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Antibody- and Cell-Mediated Immune Responses of *Actinobacillus pleuropneumoniae*-Infected and Bacterin-Vaccinated Pigs

S. E. FURESZ, B. A. MALLARD,* J. T. BOSSÉ, S. ROSENDAL, B. N. WILKIE, AND J. I. MACINNES

Department of Pathobiology, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada NIG 2W1

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Current porcine pleuropneumonia bacterins afford only partial protection by decreasing mortality but not morbidity. In order to better understand the type(s) of immune response associated with protection, antibody- and cell-mediated immune responses (CMIR) were compared for piglets before and after administration of a commercial bacterin, which confers partial protection, or a low-dose (10^5 CFU/ml) aerosol challenge with *Actinobacillus pleuropneumoniae* CM5 (LD), which induces complete protection. Control groups received phosphate-buffered saline or adjuvant. Serum antibody response, antibody avidity, delayed-type hypersensitivity (DTH), and lymphocyte blastogenic responses were measured and compared among treatment groups to the lipopolysaccharide (LPS), capsular polysaccharide (CPS), hemolysin (HLY), and outer membrane proteins (OMP) of *A. pleuropneumoniae*. Peripheral blood lymphocytes and sera were collected prior to and following primary and secondary immunization-infection and high-dose *A. pleuropneumoniae* CM5 (10^7 CFU/ml) aerosol challenge. Serum antibody and DTH, particularly that to HLY, differed significantly between treatment groups, and increases were associated with protection. LD-infected piglets had higher antibody responses ($P \leq 0.01$) and antibody avidity ($P \leq 0.10$) than bacterin-vaccinated and control groups. Anti-HLY antibodies were consistently associated with protection, whereas anti-LPS and anti-CPS antibodies were not. LD-infected animals had higher DTH responses, particularly to HLY, than bacterin-vaccinated pigs ($P \leq 0.03$). The LD-infected group maintained consistent blastogenic responses to HLY, LPS, CPS, and OMP over the course of infection, unlike the bacterin-vaccinated and control animals. These data suggest that the immune responses induced by a commercial bacterin are very different from those induced by LD aerosol infection and that current bacterins may be modified, for instance, by addition of HLY, so as to stimulate responses which better reflect those induced by LD infection.

Actinobacillus pleuropneumoniae can cause a frequently fatal, severe, necrotic, fibrinous pneumonia in pigs (40). In the acute form of the disease, pigs develop severe respiratory distress, cyanosis, vomiting, and fever and die within 24 to 48 h. Survivors can become chronically infected, with the organism residing in their tonsils and in focal pulmonary lesions (40). These animals develop immunity to *A. pleuropneumoniae* and are solidly protected against further challenge but may infect other animals, and economic returns are reduced due to decreased feed conversion, low weaning rates, and reduced market value (23). Current commercial vaccines can reduce mortality but do little to prevent morbidity or development of the carrier state (15).

Antibodies against the 104-kDa hemolysin (HLY) (12), lipopolysaccharide (LPS) (14), capsular polysaccharide (CPS) (14), and certain outer membrane proteins (OMP) (10, 32) all appear to be associated with partial protection; however, there are also data to suggest that at low concentrations, HLY and some OMP depress immune function in in vitro assays (7, 23, 33). Delayed-type hypersensitivity (DTH), an important indicator of cell-mediated immune response (CMIR), has not been previously evaluated with respect to *A. pleuropneumoniae* in pigs, although it was examined in mice using the footpad reaction (31).

Various purified and semipurified antigens of *A. pleuropneumoniae* have been tested as vaccines. CPS affords only partial protection when used alone as a vaccine against homologous challenge in both mice and pigs (38). The same is true when OMP are used as a vaccine (36). Devenish et al. (12) demonstrated complete protection against homologous challenge in pigs when gel-purified HLY was used as a vaccine but only if neutralizing antibody titers exceeded 10,000. Recombinant HLY vaccines induce partial protection which can be improved upon the addition of recombinant transferrin binding protein (39).

Current commercial vaccines against *A. pleuropneumoniae* sold in North America typically contain three or four chemically inactivated serotypes (2). Since these commercial bacterins offer only partial protection, while animals that survive natural infection are completely protected, we hypothesized that by comparing the immune responses induced by infection with those induced by bacterin vaccination, it would be possible to identify which antigens and type(s) of immune response were associated with protection. To test this hypothesis, we examined the role of antibody and aspects of CMIR to various antigens of *A. pleuropneumoniae*. This information could preferentially be used to improve vaccines so that they could elicit immune responses more closely resembling those which occur following natural infection. Results indicate that high antibody response and avidity, as well as DTH responses, especially to HLY, are associated with protection, while increased blastogenic responses to HLY, LPS, CPS, and OMP are not.

* Corresponding author. Mailing address: Department of Pathobiology, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada NIG 2W1. Phone: (519) 824-4120, ext. 4736. Fax: (519) 767-0809. E-mail: bmallard@ovcnet.uoguelph.ca.

MATERIALS AND METHODS

Bacteria. *A. pleuropneumoniae* CM5, a serotype 1 strain, was isolated from a pig with a natural case of pleuropneumonia (36). For these studies, pleural fluid

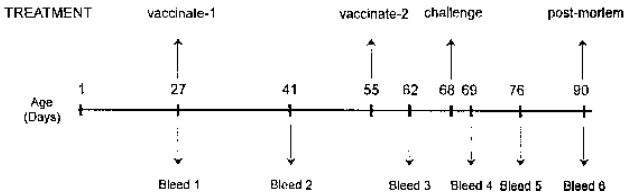

FIG. 1. Immunization, infection, and bleeding schedule for CDCD piglets before and after challenge with *A. pleuropneumoniae.*

from a pig experimentally infected with *A. pleuropneumoniae* CM5 was stored frozen at −70°C and used to prepare challenge inocula (36). The organism was cultured on Trypticase soy agar supplemented with 5% (wt/vol) heated bovine blood (80°C for 10 min) and 0.01% nicotinamide adenine dinucleotide (chocolate blood agar plus V factor [CAV]).

Experimental design. To evaluate protection of pigs against *A. pleuropneumoniae*, LD-infected, bacterin-vaccinated, adjuvant control, and phosphate-buffered saline (PBS) control treated pigs were compared for both antibody and aspects of CMIR to various *A. pleuropneumoniae* antigens. The schedule for vaccination-infection and bleeding is shown in Fig. 1. Peripheral blood for serum antibody measurement and whole blood for lymphocyte culture was collected by retro-orbital sinus puncture before and after each immunization-infection (bleed 1, 27 days of age; bleed 2, 41 days of age; bleed 3, 62 days of age; bleed 4, 69 days of age; bleed 5, 76 days of age; bleed 6, 90 days of age). The two control groups and the bacterin-vaccinated group were housed in the same isolation room, while the LD-infected group was housed in a separate room. At 68 days of age, the piglets were exposed to a high dose of $\sim 10^7$ CFU of *A. pleuropneumoniae* CM5 per ml by aerosol (41). All pigs were bled ~12 h after high-dose challenge (bleed 4, 69 days of age) and monitored for clinical signs. Animals with signs of respiratory distress were immediately euthanized for necropsy. Those which survived challenge were bled at 76 days of age (bleed 5) and bled and euthanized at 90 days of age (bleed 6). Lung lesions were evaluated by using a scoring system based on a total possible score of 9, with 1 point given for each of the following: pleural effusion, fibrinous adhesions, tracheal froth, and pericarditis. Points were also given for the percentage of lung volume with consolidation, hemorrhage, and adhesions determined visually: a score of 1 for less than 5%, 2 for 5 to 25%, 3 for 25 to 50%, 4 for 50 to 75%, and 5 for greater than 75% lung damage. Lungs were scored blind for the bacterin-vaccinated and the control groups, but due to the fact that the LD-infected piglets did not become ill following high-dose challenge and were euthanized 3 weeks later, their lungs could not be scored without knowledge of treatment group. All animal experiments were conducted in accordance with the guidelines of the Canadian Council on Animal Care (1993).

Animals and immunization schedule. Five litters of caesarian-derived, colostrum-deprived (CDCD) Yorkshire piglets (n = 49) were housed in isolation rooms designed for biosecurity requirement level II (Isolation Unit, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada). The piglets were hand reared and fed a powdered milk replacer (Launch Shur Gain; Canada Packers Inc.) and a colostrum supplement, both of bovine origin (VitalShur Gain; Canada Packers Inc.), until weaned at about 2.5 weeks of age. At 4 weeks of age, piglets were randomly assigned to two control groups and two test groups. The first test group (n = 11) received by intramuscular (i.m.) injection at 27 and 55 days of age 2 ml of a commercial *A. pleuropneumoniae* bacterin which contained formalin-inactivated serotypes 1, 2, 5, and 7 according to the manufacturer's recommendations (Fig. 1). The second test group (n = 14) received two LD aerosol exposures of *A. pleuropneumoniae* CM5 at 27 and 55 days of age. Aerosols were generated with a DeVilbiss nebulizer (The DeVilbiss Company, Somerset, Pa.), creating an aerosol of particles ranging in diameter from 0.5 to 5.0 μm, and exposure was for 20 min in an enclosed chamber (41). A total volume of 75 ml of $\sim 10^5$ CFU/ml diluted in PBS (pH 7.4) was used to generate the aerosol by nebulizing for 10 min, after which exposure was continued for another 10 min. All LD-infected pigs were given penicillin i.m. twice daily when clinical signs developed. One control group received 2 ml of PBS, pH 7.4, i.m. (n = 11), and the other (n = 13) received 2 ml of aluminum hydroxide, the adjuvant used in the commercial bacterin, i.m.

Antigen preparation. CPS, LPS, OMP, and HLY antigens were extracted from cultures of *A. pleuropneumoniae* CM5 for use in enzyme-linked immunosorbent assay (ELISA), DTH, and lymphocyte blastogenesis assays. CPS was extracted by a hot phenol method as previously described (3) and centrifuged (100,000 × *g* for 16 h) to remove LPS. Total carbohydrates in the extract were determined by the anthrone assay (17) with glucose as the standard. The protein concentration was determined by the Bradford method (5) using bovine serum albumin as the protein standard, and endotoxin content was measured using a KDO (2-keto-3-deoxyoctonate) assay (17) using synthetic KDO (Sigma Chemical Co., St. Louis, Mo.) as the reference standard.

LPS was extracted by the method of Darveau and Hancock (9) and analyzed for protein and endotoxic content.

Modification of the method of Bossé et al. (4) was used to prepare HLY (Apx I and Apx II, which copurify from serotype 1) for use in the ELISA. *A. pleuropneumoniae* CM5 was grown for 18 h at 37°C on a CAV agar plate, then washed off with RPMI 1640 plus 2.5% fetal calf serum (FCS) (lot SF80823; Bocknek Laboratories Inc.,Toronto, Ontario, Canada) and 2.5 mM $CaCl_2$ in water. The cells were then incubated with shaking for 4 h at 37°C, centrifuged (10,000 × *g*, 10 min, 4°C), and filter sterilized with a 0.2-μm-pore-size polycarbonate filter (Nalgene Labware Division, Nalge/Sybron Corp., Rochester, N.Y.). The resultant HLY preparation was tested for hemolytic activity as described previously by Rosendal et al. (37) using spectrophotometric determinations of hemoglobin in the test and 100% lysis control suspensions of bovine erythrocytes in PBS. Hemolytic units per milliliter were defined as the optical density (OD) of the first dilution below 50% lysis, divided by the OD value of the 50% lysis control, multiplied by the dilution factor of the first tube below the 50% lysis control.

HLY (Apx I and Apx II) for use in lymphocyte blastogenesis and DTH tests was produced as described by Devenish and Rosendal with modifications (11). *A. pleuropneumoniae* CM5 was incubated in RPMI 1640 plus 2.5% FCS for 20 h and following centrifugation, ammonium sulfate (Sigma Chemical Co., St. Louis, Mo.) was added to 80% saturation. The precipitated proteins were collected by centrifugation (21,000 × *g* for 45 min, 4°C) and resuspended in 1/30 of the original volume of RPMI 1640 in 0.02 M Tris buffer (pH 7.4) containing 10 mM $CaCl_2$. This material was dialyzed first against PBS (12 h, 4°C) and then against distilled water (12 h, 4°C). Following dialysis, the solution was lyophilized (48 h in a model Freezemobile II; The VirTis Co., Inc., Gardiner, N.Y.) and tested for hemolytic activity, protein content, and endotoxin content as described above.

OMP were prepared according to the method of Deneer and Potter (10) using 2% Sarkosyl (sodium *N*-laurylsarcosinate; Sigma Chemical Co.) in 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (pH 7.4). Bacteria were grown overnight at 37°C in 1 liter of RPMI 1640 containing 2.5% FCS and 0.01% NAD. Cells were harvested by centrifugation at 10,000 × *g* for 20 min at 4°C and stored at −20°C. Cell pellets were thawed and suspended in 10 mM HEPES buffer, pH 7.4 (1 g [wet weight] in 18 ml), sonicated for 5 min on ice, and centrifuged (10,000 × *g*, 20 min at 4°C) to remove unbroken cells. The supernatant was collected, mixed with 2% Sarkosyl in 10 mM HEPES buffer (pH 7.4) at a ratio of 3:1, and incubated at room temperature with shaking for 10 min. Pellets were collected by centrifugation at 100,000 × *g* for 1 h at 4°C and resuspended in 10 mM HEPES buffer. An equal volume of 2% Sarkosyl was added, and the suspension was incubated at room temperature for 30 min and centrifuged (100,000 × *g*, 1 h at 4°C), and the pellets were suspended in pyrogen-free distilled water. The protein and endotoxin contents of the OMP were determined as described above.

ELISA. Immunoglobulin G (IgG) antibody responses to LPS, CPS, and HLY antigens were determined according to the procedure of Bossé et al. (4). Briefly, sera were diluted 1:50, 1:200, 1:800, and 1:3,200 in 96-well flat-bottom polystyrene plates (Nunc; GIBCO Laboratories, Grand Island, N.Y.) by the quadrant system described by Wright (43) and IgG antibody was detected by using a biotinylated mouse monoclonal anti-porcine IgG (H+L), a generous gift from Klaus Nielsen and Martin Nemec (ADRI, Ottawa, Ontario, Canada). Pooled positive control sera and pooled negative control sera were included on all plates. The positive serum pool was obtained from 10 pigs which had been exposed to three aerosol doses of CM5. The negative serum pool was obtained from 10 gnotobiotic pigs. Duplicate samples were run for all dilutions, and the 1:200 dilution was used for all statistical analyses, since other dilutions provided no added information. ELISA plates were read on a 96-well reader (Bio-Tek Instruments, Winooski, Vt.) at ODs of 405 and 630 nm when the positive control well reached a value of 0.999. There were no positive or negative cutoffs for these ELISAs because values between serum samples were compared and analyzed as continuous variables.

Antibody avidity. Indices of antibody avidity to LPS and CPS were determined by a modified ammonium thiocyanate ion (SCN^-) elution ELISA described previously by Appleyard et al. (1). The standard ELISA procedure was followed as described above with the following modifications. Serum samples were diluted 1:50 and were tested in quadruplicate in the presence of SCN^-. After washing and before addition of the secondary antibody, ammonium thiocyanate (NH_4SCN) (Fisher Scientific Co., Unionville, Ontario, Canada) in PBS was added to all wells for each test sample in the following concentrations: 0, 0.25, 0.5, 1, 2, and 4 M. After 15 min, plates were washed with PBS containing 0.05% (vol/vol) Tween 20. All steps were at room temperature. Color was developed and was read as described above. A control plate was also prepared to ensure that avidity was independent of the amount of antibody. To do this a set of serum samples were diluted (1:50, 1:200, and 1:800), and each dilution was exposed to the different SCN^- concentrations (0, 0.25, 0.5, 1, 2, 4, 6, and 8 M) and the avidity index was calculated. The index remained the same regardless of the initial dilution of the serum sample (data not shown).

The avidity index was calculated as follows: the mean OD values obtained from each serum sample were expressed as a percentage of the control treatment (O M SCN^-) and were then converted to $\log_{10}$. By standard statistical procedures, the value 2 was subtracted from all (log%) datum points, and a quadratic regression line ($ax^2 + bx + c$) was calculated, forcing the origin to zero (1). The avidity index was estimated by solving the equation for an OD of 50% relative to the zero molar SCN^- treatment. Only sera having an OD greater than the positive cutoff of 0.05 OD unit at the 0 M SCN^- treatment and an elution curve slope of less than −0.2 (slope is negative) were used. This eliminated those sera which had no detectable antibodies.

Since antibody to HLY was detected by using an antigen capture system, it was not possible to determine avidity indices for anti-HLY antibodies.

DTH. To test for the ability of pigs to mount a DTH response to various antigens, 12.5 μg of each antigen (LPS, CPS, OMP, and HLY) was diluted in 0.5 ml of sterile PBS (pH 7.4) and injected intradermally (i.d.). Each antigen was injected into a separate site on the inside of both thighs at day 67, just prior to bleed 4 (25). Another site received 0.5 ml of PBS and was used as a negative control. Increase in double-fold skin thickness was measured 24 h later with Harpenden calipers (Creative Health Products, Plymouth, Mich.) and expressed as

$$\% \text{ thickness increase} = \left[\frac{\text{test site thickness} - \text{pretest thickness}}{\text{pretest thickness}}\right] - \left[\frac{\text{control site thickness} - \text{precontrol thickness}}{\text{precontrol thickness}}\right] \times 100$$

Percent increase in skin thickness was evaluated in a subset of pigs at 6, 12, 24, 48, and 72 h to ensure type of hypersensitivity (i.e., type IV) and time of peak response. Skin thickness responses were consistent with DTH, and the peak increase in skin thickness was observed at 24 h postimmunization, the time used for all subsequent measurements. For this test, 10 piglets were assigned to the LD-infected treatment group, 6 piglets were assigned to the bacterin-vaccinated group, 9 piglets were assigned to the PBS control group, and 8 piglets were assigned to the adjuvant control group.

Mitogen- and antigen-induced lymphocyte blastogenesis. Peripheral blood lymphocyte (PBL) blastogenesis was evaluated six times during the immunization-infection schedule by using the T-cell mitogen concanavalin A (ConA) and *A. pleuropneumoniae* CM5 antigens LPS, CPS, OMP, and HLY, according to a modified procedure of Mallard et al. (26). PBL were separated by Ficoll-Hypaque (specific gravity, 1.077; 260 mosmol) and suspended in RPMI 1640 plus 10% FCS (lot SF80823; Bocknek Laboratories Inc.) at 4×10^6 cells/ml. For each pig tested, one 96-well tissue culture plate (Nunclon; GIBCO Laboratories) was used. Fifty microliters of cell suspension was added to all wells. Wells serving as unstimulated controls received 200 μl of medium alone (six replicates per pig). Other wells received 100 μl of either LPS (25 μg/ml), CPS (25 μg/ml), OMP (25 μg/ml), or HLY (10 or 25 μg/ml) and either an additional 100 μl of medium or 100 μl of ConA (10 μg/ml) (24, 30). Wells serving as positive controls received only 100 μl of culture medium plus 100 μl of ConA (10 μg/ml) (six replicates per pig). Plates were incubated for 48 h at 37°C (5% CO_2), and the cells were then pulsed with 20 μl of tritiated thymidine per well (25 μCi/ml; Dupont, New Products, Boston, Mass.) in RPMI 1640. Preliminary experiments compared PBL proliferation at 2 and 4 days prior to addition of tritiated thymidine; however, 2 days was determined as optimal. Following incubation for 24 h (37°C, 5% CO_2 added), all plates were wrapped in Parafilm M (American National Can, Greenwich, Conn.) and stored frozen (−20°C) until harvesting to avoid variation due to freezing and thawing. Cells from thawed plates were harvested on glass fiber filters with an automated cell harvester (Skatron Cell Harvester System; Wallac, Turku, Finland). Tritiated thymidine incorporation was then determined by liquid scintillation counting with a β-plate counter (model LKB 1205; Fisher Scientific Co.).

Statistical analyses. The Univariate procedure of SAS (18) was used to determine normality of distributions for each data set, and natural log transformations were performed when necessary. The general linear model procedure of SAS was used for analysis of variance (ANOVA) and to determine least-square means (LS means).

TABLE 1. Mortality, mean time until death, and mean lung lesion scores after high-dose challenge with *A. pleuropneumoniae* for various treatment groups

Treatment group	Mortality (no. of pigs/total)	Time to death (h)	Mean lesion score ± SD
LD infected	0/18	NA[a]	1.72 ± 1.41
Bacterin	20/20	15.5	5.85 ± 1.27
PBS control	18/18	14.7	5.72 ± 1.9
Adjuvant control	13/13	16.5	5.67 ± 2.1

[a] NA, not applicable.

In previous studies (6, 25) spontaneous blastogenesis (i.e., counts per minute when no mitogen or antigen was added) accounted for a substantial proportion of the variation in the lymphocyte blastogenic responsiveness. Therefore, counts per minute from spontaneous blastogenic activity of lymphocytes were used to covariately adjust antigen-stimulated or mitogen-stimulated counts per minute. The effect of treatment on lymphocyte blastogenesis was examined by using the following statistical model: $Y_{ijklm} = \mu + \text{litter}_i + \text{treatment}_j + \text{bleed}_k + (\text{treatment} \times \text{bleed})_{jk} + (\text{litter} \times \text{treatment})_{ij} + \text{animal}(\text{litter} \times \text{treatment})_{ijl} + b_1(\text{cov})_{ijkl} + e_{ijklm}$ where Y_{ijklm} is the response of piglet I in litter i at time k for treatment j, μ is the population mean of a response to treatment, litter_i is the effect of litter i, treatment_j is the effect of treatment j, bleed_k is the effect of time k, $(\text{treatment} \times \text{bleed})_{jk}$ is the effect of treatment by bleed interaction, $(\text{litter} \times \text{treatment})_{ij}$ is the effect of litter by treatment interaction, animal(litter × treatment) is the effect of an animal nested within a litter by treatment, $b_1(\text{cov})$ is the regression coefficient for the spontaneous lymphocyte blastogenic activity covariate (cov), and e_{ijklm} is a random error term. The model used to evaluate DTH was $Y_{ijk} = \mu + \text{litter}_i + \text{treatment}_j + (\text{treatment} \times \text{litter})_{ij} + e_{ijk}$. The model used to evaluate antibody avidity was $Y_{ijkl} = \mu + \text{treatment}_i + \text{bleed}_j + (\text{treatment} \times \text{bleed})_{ij} + \text{animal}(\text{treatment})_{ik} + e_{ijkl}$. The model used to evaluate antibody response was $Y_{ijklm} = \mu + \text{litter}_i + \text{treatment}_j + \text{bleed}_k + (\text{treatment} \times \text{bleed})_{jk} + (\text{litter} \times \text{treatment})_{ij} + \text{animal}(\text{litter} \times \text{treatment})_{ijl} + e_{ijklm}$.

The statistical models were modified slightly depending on the nature of the dependent variable. For instance, no effect of bleed is included in the DTH analysis since it was only evaluated at one point (day 68). Significance was reported as $P \leq 0.05$, and trends were reported as $P \leq 0.10$.

RESULTS

Lung lesion scores. All LD-infected pigs and none of the bacterin-vaccinated or control animals survived high-dose aerosol challenge with *A. pleuropneumoniae* (Table 1).

Animals which became sick following challenge all had to be euthanized between 12 and 24 h, and their lungs had extensive areas of fibrinonecrotic and hemorrhagic pleuropneumonia (Fig. 2A). Lung lesion scores for the bacterin-vaccinated and control animals were not statistically significantly different (Table 1). The LD-infected pigs showed no clinical signs following

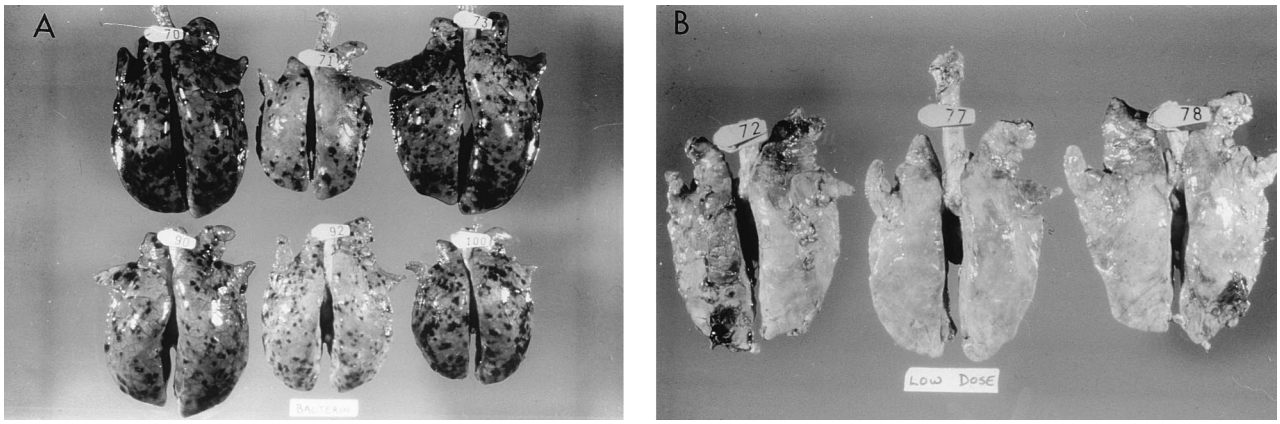

FIG. 2. Representative lungs of CDCD piglets after high-dose aerosol challenge with *A. pleuropneumoniae*. (A) Lungs of bacterin-vaccinated piglets; (B) lungs of LD-infected piglets.

TABLE 2. ANOVA of treatment effects on immune response parameters of CDCD piglets after immunization-infection with *A. pleuropneumoniae*

Immune response traits	r^{2a}	*P* value for the following sources of variation[b]:				
		Litter	Trmt[c]	Bleed	Trmt × bleed	Animal(litter × trmt)
IgG-HLY[d]	0.821	0.0001	0.0001	0.0001	0.0001	0.0006
IgG-LPS	0.687	NS[e]	0.037	0.0001	0.0001	0.0001
IgG-CPS	0.664	NS	NS	0.0001	0.0096	0.0001
IgG-LPS avidity[f]	0.674	NT[g]	NS	NS	NS	0.0021
IgG-CPS avidity	0.777	NT	NS	NS	NS	0.0028
DTH-HLY[h]	0.473	NS	0.0059	NT	NT	NT
DTH-LPS	0.575	0.0576	NS	NT	NT	NT
DTH-CPS	0.626	0.0107	0.0258	NT	NT	NT
DTH-OMP	0.525	0.0066	NS	NT	NT	NT
Blasto-HLY[i]	0.509	0.0008	NS	0.0001	0.0001	0.0001
Blasto-LPS	0.603	0.0001	0.0378	0.0001	0.0001	0.0001
Blasto-CPS	0.562	0.0001	NS	0.0001	0.0001	0.0001
Blasto-OMP	0.369	0.0287	NS	0.0001	0.0006	0.0001

[a] Coefficient of determination.
[b] Probability of significant difference. Significance ($P > F$, where F is the variance ratio) was as determined by the SAS general linear model.
[c] Trmt, treatment.
[d] IgG ELISA response as determined by OD.
[e] NS, not significant.
[f] Antibody avidity as determined by thiocyanate ion elution ELISA.
[g] NT, not tested because inappropriate for these parameters.
[h] DTH measured by percent increase in skin thickness in response to antigens.
[i] Lymphocyte blastogenesis with LPS, HLY, CPS, and OMP.

challenge and significantly lower ($P \leq 0.001$) lung lesion scores (Table 1) than the bacterin-vaccinated and control animals (Fig. 2B). Five LD-infected piglets, part of another aspect of this research thus not included in these experiments, were also euthanized 24 h following high-dose challenge and used to show that the average lung lesion scores for these animals were not statistically significantly different from the scores for those animals that were euthanized at the end of the experiment (1.72 ± 1.41 versus 2.0 ± 1.22).

ANOVA. ANOVA for each trait is summarized in Table 2. The models explained between 36.9 and 82.1% of the variation in the immune response parameters examined. The individual pig [animal(litter × treatment)] always had a significant influence on antibody and CMIR ($0.0028 \leq P \leq 0.0001$), while litter was not significant for LPS and CPS antibody responses or for DTH to HLY antigen. The effect of treatment within time (treatment × bleed) accounted for a significant proportion of the variation in antibody and PBL blastogenesis but not for antibody avidity.

IgG ELISA. Antibodies to HLY, CPS, or LPS antigens were not detected in any animal prior to treatment. After the first LD infection (bleed 2) a significant ($P \leq 0.05$) increase in antibody response to the HLY antigen was detected (5 of 14 piglets responded), but there was no antibody response to LPS or CPS detected (Fig. 3). After the second LD infection (bleed 3) there was a significant ($P \leq 0.05$) increase in antibody response to HLY (14 of 14 responded) and LPS (6 of 14 responded) but not CPS. Minimal antibodies were detected in bacterin-vaccinated (3 of 11 responded) animals, and none were detected in control animals following two treatments (Fig. 3). Eight piglets in the LD treatment group had significant HLY antibody responses at bleed 3 but only developed significant IgG antibody responses to LPS or CPS after high-dose challenge (bleed 4).

IgG antibody avidity. Only animals in the LD-infected and bacterin-vaccinated groups which developed antibodies to LPS and CPS were tested for antibody avidity. The LD-infected animals increased antibody avidity earlier (bleed 2 versus bleed 3) than bacterin-vaccinated pigs (Fig. 4) and had higher but not significantly higher avidity indices at bleeds 3 and 4 for CPS and at bleed 4 for LPS. Avidity was measured after bleed 4 only for the LD treatment group, since it was the only treatment group to survive high-dose challenge. Antibody avidity to LPS continued to increase at bleed 5 and returned to bleed 4 values by bleed 6. Antibody avidity to CPS remained unchanged at bleed 5 but increased significantly ($P \leq 0.05$) at bleed 6.

DTH. DTH was measured on day 68 immediately prior to high-dose challenge (Fig. 5). There were significant ($P \leq 0.05$) differences in DTH response between LD-infected animals (8 of 10 responded) and bacterin-vaccinated (2 of 7 responded) animals for HLY. These differences approached significance for LPS ($P \leq 0.08$) and CPS ($P \leq 0.09$), with the pigs in the LD treatment group (6 of 10 responded to LPS and CPS) having the higher responses (Fig. 5). The DTH responses to OMP in the LD-infected, bacterin-vaccinated, or control animals did not differ significantly.

PBL blastogenesis. The LD-infected group maintained a consistent blastogenic response over the course of infection, while bacterin-vaccinated and control animals tended to have large changes in response. Differences between the LD-infected group and other treatments were not significant except at bleed 2 for responses to CPS and bleed 3 for responses to LPS when the LD-infected piglets had significantly ($P \leq 0.05$) lower proliferative responses than bacterin-vaccinated piglets (Fig. 6). These proliferative responses were also significantly different ($P \leq 0.05$) from that of lymphocytes cultured in medium alone. Differences between the treatment groups with respect to ConA blastogenesis at the different time points were significant only at bleed 2, when the LD-infected group value was significantly ($P \leq 0.05$) higher than that of the adjuvant-treated control group. When antigen plus ConA was used to stimulate lymphocytes, there were no significant differences between groups.

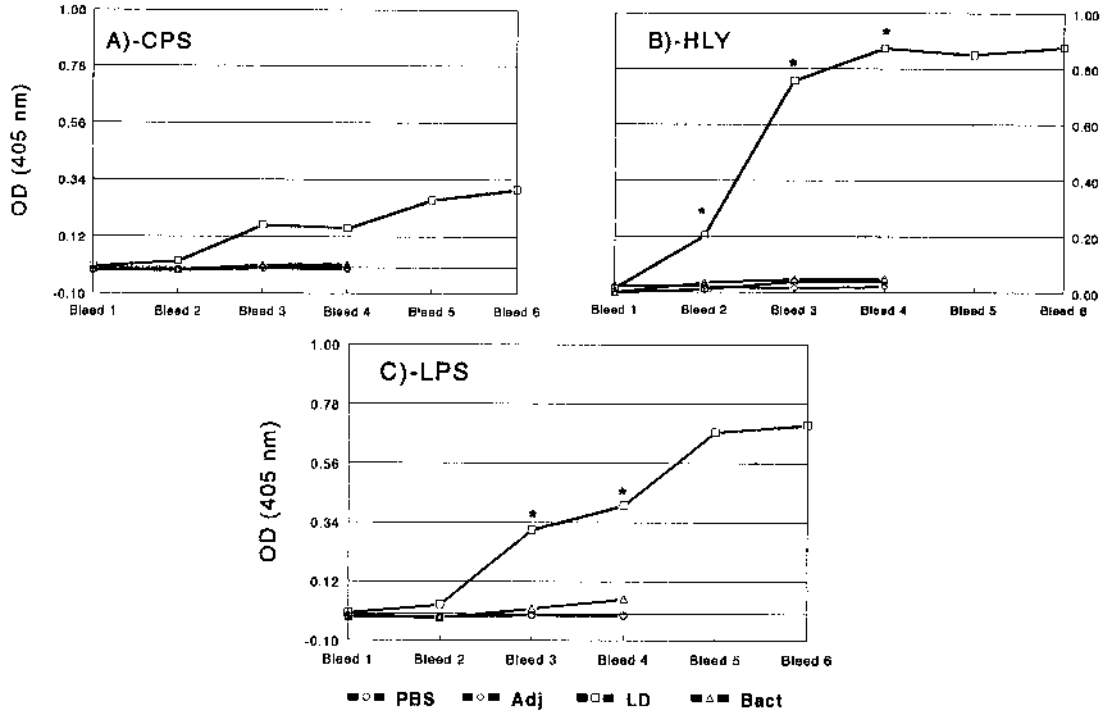

FIG. 3. LS means of serum IgG antibody responses to *A. pleuropneumoniae* antigens. CPS (A), HLY (B), and LPS (C) were measured by ELISA. Stars indicate significant differences ($P \leq 0.05$) between treatments at those time points.

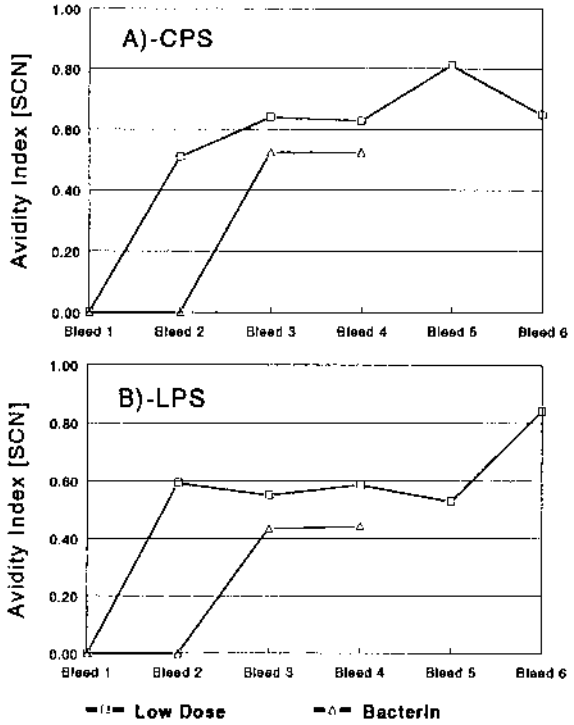

FIG. 4. LS means of indices of serum IgG antibody avidity to *A. pleuropneumoniae* antigens. CPS (A) and LPS (B) were measured by a thiocyanate ion elution ELISA. An avidity index of zero indicates that there was no antibody response at that time point and therefore no antibody avidity.

DISCUSSION

Following challenge of pigs with a high dose of aerosolized *A. pleuropneumoniae* there was 100% mortality in the bacterin-vaccinated and unvaccinated control groups and none in pigs previously exposed to LD infection (Table 1).

Significant increases in serum antibody response and DTH of pigs in the LD-infected group were associated with protection. Antibody avidity was consistently higher in the LD-infected group than in bacterin-vaccinated and control pigs, but these differences were not significant at bleeds 3 and 4. Lymphocyte blastogenic responses of LD-infected pigs were consistent over time and were either the same as or lower than those for pigs in other treatment groups, but differences were generally not statistically significant.

There are several possible explanations for higher antibody responses in the LD-infected treatment group than in the bacterin-vaccinated animals. First, it was subsequently determined that the bacterin contained little or no detectable HLY (35), and although the bacterin contained CPS and LPS (34), it is conceivable that the formalin inactivation process denatured the antigens so that any antibodies produced would not recognize the native antigens. Secondly, higher serum antibody responses were achieved in the LD-infected animals, although the immunization was by the aerosol route. Location of antibody response has been shown to be related to the size and composition of the immunizing particles (42). Small particles (0.5 to 3.0 μm) are able to enter the alveolar spaces from which they can gain access to lymphatics, blood vessels, or both and reach regional or systemic lymph nodes, thus inducing both local and systemic antibody responses (19).

Although lung lavage samples were not collected during these studies to examine local antibody responses, Bossé et al. (4) reported both IgA and IgG antibody responses to HLY, CPS, and LPS in the lungs of pigs 21 days after infection with

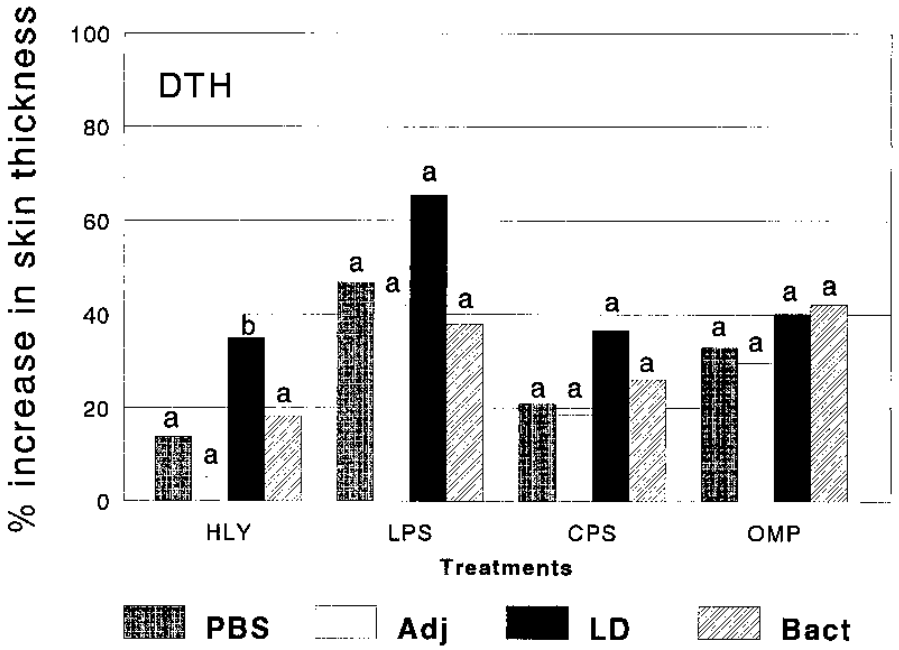

FIG. 5. LS means of DTH responses, measured as percent increase in skin thickness, to the *A. pleuropneumoniae* antigens HLY, LPS, CPS, and OMP. Measurements were taken 24 h postinjection for the four treatment groups. Different letters above bars indicate significant differences ($P \leq 0.05$) between treatments for each antigen.

a low dose of aerosolized *A. pleuropneumoniae*, unlike with control animals. Passive transfer of immune sera containing predominantly IgG to naive piglets by the intraperitoneal and i.m. routes followed by challenge with *A. pleuropneumoniae* provided complete protection, and the serum IgG-associated antibody titers were similar to those of LD-infected convalescing piglets (4). It was concluded that serum IgG antibodies were involved in protection against porcine pleuropneumonia. Although an aerosol vaccine would have been beneficial for comparison purposes, it is possible to provide complete protection by systemic immunization, since IgG is the protective antibody isotype.

Some of the LD-infected piglets only developed antibodies to HLY prior to high-dose challenge and they were completely protected, which suggests that anti-HLY antibodies may contribute more to protection than anti-LPS and -CPS. These

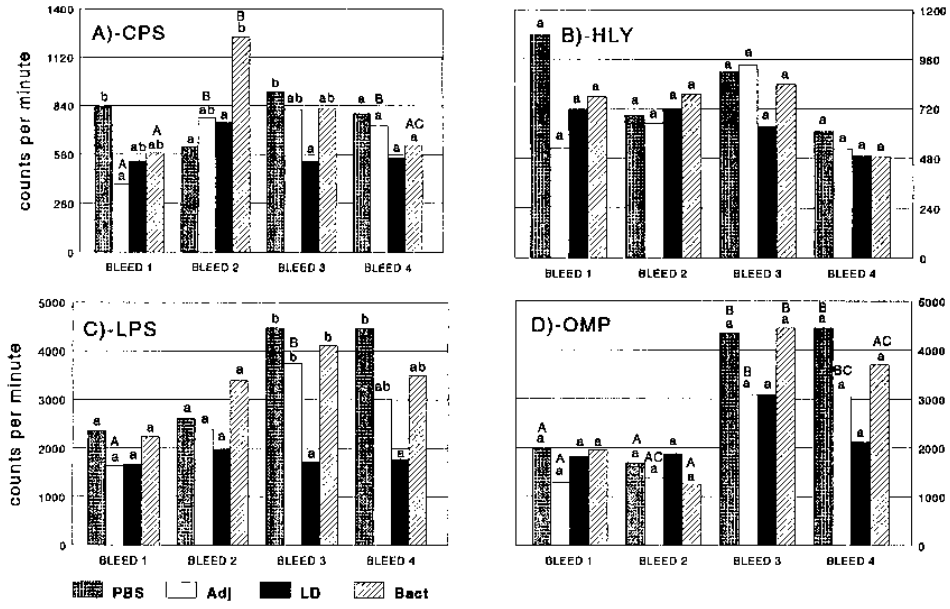

FIG. 6. LS means of peripheral blood proliferation (counts per minute) following stimulation with *A. pleuropneumoniae* antigens CPS (A), HLY (B), LPS (C), and OMP (D). Different lowercase letters above bars indicate significant differences ($P \leq 0.05$) between treatments at one time point. Different uppercase letters indicate differences ($P \leq 0.05$) over time for each treatment group.

results support previous findings (4, 12) that antibody to *A. pleuropneumoniae* antigens, particularly HLY, is associated with protection.

DTH is considered a measure of CMIR, which offers the advantage of being a quantifiable in vivo response reflecting afferent and efferent events in CMIR. DTH has not been reported previously with *A. pleuropneumoniae* antigens in pigs, likely because this bacterium is extracellular and immunity is largely governed by appropriate antibody responses. However, effective antibody responses normally require help from T lymphocytes (21), and DTH is essentially a measure of $CD4^+$ T-cell activity (13, 20). In addition, recent reports suggest that certain T-cell subsets have direct antibacterial activity against extracellular pathogens (22) and that some extracellular pathogens require CMIR for protection (27, 28). It has also been shown that CMIR plays a role in bacterial clearance in *Bordetella pertussis* infections (29), maybe because *B. pertussis* has been shown to survive up to 3 days in human alveolar macrophages and thus has an intracellular stage (16). An intracellular stage has not been noted for *A. pleuropneumoniae*, although it can survive at least 4 h inside porcine alveolar macrophages (8) so that CMIR may activate macrophages, thus contributing to protection in this disease.

In this study DTH associated positively with antibody response. All LD-infected animals developed anti-HLY antibodies by bleed 3, and 6 of 10 responded to HLY as measured by DTH. The LD-infected piglets developed higher DTH responses than the bacterin-vaccinated animals to CPS ($P \leq 0.09$), LPS ($P \leq 0.08$), and HLY ($P \leq 0.03$) but not to OMP. Future vaccines developed to combat *A. pleuropneumoniae* may seek to evoke antibody and/or DTH responses to relevant antigens.

Historically lymphocyte blastogenic responses to antigens and mitogens are considered measures of CMIR, but correlations with other indicators of CMIR such as DTH have not always been found. However, nonspecific blastogenic responses following culture with phytomitogens are likely of limited value, since mitogens, such as ConA, bind not to T-cell receptors but to various sugar moieties on lymphocyte surfaces. Although blastogenic response to antigen is clonally specific, T- and B-cell responses cannot be clearly differentiated when unseparated PBL are used as the culture system. In this study, the lack of in vitro lymphocyte proliferation in response to *A. pleuropneumoniae* antigens in the LD-infected group compared to that in bacterin-vaccinated and control animals was opposite to the observed DTH response to the same antigens. The decreased response or nonresponse in the blastogenesis assay in the LD-infected group is difficult to interpret but could be related to differences in antigen composition between the bacterin and LD infection or the route of delivery of the antigen dose, or they may simply indicate that the response measured by blastogenesis is not necessary for protection in LD infection. It is also possible that blastogenesis is actually indicative of a nonprotective or detrimental response and therefore not seen in the LD aerosol-infected group. Another interpretation could be that the LD-infected pigs responded more quickly than the bacterin-vaccinated and control groups and thus at the time of ^{3}H-thymidine incorporation (48 h) the lymphocytes were no longer dividing.

When antigen plus ConA was used in blastogenesis cultures, no significant differences were found; thus, there was no evidence by this assay system to support the hypothesis that HLY, OMP, LPS, or CPS antigens of *A. pleuropneumoniae* are associated with immunodepression.

Fenwick and Osburn (15) reported that lymphocyte proliferation in LD-infected pigs was significantly greater than that in bacterin-vaccinated pigs in response to LPS but not in response to CPS. However, both of these treatment groups responded to CPS and LPS significantly more strongly than did the PBS-vaccinated controls. These discrepancies could be due to several differences in the infection-vaccination protocols. First, comparatively few piglets were used in the Fenwick and Osburn study and only one time point was examined during the immunization-infection schedule (approximately equivalent to bleed 3). Also, no high-dose challenge was performed, and this was associated with the most dramatic differences between the treatment groups for two of the antigens (LPS and OMP). Finally, there were differences in lymphocyte assay conditions, such as length of culture and cell concentration per well.

The present study indicates that there are significant differences in both antibody and DTH responses between LD-infected animals and bacterin-vaccinated piglets. High antibody responses and DTH responses, especially to HLY, are associated with protection, while increased blastogenic responses are not. This information may be useful in modifying current commercial bacterins so an immune response which mimics that seen following natural infection can be induced.

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