INFECTION AND IMMUNITY, Feb. 1992, p. 479–484
0019-9567/92/020479-06$02.00/0

Vol. 60, No. 2

Protective Local and Systemic Antibody Responses of Swine Exposed to an Aerosol of *Actinobacillus pleuropneumoniae* Serotype 1

JANINE T. BOSSÉ,[1]* ROGER P. JOHNSON,[2] MARTIN NEMEC,[3] AND SØREN ROSENDAL[1]

Department of Veterinary Microbiology and Immunology, University of Guelph, Guelph, Ontario N1G 2W1,[1] Health of Animals Laboratory, Agriculture Canada, Guelph, Ontario N1G 3W4,[2] and Animal Diseases Research Institute, Nepean, Ontario K2H 8P9,[3] Canada

Received 8 July 1991/Accepted 13 November 1991

The isotype-specific antibody responses in serum and in nasal and pulmonary lavage fluids of swine following aerosol immunization with an attenuated strain of *Actinobacillus pleuropneumoniae* serotype 1, strain CM5A, was investigated. The presence of immunoglobulin G (IgG), IgA, and IgM with specificities for capsular polysaccharide, lipopolysaccharide, and hemolysin was determined by enzyme-linked immunosorbent assay by using purified antigens. Strain CM5A induced serum antibodies of each isotype to the three antigens. The serum antibody response was sustained and typical of persistent antigenic stimulation. The specific IgM response decreased and the specific IgG response increased after challenge with strain CM5. IgA specific for the three antigens was detected in nasal secretions from all immune pigs, whereas specific IgG could only be detected in samples contaminated with blood. Both IgA and IgG specific for each of the antigens were detected in pulmonary lavage samples. There was no significant increase in specific IgA in nasal secretions; however, levels of lipopolysaccharide-specific and hemolysin-specific IgG and IgA in pulmonary secretions rose after aerosol challenge with strain CM5. Passive transfer of immune swine serum resulted in protection against pleuropneumonia and in levels of specific serum IgG which were similar to those in actively immunized pigs. It is concluded that specific serum IgG antibodies are important in protection from porcine pleuropneumonia.

Pleuropneumonia is a disease of pigs which is of considerable economic importance worldwide. Infection with the causative bacterium *Actinobacillus pleuropneumoniae* results in protective immunity (25, 26). Vaccination with whole-cell bacterins, capsular extracts, lipopolysaccharides (LPS), and outer membrane proteins may reduce mortality and morbidity but do not prevent infection or the development of chronic disease and do not cross-protect against all serotypes (14, 16, 28, 32). It is not known whether the failure of these vaccines to provide complete protection is due to lack of sufficient quantities of antibodies, lack of appropriate antigen-specific antibodies, lack of appropriate isotypes, lack of local antibody response, or a combination of these factors.

Several virulence factors of *A. pleuropneumoniae*, including capsular polysaccharides (CPS), LPS, and a 104-kDa protein hemolysin (HLY), have been described, and their ability to induce serum antibodies has been investigated (10, 15, 18, 19, 21, 30, 32). However, the antigens which elicit protective immunity have not been clearly identified.

Aerosol and oral exposure of pigs to CM5A, an attenuated strain of *A. pleuropneumoniae* serotype 1, have been shown to protect pigs from infection with the virulent strain CM5 (22). Aerosol exposure results in deposition of bacteria throughout the lungs (31), which would be expected to stimulate both local and systemic immune responses (20). Characterization of the attenuated strain, CM5A, revealed that it differs from the virulent parent strain only in that it produces a thinner capsule (31). Since CM5A produces all of the antigenic components of *A. pleuropneumoniae* serotype 1, aerosol immunization of pigs with this strain would allow investigation of the local and systemic antibody responses to an infection which leads to protection.

The objective of this study was to characterize the antibody response in serum and in nasal and pulmonary secretions after aerosol immunization with strain CM5A and subsequent aerosol challenge with strain CM5. Titers of immunoglobulin G (IgG), IgM, and IgA antibodies specific for CPS, LPS, and HLY were determined by isotype-specific enzyme-linked immunosorbent assays (ELISAs) with purified antigens. In addition, the role of serum antibodies in immunity to porcine pleuropneumonia was investigated by passive immunization of pigs with serum antibodies obtained from actively immunized pigs.

MATERIALS AND METHODS

Bacteria. Strain CM5 of *A. pleuropneumoniae* serotype 1 was isolated from frozen pleural fluid of a natural case of pleuropneumonia (29). The strain was grown only once in vitro to obtain a pure culture. The attenuated strain CM5A was derived by passaging strain CM5 70 times in vitro (31). Both strains were cultured on CAV agar consisting of Trypticase soy agar supplemented with 5% heated bovine blood (80°C for 10 min) and 0.01% NAD. Strain CM5A Nal^r was obtained by growing strain CM5A on CAV agar with 20 mg of nalidixic acid (CAV-Nal) per ml.

Active immunization. A litter of 12 pigs delivered by caesarean section and maintained in isolation was divided into two groups of six pigs housed in separate rooms. At 6 weeks of age nasal and pharyngeal swabs were taken for bacterial culture and blood, nasal, and lung lavage samples were taken for serological analysis. Nasal and lung lavage samples were collected under halothane anesthesia. Each nostril was flushed with 12.5 ml of warm (37°C) phosphate-

* Corresponding author.

buffered saline (PBS; pH 7.1) injected through a 5-cm-long tube and collected in a beaker as it drained from the nostrils. For pulmonary lavage, plastic tubing was attached to the external end of the cuffed endotracheal tube which was advanced down the trachea to the level of the heart. The cuff was inflated, and 50 ml of warm PBS was injected into the lungs while the pig was in sternal recumbency. The lavage fluid was collected by suction into a syringe attached to the tubing while the pig was placed on a slant, with its head down. Three days after lavage sampling (day 0), one group was placed in a chamber and exposed to an aerosol of 75 ml of a saline suspension of 2×10^6 CFU of strain CM5A Nal^r per ml (32, 33). Pharyngeal swab samples were collected every 3 or 4 days and cultured for bacteria. Blood samples for serology were taken weekly. On day 21 nasal and lung lavage samples were again collected from all pigs. One immunized pig died in recovery from the lavage procedure. On day 26 all the pigs were placed in one chamber and exposed to an aerosol of strain CM5 (75 ml containing 10^7 CFU/ml). Pigs which died after challenge were necropsied immediately. On day 41 nasal and lung lavage samples were collected from all surviving pigs prior to euthanasia and necropsy. The volume of PBS for lung lavage on day 41 was increased from 50 to 100 ml to accommodate the increase in size of the pigs.

Serum from blood collected at necropsy from each of the immunized pigs was pooled and treated with ammonium sulfate to precipitate immunoglobulins. The precipitate was dissolved in PBS to 1/10 of the original serum volume, dialyzed against PBS, and freeze-dried.

Passive immunization. One litter of six pigs delivered by caesarean section and maintained in isolation were divided into two groups of three pigs each. At eight weeks of age the pigs in one group were each given 120 ml of the reconstituted immunoglobulins (approximately equivalent to 1,200 ml of serum) divided equally between intraperitoneal and intramuscular routes. The following day both groups were challenged with an aerosol of 75 ml of a suspension of 7×10^6 CFU of strain CM5 per ml. Pigs which died were necropsied immediately, and surviving pigs were euthanized 5 days later and necropsied. Serum from blood samples collected before immunization and immediately before challenge was assayed for antibodies by ELISAs.

Processing of serum and lavage samples. Blood was collected by jugular venipuncture with vacutainers (Becton-Dickinson, Mississauga, Ontario, Canada). Clotted blood was centrifuged, and the serum was removed and stored at −20°C in 0.5-ml aliquots.

Each fresh lavage sample was cultured for bacteria by using CAV agar, CAV-Nal agar, and MacConkey agar. The samples were centrifuged at $1,000 \times g$ for 10 min and the supernatants were collected. Total and differential counts of leukocytes in the cell pellets of lung washings were determined. Supernatants were dialyzed against distilled water, analyzed for protein (3) with bovine serum albumin as the standard, and tested for hemoglobin content with Multistix-SG test strips (Miles Scientific, Etobicoke, Ontario, Canada). The samples were subsequently freeze-dried and stored in sealed vials at room temperature until reconstituted for antibody evaluation.

Antigen preparations. The CPS, LPS, and HLY antigens were extracted from cultures of strain CM5. The CPS was extracted as previously described (1, 2) and centrifuged at $100,000 \times g$ for 16 h to remove LPS. Neutral sugars in the extract were determined by the method of Dubois (12), with D-galactose as the standard. Protein concentration was determined (3) by using bovine serum albumin as the standard, and endotoxin content was measured with the *Limulus* amebocyte lysate assay (Multi-test Limulus Amebocyte Lysate Pyrogent, lot 9L1520; Mallinckrodt Inc., St. Louis, Mo.) and *Escherichia coli* O55:B5 endotoxin as reference.

LPS was extracted by the method of Darveau and Hancock, as previously described (7, 31), and analyzed as described above for protein content and endotoxic activity. HLY antigen was prepared and standardized as described previously (8, 10).

ELISA. Mouse monoclonal antibodies (MAb) of IgG, isotype specific for porcine IgG, IgM, and IgA, were conjugated to biotin (Vector Laboratories, Burlingame, Calif.) following the protocol provided by the manufacturer. The working dilution and specificity of each MAb was determined by ELISA by using purified porcine immunoglobulins to coat the plates (Immulon II flat bottom, lot CM521229; Dynatech Laboratories, Chantilly, Va.).

Optimal coating concentrations for the CPS and LPS antigens were determined by checkerboard titration. For the CPS and LPS, wells of flat-bottom polystyrene microtiter plates (Microwell, 2-69620, lot 1989 VII06; Nunc, Roskilde, Denmark) were loaded with 100 μl of antigen diluted in coating buffer (1.59 g of Na_2HCO_3, 2.93 g of $NaHCO_3$, 1.0 g of $MgCl_2 \cdot 6H_2O$ in 1 liter of H_2O, pH 9.6) per well. The CPS antigen was used at a concentration corresponding to 0.125 μg of galactose per ml. The LPS antigen was used at a concentration of 0.625 μg/ml (wt/vol).

The HLY antigen was captured out of crude culture supernatant containing 100 hemolytic units/ml onto wells of polystyrene plates (Immulon II flat-bottom plates, lot CM521229; Dynatech Laboratories) coated with 100 μl of a 1:2,000 dilution of rabbit antiserum specific for the 104-kDa hemolysin per well, as previously described (10, 11).

All plates were coated with antigen overnight at 4°C. Plates not used immediately were sealed in Parafilm and stored at 4°C until used (up to 1 week). Prior to addition of samples, all plates were washed four times with PBS containing 0.05% Tween 20 (PBS-T). For the CPS and LPS ELISAs, samples and conjugates were diluted in PBS-T. For the HLY ELISAs, samples and conjugates were diluted in PBS-T containing 10% normal rabbit serum. For all ELISAs, the enzyme conjugate was streptavidin-peroxidase (lot 90803452; Zymed Laboratories, Inc., San Francisco, Calif.) diluted in PBS-T.

Serum samples. Test sera were diluted twofold in 100-μl volumes, commencing at 1:25 to 1:100, depending on isotype and antigen. Sera were initially diluted 1:25 for detection of IgA antibodies to all antigens, 1:50 for detection of IgG heavy plus light chains (H+L) and IgG to all antigens, 1:25 for IgM to CPS and LPS, and 1:100 for IgM to HLY. Duplicate samples were tested on separate plates, and all plates were washed four times with PBS-T between reagent steps. Plates were incubated with serum dilutions for 2 h at room temperature, and bound antibodies were detected with peroxidase-labelled rabbit anti-swine IgG (H+L) (lot E244; Miles Scientific, Naperville, Ill.) diluted 1:2,000 or with biotinylated MAbs specific for porcine IgG (1:3,000), IgM (1:4,000), and IgA (1:5,000) which were followed by streptavidin-peroxidase (1:1,000). Plates were incubated at room temperature for 1 h with either peroxidase-labelled or biotinylated second antibodies and in the latter case for an additional 30 min with streptavidin-peroxidase. The hydrolysis of H_2O_2 by bound peroxidase was determined in the presence of 2,2′-azinobis-(3-ethylbenzthiazoline sulfonic acid)diammonium salt (ABTS tablets, 50 mg, lot 61829301;

ABTS buffer, lot 16552501; Boehringer Mannheim, Dorval, Quebec, Canada) with a Microplate Autoreader (Bio-Tek Instruments, Winooski, Vt.) at a wavelength of 405 nm. The absorbance of a target well (the lowest dilution of the pooled positive control serum) was monitored until the optical density (OD) reached 1.000, at which time the plates were read.

Pooled positive control sera and pooled negative control sera were included on all plates. Four positive control sera were obtained from pigs which, in a previous experiment, were exposed to an aerosol of CM5A and then to aerosol challenge with CM5 (22). The negative serum pool was obtained from six gnotobiotic swine.

The average ODs of duplicate wells for each dilution of test sera were calculated. Titers were expressed as the highest dilution ($-\log_2$ values) at which the averaged OD exceeded the negative cutoff values, which were determined for each antigen-isotype combination as the mean + 2 standard deviations (SD) of all preimmune sera tested in duplicate at the lowest test dilution. Test sera which gave OD values below the negative cutoff were assigned a value of 1. These data were used to calculate mean titers for each experimental group.

Nasal and lung lavage samples. Test samples standardized to 1.0 mg of protein/ml in PBS were diluted twofold in duplicate commencing at 1:5 (IgG, nasal IgA, and lung IgM) or 1:10 (lung IgA). Specific antibodies reacting with each of the antigens were detected with biotinylated MAbs specific for porcine IgG (1:1,500), IGM (1:2,000), and IgA (1:2,500), which was followed by streptavidin-peroxidase (1:2,000). Since no positive control nasal or lung lavage samples were available, the positive control included on each plate was the pooled positive serum (see above) diluted from 1:25 for IgA plates or 1:400 for IgG plates. Negative cutoff values were determined as the mean + 2 SD of all preimmune samples tested in duplicate at 1:5 dilution. Mean titers were determined as for serum samples.

RESULTS

Active immunization. All pigs exposed to strain CM5A Nal^r went off feed for 1 day but did not develop clinical signs of pneumonia. No clinical signs were observed in any of these immunized pigs after challenge with strain CM5 on day 26. At necropsy (day 41), two pigs had several areas (0.5 cm diameter) of pulmonary necrosis, but total lung involvement did not exceed 1%. Diffuse fibrotic adhesions were found on the diaphragmatic lobes of all immunized pigs, and two pigs also had diffuse fibrotic pericarditis. Necropsy of the pig which died following prechallenge lung lavage revealed only mild diffuse adhesive pleuritis on the diaphragmatic lobes, and death was attributed to complications arising from anesthesia and the lavage procedure.

All nonimmunized pigs died within 24 h of exposure to strain CM5 and at necropsy had extensive necrotizing fibrinohemorrhagic pleuropneumonia.

Bacteria interpreted as normal flora were isolated in large numbers from nasal washings and tonsils and in low numbers from lung lavage samples collected before exposure to strain CM5A Nal^r. Subsequently, strain CM5A Nal^r was isolated from the tonsils of the exposed pigs throughout the experiment but not from nasal or lung lavage samples. At necropsy, CM5A Nal^r was isolated from nasal swabs from two pigs and also from the focal pneumonic lesions in two pigs. Interestingly, the challenge strain, CM5 Nal^s, was not recovered from any of the immunized pigs after challenge.

In the nonimmunized group, only bacteria of the normal flora were isolated from samples collected before challenge. At necropsy, large numbers of challenge strain CM5 Nal^s were isolated from the airways of all pigs and also from the spleens of two pigs.

The volume of nasal lavage fluid recovered from each pig was 80% or more of the instilled 25-ml volume. Mean protein concentrations of the 12 samples on day 0 and on day 21 were 25.4 ± 6.86 and 32.1 ± 4.65 μg/ml, respectively. Samples from immunized pigs on day 21 did not differ significantly in protein concentration from those of nonimmunized pigs (Student's t test, $P < 0.05$). For the samples collected from the five immunized pigs after challenge (day 41), the mean protein concentration was 59.2 ± 21.6 μg/ml, which was significantly higher than for previous samples ($P < 0.05$).

Approximately 50 to 60% of the lung lavage volumes were recovered. Mean protein concentrations were 97.2 ± 32.4, 102 ± 36.6, and 86.8 ± 18.8 μg/ml on days 0, 21, and 41, respectively. Prior to exposure (day 0) the mean cell count for all 12 pigs was $8.8 \times 10^5 \pm 3.6 \times 10^5$ nucleated cells per ml, of which 83 (±10%) were macrophages and 13 (±9%) were neutrophils. Similar cytological results were obtained for the 12 lung washings on day 21 (before challenge) and the 5 samples on day 41. Mean protein concentration and mean cell counts for immunized pigs on day 21 did not differ significantly from those of nonimmunized pigs nor were there any significant differences in these parameters because of sampling times ($P < 0.05$).

Passive immunization. The three immunized pigs survived challenge without any clinical signs and at necropsy did not have evidence of pleuropneumonia. One pig had a 1-cm lesion of focal subacute pneumonia. The three nonimmunized pigs died within 24 h of challenge and had typical lesions of extensive fibrinohemorrhagic pleuropneumonia. Strain CM5 was isolated in large numbers from the airways of these pigs but in lesser numbers and only from the tonsils of the three passively immunized pigs.

ELISA antigens. The CPS antigen, at its working concentration in ELISA, contained 5 ng of protein per ml and caused gelation of *Limulus* amebocyte lysate when undiluted and diluted 10^{-1} but not at higher dilutions. The LPS antigen was insoluble in water but soluble in triethylamine. The working concentration (0.625 μg/ml) had 8 ng of protein per ml and caused gelation of *Limulus* amebocyte lysate at dilutions of $\leq 10^{-4}$.

Serum antibodies in actively immunized pigs. Results of ELISAs of preexposure sera were very similar to those of the negative control sera. Aerosol exposure to the attenuated strain induced antibodies of each isotype (IgG, IgA, and IgM) reactive with the three ELISA antigens used (Table 1). Titers of IgG antibodies increased between day 7 and day 21 and only slightly after challenge. Highest titers of IgM antibodies were present on day 14, and IgA antibodies were highest on day 14 or 21. Titers of both these isotypes decreased after challenge with strain CM5, while total antibody activity (determined with anti-IgG H+L-specific conjugate) remained the same or increased slightly.

Local antibody response. Assays for antibodies in nasal washings were limited to IgG and IgA isotypes because of the small quantity of protein in these samples. IgA antibodies specific for CPS, LPS, and HLY were detected in samples from all immunized pigs collected on day 21, and titers were not significantly different 15 days after challenge (Table 2). Mean titers of specific IgG antibodies were generally low, and individual animal data (not shown) indicated

TABLE 1. Serum antibody responses detected by ELISA after aerosol immunization of pigs with *A. pleuropneumoniae* CM5A Nal^r

Isotype	Time[a]	Mean serum antibody titer ± SD[b] to:		
		CPS	LPS	HLY
IgG (H+L)	7	7.03 ± 3.34	5.26 ± 3.19	1.77 ± 1.73
	14	8.53 ± 3.68	7.37 ± 3.07	8.47 ± 1.07
	21	8.37 ± 3.36	8.64 ± 1.63	10.81 ± 1.95
	33	9.44 ± 0.98	9.44 ± 0.98	12.24 ± 1.50
	41	10.24 ± 1.10	9.64 ± 1.50	12.24 ± 1.50
IgG	7	4.82 ± 3.86	1.00 ± 0.00	2.11 ± 2.47
	14	7.70 ± 3.54	8.97 ± 1.49	8.97 ± 1.49
	21	8.87 ± 3.63	9.81 ± 0.90	9.81 ± 0.90
	33	10.64 ± 1.10	11.04 ± 0.80	10.64 ± 1.10
	41	10.24 ± 1.50	11.24 ± 1.20	11.04 ± 1.50
IgM	7	6.97 ± 2.05	5.26 ± 3.30	4.32 ± 3.37
	14	7.31 ± 1.37	6.37 ± 2.43	6.53 ± 2.54
	21	5.97 ± 0.75	5.97 ± 0.94	2.88 ± 2.66
	33	5.04 ± 0.49	5.44 ± 1.17	3.26 ± 2.76
	41	3.91 ± 1.46	4.31 ± 1.83	1.00 ± 0.00
IgA	7	3.82 ± 3.05	3.93 ± 2.33	2.55 ± 2.26
	14	7.47 ± 1.07	6.64 ± 1.53	5.37 ± 2.16
	21	7.31 ± 0.75	5.87 ± 2.28	5.70 ± 2.18
	33	7.04 ± 0.49	5.44 ± 0.98	6.24 ± 0.49
	41	5.84 ± 0.75	5.04 ± 0.49	5.11 ± 2.10

[a] Time is in days postimmunization with strain CM5A Nal^r.
[b] Antibody titers are reported as mean $-\log_2$ values ± SD; values for preimmune samples are 1.00. Pigs were challenged with strain CM5 on day 26.

that the presence of this isotype was related to blood contamination. IgG antibodies specific for CPS and LPS were detectable only in nasal washings of two pigs which contained hemoglobin. Similarly, HLY-specific IgG antibodies were present at low titers in samples with traces of hemoglobin and at higher titers in the two pigs in which there was clear evidence of hemoglobin contamination.

Lung lavage samples collected 21 days after immunization contained IgA and IgG antibodies specific for the three antigens (Table 3). Titers of IgA antibodies were generally higher than IgG antibodies, and titers of both isotypes increased after challenge. Samples from all immunized pigs contained IgM antibodies to HLY both before and after challenge (titers, ≤3.32), whereas IgM antibodies to CPS and LPS were detected only in lung washings from one pig before challenge (titers, 2.32 for both antigens) and were not detected after challenge.

TABLE 2. Nasal antibody responses detected by ELISA after aerosol immunization of pigs with *A. pleuropneumoniae* CM5A Nal^r

Isotype	Time[a]	Mean nasal antibody titer ± SD[b] to:		
		CPS	LPS	HLY
IgA	21	5.82 ± 1.61	3.99 ± 0.94	4.99 ± 1.25
	41	4.26 ± 1.90	2.66 ± 0.91	4.52 ± 1.17
IgG	21	1.39 ± 0.86	1.22 ± 0.49	2.21 ± 0.95
	41	1.26 ± 0.53	1.53 ± 0.65	5.12 ± 1.47

[a] Time is in days postimmunization with strain CM5A Nal^r.
[b] Antibody titers are reported as mean $-\log_2$ values ± SD; values for preimmune samples are 1.00. Pigs were challenged with strain CM5 on day 26.

TABLE 3. Pulmonary antibody responses detected by ELISA after aerosol immunization of pigs with *A. pleuropneumoniae* CM5A Nal^r

Isotype	Time[a]	Mean pulmonary antibody titer ± SD[b] to:		
		CPS	LPS	HLY
IgA	21	6.15 ± 0.69	6.99 ± 0.47	5.99 ± 0.94
	41	6.52 ± 0.75	8.52 ± 1.17	7.52 ± 0.98
IgG	21	2.60 ± 1.02	2.99 ± 1.11	5.15 ± 0.69
	41	3.72 ± 1.02	4.72 ± 0.49	8.12 ± 0.75
IgM	21	1.22 ± 0.49	1.22 ± 0.49	2.49 ± 0.37
	41	1.00 ± 0.00	1.00 ± 0.00	2.92 ± 0.49

[a] Time is in days postimmunization with strain CM5A Nal^r.
[b] Antibody titers are reported as mean $-\log_2$ values ± SD; values for preimmune samples are 1.00. Pigs were challenged with strain CM5 on day 26.

Antibodies in passively immunized pigs. Titers of IgG specific for CPS, LPS, and HLY in the concentrated immunoglobulin fraction of the pooled immune serum were 15.29, 13.29, and 16.29, respectively. Serum titers in the three passively immunized pigs 1 day after receiving this immunoglobulin fraction (Table 4) were very similar to those in actively immunized pigs 3 weeks after aerosol exposure (Table 1). Virtually all specific antibody activity detectable with the IgG H+L-specific conjugate was attributable to IgG antibodies (Table 4).

DISCUSSION

Comparative studies of strains CM5 and CM5A have revealed that the two strains do not differ except in the apparent amount of capsule (31). The thinner capsule on strain CM5A would presumably make it more susceptible to defensive host clearance functions of the lung. The exposure of pigs to an aerosol of strain CM5A confirmed previous studies that this strain does not cause extensive pleuropneumonia (22, 31). The mild pulmonary lesions observed in two of the exposed pigs and the diffuse pleuritis in all pigs, including the pig which died prior to challenge with strain CM5, suggest that strain CM5A is not completely avirulent or that caesarian-derived and isolation-maintained pigs are more susceptible than the specific pathogen-free pigs used previously. In addition, strain CM5A was able to persist in tonsils and chronic lung lesions (two pigs) in the face of an immune response. Although tonsils are capable of producing

TABLE 4. Serum antibody responses detected by ELISA after passive immunization with an immunoglobulin-rich fraction of sera from pigs immunized with an aerosol of *A. pleuropneumoniae* CM5A Nal^r and challenged with an aerosol of strain CM5

Isotype	Time[a]	Mean serum antibody titer ± SD[b] to:		
		CPS	LPS	HLY
IgG (H+L)	1	8.31 ± 0.58	7.64 ± 0.00	11.97 ± 0.58
IgG	1	8.97 ± 0.58	9.31 ± 0.58	9.31 ± 0.58
IgM	1	2.21 ± 2.10	1.00 ± 0.00	1.00 ± 0.00
IgA	1	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00

[a] Time is in days postimmunization with strain CM5A Nal^r.
[b] Antibody titers are reported as mean $-\log_2$ values ± SD; values for preimmune samples are 1.00. Pigs were challenged with strain CM5 on day 26.

IgG, IgA, and IgM antibodies (4, 5), they do not appear to be effective in clearing tonsillar infections.

This study also confirmed that exposure to strain CM5A stimulates protective immunity against severe pleuropneumonia caused by the virulent strain CM5. Furthermore, the challenge strain was not isolated from any actively immunized pigs, suggesting that exposure to CM5A stimulated effective protection against infection as well as disease. Such findings again raise the question of whether live vaccines are more efficacious than inactivated bacterial vaccines or subunit vaccines in the control and prevention of pleuropneumonia (22). Strain CM5A could potentially be used as a live vaccine; however, further studies are required to assess whether this strain remains attenuated after passage in pigs. It may be possible to introduce a stable mutation which would limit the replication of strain CM5A in vivo. Further studies are also required to determine whether exposure to CM5A, like natural infection, results in cross-protection.

The ELISA procedures utilized in this study allowed measurement of specific IgG, IgA, and IgM antibody responses of pigs to three potentially important antigens of *A. pleuropneumoniae*. In cross-titrations the biotinylated monoclonal antibodies against porcine isotypes were found to be highly specific (1). The *A. pleuropneumoniae* antigens were well-defined biochemically. Results of the *Limulus* amebocyte lysate test showed that there was little LPS contamination in the CPS antigen, since 1,000 times more of the CPS preparation was required to cause gelation of the lysate compared with LPS. The CPS and LPS preparations contained contaminating proteins which at working dilutions probably did not affect the specificity of the tests, especially as the polystyrene plates used for both the CPS and LPS ELISAs were low-protein-binding plates.

As shown previously (10), the use of a highly specific polyclonal rabbit antiserum to capture HLY out of crude culture supernatant provided a rapid and easy method of assaying for antibodies to HLY (10).

Active immunization with CM5A elicited antibodies against CPS, LPS, and HLY in serum and respiratory tract secretions. The serum antibody response included IgM, IgG, and IgA antibodies. The initial rise and then decline of IgM antibodies and the sustained IgG response were typical of systemic antibody responses to natural infections and live vaccines. The IgG response may also result in part from prolonged immune stimulation due to persistent infection. None of the antigens appeared to be more immunogenic, although the CPS and LPS antigens induced stronger early responses (day 7) than HLY.

Nasal lavage samples collected from all immunized pigs 21 days after exposure to strain CM5A contained specific IgA antibodies to the three antigens. These antibodies presumably were produced locally, since detection was not influenced by the amount of hemoglobin in the samples, which was used as an indication of blood contamination. Specific IgG antibodies in nasal lavage samples were, on the other hand, likely derived from blood since significant amounts were detected only in samples with hemoglobin. Although nasal lavage samples were not assayed for specific IgM, antibodies of this isotype would have been present at very low levels because porcine nasal secretions contain only trace amounts of IgM (23).

Specific IgG and IgA antibodies to CPS, LPS, and HLY were also present in pulmonary lavage samples collected on day 21 from the immunized pigs. Only trace amounts of IgM specific for one antigen, HLY, were detected. Specific IgA titers were greater than IgG titers, suggesting tracheobronchial rather than bronchoalveolar origin of the lavage samples (23, 24). However, as total IgA and IgG concentrations were not measured and the ELISAs were standardized for each individual isotype, comparisons between tests must be made with caution. Also, differences between titers of these isotypes may reflect differences in their affinities and the affinities of the isotype-specific second antibodies.

Interpretation of lavage data is complicated also by the dilution factor introduced during collection of samples and the method of standardization (6). In this study wash volumes were kept constant at each sampling time, and the amounts recovered were reasonably consistent. All washings were standardized according to protein concentration, as reported previously (5, 6, 13). It may have been preferable to use albumin as a standard since it is not synthesized or concentrated in the lungs (6). However, sample volumes were not sufficient to allow this.

Cell concentrations in lung washings from immunized pigs 21 days after exposure to strain CM5A were not significantly different from those in samples taken before exposure or in corresponding samples from nonimmunized pigs. An increase in neutrophil counts, indicative of inflammation, may have been found if sampling had been conducted closer to the time of exposure. Nevertheless, these results suggest that any inflammation caused by the attenuated strain in the lungs was transient and quickly resolved. Similarly, any inflammation or injury caused by the virulent strain in immunized pigs appeared to be transient, for this parameter was not elevated in samples collected 15 days after challenge.

From the passive transfer experiment, it is evident that serum antibodies confer effective protection against pleuropneumonia. This corroborates the findings of Inzana et al. (17), who showed that passive transfer of antiserum from pigs convalescent to challenge with live virulent *A. pleuropneumoniae* prevented development of pulmonary lesions. Antibodies specific for CPS, LPS, and HLY antigens were detected in sera of the passively immunized pigs at titers seen in convalescent immune pigs. Virtually all specific antibody activity was attributable to IgG antibodies, which was anticipated after preparation of the transferred immunoglobulin fraction with ammonium sulfate.

Since the immune serum for passive transfer was obtained from pigs immunized with live organisms, it is likely antibodies specific for other bacterial antigens were also present. Therefore, the relative importance of individual antigens in eliciting protection against pleuropneumonia could not be determined. Others, however, have found that antibodies to individual virulence factors of *A. pleuropneumoniae* contribute to resistance. Antibodies to capsular antigens will prevent fatal pleuropneumonia but not localized lung lesions (17, 32), and similar results have been reported for antibodies to LPS (14). Also, by inducing antibodies specific for HLY antigen, Devenish et al. (9) showed recently that pigs with very high titers of neutralizing antibodies were protected against pleuropneumonia. Antibodies to outer membrane proteins probably also play a role in protection (27) and were undoubtedly induced in our study, although we did not specifically determine their presence.

In conclusion, the study provides evidence that pigs exposed to an aerosol of an attenuated strain of *A. pleuropneumoniae* serotype 1 develop protective humoral immunity. Antibodies to CPS, LPS, and HLY were present in serum and in nasal and pulmonary lavage samples. The serum antibody response included IgM, IgG, and IgA antibodies, although the IgM response decreased over time. In

nasal washings the predominant isotype was IgA, whereas in lung washings both IgA and IgG antibodies were present. Transfer of an IgG-rich fraction of convalescent serum conferred effective passive protection against *A. pleuropneumoniae*, providing strong evidence of the importance of specific serum IgG antibodies in resistance to porcine pleuropneumonia.

ACKNOWLEDGMENTS

We thank K. Nielsen, ADRI, Nepean, Ontario, Canada, for advice and assistance with production of MAbs.

The research was supported by grants from the Ontario Pork Industry Improvement Program, Ontario Ministry of Agriculture and Food, and the Ontario Pork Producers Marketing Board.

REFERENCES

1. **Bossé, J. T.** 1991. M.Sc. thesis. University of Guelph, Guelph, Ontario, Canada.
2. **Bossé, J. T., R. P. Johnson, and S. Rosendal.** 1990. Capsular polysaccharide antigens for detection of serotype specific antibodies to *Actinobacillus pleuropneumoniae*. Can. J. Vet. Res. **54**:320–325.
3. **Bradford, M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Anal. Biochem. **95**:248–254.
4. **Bradley, P. A., F. J. Bourne, and P. J. Brown.** 1976. The respiratory tract immune system in the pig. II. Associated lymphoid tissues. Vet. Pathol. **13**:90–97.
5. **Charley, B.** 1977. Local immunity in the pig respiratory tract. I. Cellular and humoral immune responses following swine influenza infection. Ann. Inst. Pasteur Microbiol. **128**:95–107.
6. **Daniele, R. P.** 1988. The secretory immune system of the lung, p. 115–126. *In* R. P. Daniele (ed.), Immunology and immunologic diseases of the lung. Blackwell Scientific Publications, Boston.
7. **Darveau, R., 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.
8. **Devenish, J., and S. Rosendal.** 1989. Identification of the heat-labile hemolysin of *Actinobacillus pleuropneumoniae* serotype 1. Can. J. Vet. Res. **53**:251–254.
9. **Devenish, J., S. Rosendal, and J. T. Bossé.** 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. Bossé, 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. **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.
13. **Duncan, J. R., and R. G. Thomson.** 1970. Influence of immunization procedures on upper respiratory tract immunity in cattle. Can. J. Comp. Med. **34**:94–100.
14. **Fenwick, B. W., J. S. Cullor, B. I. Osburn, and H. J. Olander.** 1986. Mechanisms involved in protection provided by immunization against core lipopolysaccharides of *Escherichia coli* J5 from lethal *Haemophilus pleuropneumoniae* infections in swine. Infect. Immun. **53**:298–304.
15. **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.
16. **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.
17. **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.
18. **Inzana, T. J., and B. Matheson.** 1987. Serotype specificity and immunogenicity of the capsular polymer of *Haemophilus pleuropneumoniae* serotype 5. Infect. Immun. **55**:1580–1587.
19. **Inzana, T. J., G. F. Clark, and J. Todd.** 1990. Detection of serotype-specific antibodies or capsular antigen of *Actinobacillus pleuropneumoniae* by a double-label radioimmunoassay. J. Clin. Microbiol. **28**:312–318.
20. **Kaltreider, H. B.** 1976. Initiation of immune responses in the lower respiratory tract with red cell antigens, p. 73–97. *In* C. H. Kirkpatrick and H. Y. Reynolds (ed.), Immunologic and infectious reactions in the lung. Marcel Dekker, Inc., New York.
21. **Kamp, E. M., and L. A. M. G. van Leengoed.** 1989. Serotype-related differences in production and type of heat-labile hemolysin and heat-labile cytotoxin of *Actinobacillus* (*Haemophilus*) *pleuropneumoniae*. J. Clin. Microbiol. **27**:1187–1191.
22. **MacInnes, J. I., and S. Rosendal.** 1988. Prevention and control of *Actinobacillus* (*Haemophilus*) *pleuropneumoniae* infection in swine: a review. Can. Vet. J. **29**:572–573.
23. **Morgan, K. L., and F. J. Bourne.** 1980. Immunoglobulin levels in porcine nasal and tracheal secretions—the influence of the method of collection. J. Immunol. Methods **37**:165–173.
24. **Morgan, K. L., A. M. Hussein, T. J. Newby, and F. J. Bourne.** 1980. Quantification and origin of the immunoglobulins in porcine respiratory tract secretions. Immunology **41**:729–736.
25. **Nielsen, R.** 1979. *Haemophilus parahaemolyticus* serotypes: pathogenicity and cross immunity. Nord. Veterinaermed. **31**: 407–413.
26. **Nielsen, R.** 1984. Haemophilus pleuropneumoniae serotypes: cross protection experiments. Nord. Veterinaermed. **36**:221–234.
27. **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.
28. **Rapp, V. J., and R. F. Ross.** 1988. Immunogenicity of outer membrane components of *Haemophilus* (*Actinobacillus*) *pleuropneumoniae*. Can. Vet. J. **29**:585–587.
29. **Rosendal, S., D. S. Carpenter, W. R. Mitchell, and M. R. Wilson.** 1981. Vaccination against pleuropneumonia of pigs caused by *Haemophilus pleuropneumoniae*. Can. Vet. J. **22**:34–35.
30. **Rosendal, S., J. Devenish, J. I. MacInnes, J. H. Lumsden, S. Watson, and H. Yun.** 1988. Evaluation of heat-sensitive, neutrophil-toxic, and hemolytic activity of *Haemophilus* (*Actinobacillus*) *pleuropneumoniae*. Am. J. Vet. Res. **49**:1053–1058.
31. **Rosendal, S., and J. I. MacInnes.** 1990. Characterization of an attenuated strain of *Actinobacillus pleuropneumoniae*, serotype 1. Am. J. Vet. Res. **51**:711–717.
32. **Rosendal, S., O. P. Miniats, and P. Sinclair.** 1986. Protective efficacy of capsule extracts of *Haemophilus pleuropneumoniae* in pigs and mice. Vet. Microbiol. **12**:229–240.
33. **Sebunya, T. N. K., J. R. Saunders, and A. D. Osborne.** 1983. Dose response relationship of *Haemophilus pleuropneumoniae* aerosols in pigs. Can. J. Comp. Med. **47**:54–56.