of both high ELISA and high neutralizing antibody titers to the 104-kDa hemolysin, and the absence of antibodies to CPS and LPS, in the prechallenge sera of test pigs confirms the purity of the injected protein and that the protective response observed to challenge was the result of humoral antibodies to the hemolysin only. We have also found that passive transfer of

convalescent sera confers protection, supporting the importance of humoral immunity in protection. Immunization of pigs with the purified denatured 104-kDa hemolysin of serotype 1 *A. pleuropneumoniae* offered complete protection against challenge with virulent bacteria in two pigs with neutralizing antibody titers higher than 1:10,000. The protection in pigs with lower titers was partial only but seemed to be inversely correlated to the neutralizing titers. It may be tentatively suggested that a hemolysin-neutralizing antibody titer of at least 1:10,000 would protect against disease, but a larger group of animals would have to be tested to more accurately determine this figure.

The 104-kDa hemolysin of serotype 1 *A. pleuropneumoniae* strains has been shown to be toxic in vitro for porcine neutrophils and erythrocytes (20). This cytotoxicity may be an important feature of the pathogenesis of pleuropneumonia (1, 22). However, to date there has been no firm evidence substantiating this theory. The production of an antibody response, as demonstrated here, which specifically neutralizes the cytolytic activity of the 104-kDa hemolysin and which also produces complete protection against disease provides indirect evidence that the 104-kDa hemolysin of *A. pleuropneumoniae* serotype 1 is a virulence factor.

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