Hyperimmune Serum from Rabbits Immunized with Potassium Thiocyanate Extract of *Pasteurella multocida* Protects against Homologous Challenge

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Hyperimmune rabbit sera directed to the KSCN extract of 3:A *Pasteurella multocida* were characterized by enzyme-linked immunosorbent assay (ELISA), presolubilized cell radioimmunoprecipitation, and immunoblotting analysis. The results showed that the hyperimmune serum had a very high titer of immunoglobulin G ELISA antibody and a negligible immunoglobulin A ELISA antibody, precipitated 10 different outer membrane protein antigens by radioimmunoprecipitation, and reacted to 10 different membrane vesicle antigens of *P. multocida* by immunoblotting analysis. The hyperimmune rabbit sera were also evaluated for protective efficacy against experimental rabbit pasteurellosis by homologous challenge. Thirty-six rabbits were divided into four groups. Group 1, 2, and 3 rabbits were inoculated intranasally with hyperimmune rabbit serum, phosphate-buffered saline, or normal rabbit serum, respectively, at 24 h prior to and 24, 48, and 72 h after intranasal challenge with the virulent homologous *P. multocida* strain. Group 4 rabbits were inoculated with normal rabbit serum without challenge. Necropsies of surviving rabbits were performed 2 weeks postinfection. The mortality rates for groups 1 through 4 were 25% (3 of 12), 67% (8 of 12), 75% (6 of 8), and 0% (0 of 4), respectively. The prevalence and severity of pneumonia were significantly lower in the hyperimmune serum-treated rabbits. The prevalence of *P. multocida* colonization in lungs was significantly lower in group 1 rabbits, and the geometric mean CFU of *P. multocida* in lungs was 59,166-fold less in group 1 rabbits than in group 3 rabbits. The geometric mean CFU of *P. multocida* in nasal cavities of group 1 rabbits was significantly lower than that of group 3 rabbits. All challenged rabbits (groups 1, 2, and 3) had elevated nasal immunoglobulin A and pulmonary (lung lavage) immunoglobulin A antibody levels at necropsy (day 14 postinfection). Similarly, all challenged rabbits had elevated levels of ELISA immunoglobulin G antibody in serum at day 14 but not at day 7 postinfection, indicating that rabbits receiving hyperimmune serum can mount a specific humoral immune response against the homologous challenge *P. multocida* organisms. We concluded that hyperimmune serum directed to the KSCN extract of 3:A *P. multocida* provides significant protection against homologous challenge in rabbits.

Pasteurellosis caused by *Pasteurella multocida* is a common and serious problem in rabbits. The complications of *P. multocida* infections range from lesions in the respiratory, genital, and sensory systems to death of animals.

We are testing the efficacy of several vaccine preparations for preventing the disease in rabbits. One important aspect of vaccine development is to determine the role of humoral and cellular immunity in protection. Protection studies in cattle (7) and mice (9, 27) indicated that humoral immunity was important in protecting vaccinated animals against *P. multocida* infection. Experimental adoptive transfer studies in mice showed that mouse immune serum (9, 27) but not immune splenic cells (11) were protective against homologous challenge with *P. multocida*. Apparently, antibody plays an exclusive role in protection against pasteurellosis in mice. In chickens and turkeys, unlike in mammals, recent studies (3, 4) indicated that cell-mediated immunity plays a more important role than humoral immunity in protecting against systemic pasteurellosis.

In previous experiments, rabbits immunized intranasally (i.n.) with streptomycin-dependent 3:A *P. multocida* mutant vaccines (8, 19, 23) or *P. multocida* vaccines prepared by KSCN extraction (24; Y.-S. Lu, S. P. Pakes, L. Massey, and C. Stefanu, Infect. Immun., in press) were protected

against homologous challenge. These rabbits developed immunoglobulin G (IgG) and immunoglobulin A (IgA) antibodies specific for *P. multocida* (24; Lu et al., in press). It is not yet known to what degree these antibodies were involved in the protection elicited by these vaccines.

The purpose of this study was to determine whether the protection provided by a KSCN extract of *P. multocida* is mediated by serum humoral antibody. Rabbit hyperimmune serum produced by frequent injections of KSCN extract of *P. multocida* was characterized by enzyme-linked immunosorbent assay (ELISA), presolubilized cell radioimmunoprecipitation (PS RIP), and immunoblotting analysis. The hyperimmune serum was also evaluated for its efficacy in protecting challenged rabbits against lesion development, nasal colonization, and colonization of lungs and nonrespiratory tissues. We also tested whether passive immunization with hyperimmune serum affects host humoral immune response to the challenge organism.

MATERIALS AND METHODS

Animals. Pasteurella-free New Zealand white female rabbits, weighing 2.7 to 3.6 kg, were purchased from Hazleton-Dutchland Laboratories, Denver, Pa. All rabbits were tested twice for the presence of *P. multocida* by swabbing nasal turbinates with a calcium alginate swab, which was streaked onto a blood agar plate and incubated for 24 to 48 h at 37°C.

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Sera were collected and tested for *P. multocida* IgG antibody by ELISA. All rabbits were free of *P. multocida* and negative for IgG antibody against *P. multocida*. Rabbits were housed under a light-dark cycle of 12 h-12 h in stainless-steel cages with wire floors and provided water and feed (Teklad Laboratory Rabbit Diet; Harlan Sprague Dawley, Inc., Madison, Wis.) ad libitum. Rooms were maintained at 20 to 21°C and 30 to 70% humidity and were ventilated at 12 to 15 air changes per h.

Experimental design. Thirty-six rabbits were divided into four groups and treated as follows. Group 1 (12 rabbits) were inoculated i.n. with hyperimmune rabbit serum and challenged. Group 2 (12 rabbits) were inoculated i.n. with sterile 0.01 M phosphate-buffered saline (PBS) and challenged. Group 3 (8 rabbits) were inoculated i.n. with normal rabbit serum and challenged. Group 4 (4 rabbits) were inoculated with normal rabbit serum and not challenged.

Rabbits in groups 1, 2, and 3 were inoculated i.n. with 1 ml of hyperimmune serum, PBS, or normal rabbit serum, respectively, at 24 h prior to and 24, 48, and 72 h after i.n. challenge with 1 ml of virulent *P. multocida* (4×10^9 CFU/ml). Serum, PBS, and the *P. multocida* inoculum were delivered dropwise with a sterile Pasteur pipette. Group 1, 2, and 3 rabbits were challenged i.n. on day 0, and the survivors were humanely killed with an overdose of pentobarbital sodium 14 days later. Group 4 rabbits were treated the same as group 3 rabbits but were not challenged. Nasal swab samples were collected from surviving rabbits of groups 1, 2, and 3 on days 4, 7, 8, 10, 12, and 14 postchallenge, and numbers of *P. multocida* were determined on TSAY (tryptic soy agar with 0.3% yeast extract). Serum samples were collected from surviving rabbits in all groups at days 7 and 14 postinfection (p.i.).

Preparation of challenge organisms. Wild-type 3:A *P. multocida* (UT-1 strain) organisms were grown from lyophilized stock and inoculated intraperitoneally into mice. The *P. multocida* organisms were reisolated from heart blood of infected mice and grown in brain-heart infusion broth with shaking at 37°C for 6 h. *P. multocida* cells were washed three times with 0.01 M PBS (pH 7.2), serially diluted, and plated on TSAY. The challenge inoculum contained 4.0×10^9 organisms per ml.

Preparation of hyperimmune and normal sera. Hyperimmune sera were collected from two pasteurized-free rabbits immunized intramuscularly with the KSCN extract (1 mg of protein per ml) of 3:A *P. multocida* in complete Freund adjuvant initially and in PBS thereafter, at biweekly to monthly intervals for a period of 12 months. Rabbit sera with high-titer ELISA antibody against the homologous KSCN extract were pooled and concentrated. The pooled immune sera were precipitated with ammonium sulfate (12) to enrich the immunoglobulin. The concentrated immune sera were then filtered (0.45- μ m-pore size; Millipore Corp.), tested for sterility, separated into 1-ml portions, and frozen until used. Normal rabbit sera collected from five pasteurized-free rabbits were pooled and concentrated in the same manner as the hyperimmune sera. The concentrated hyperimmune and normal sera were tested for IgG and IgA ELISA antibodies against the KSCN extract of 3:A *P. multocida*.

Characterization of hyperimmune serum. A PS RIP method and Western blot (immunoblot) immunoanalysis were used to identify *P. multocida* antigens reactive to the hyperimmune serum.

PS RIP method. The PS RIP method was an adaptation of the procedure described by Hansen et al. (14). Briefly, ^{125} I-labeled *P. multocida* cells (2×10^8 cpm/ml) were solu-

bilized by incubating the cells with solubilization buffer (10 mM Tris, pH 7.8, 10 mM EDTA, 150 mM NaCl, 1% [vol/vol] Triton X-100, 0.2% [wt/vol] sodium deoxycholate, 0.1% [wt/vol] sodium dodecyl sulfate [SDS]) at 39°C for 1 h, mixing every 15 min. The supernatant was collected after centrifugation of the mixture at $45,000 \times g$ for 1 h at 16°C. Mixtures containing presolubilized cells and immune rabbit serum, normal rabbit serum, or PBS were incubated on a rocking platform for 1 h at 4°C. Prewashed staphylococcal protein A suspension was added. The mixture was incubated as before and centrifuged at $12,000 \times g$ for 1 min at 25°C. The pellet containing the staphylococcal protein A-antigen-antibody complex was washed five times in solubilization buffer. The final pellet was suspended in digestion buffer (62.5 mM Tris, pH 6.8, 2% [wt/vol] SDS, 10% [vol/vol] glycerol), boiled for 5 min, and centrifuged. The supernatants were frozen at -20°C until analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). The labeled antigens precipitated by the immune serum were separated by SDS-PAGE and identified by autoradiography (15).

Western blot immunoanalysis. (i) **Preparation of *P. multocida* immunogens for Western blot analysis.** Two sources of *P. multocida* immunogens were used for Western blot analysis. The outer membrane immunogens of *P. multocida* were prepared by a procedure similar to that used to prepare *Haemophilus influenzae* outer membranes (14). The membrane vesicle immunogen of *P. multocida* was prepared by the procedure used to extract membrane vesicles of *Neisseria gonorrhoeae* (21) and *H. influenzae* (13).

(ii) **SDS-PAGE.** The SDS-PAGE procedure of Laemmli (18) was used. The samples were run on a discontinuous SDS-PAGE in 1.5-mm-thick slab gels. A 4% (wt/vol) polyacrylamide stacking gel and a 10% (wt/vol) polyacrylamide separating gel were used to separate the distinct proteins into bands. Electrophoresis was conducted at 30 mA constant current per gel at 4°C until the pyronin Y tracking dye entered the separating gel; the constant current was then increased to 45 mA per gel. Protein contents of samples were measured by the method of Bradford (6) with Bio-Rad reagent (Bio-Rad Laboratories, Richmond, Calif.). Samples were boiled with digestion buffer containing 5% (vol/vol) 2-mercaptoethanol prior to being loaded onto the gel. Labeled proteins on the gel were fixed, stained with Coomassie blue, destained, dried, and autoradiographed.

(iii) **Western blot immunoanalysis of antigens.** The SDS-PAGE-separated *P. multocida* antigens were transferred from the slab gel to nitrocellulose paper (NCP) by the methanol-Tris glycine method (26). Electroblooming was conducted in a transblot apparatus (Bio-Rad Laboratories) for 2 h at 125 V and 4°C. The gels were removed and stained with Coomassie blue to determine whether transfer was complete. The NCP strips were washed in PBS-0.05% Tween 20-1% bovine serum albumin, incubated with PBS/T (0.01 M PBS, 0.05% Tween 20, pH 7.2)-diluted rabbit immune serum or normal rabbit serum at 4°C overnight, and washed three times in PBS/T at the end of incubation. The NCP strips were incubated at 4°C for 60 min with 1:3,000-diluted goat anti-rabbit immunoglobulin (Bio-Rad Laboratories) conjugated with horseradish peroxidase and washed three times in PBS/T to remove the unattached second antibody. The NCP strips were incubated with substrate (4-chloro-1-naphthol) and H_2O_2 for color development. The enzymatic reaction was stopped by washing the strips in tap water.

Isolation of *P. multocida* from challenged rabbits. To determine the effect of hyperimmune serum on nasal colonization by virulent *P. multocida*, bilateral nasal swab samples were

collected from surviving rabbits in groups 1, 2, and 3 on days 4, 7, 8, 10, 12, and 14 p.i. Nasal swabs were vortexed in 1 ml of PBS (0.01 M, pH 7.2) for 5 min. The suspension was serially diluted 10-fold, and titers were determined on TSAY plates in triplicate at each dilution. Plates were incubated at 37°C for 24 to 48 h.

The prevalence of colonization and number of virulent *P. multocida* in nasal cavities were compared among the three groups. At necropsy, bacterial cultures were collected from nasal cavities, left and right tympanic bullae, lungs, liver, spleen, uterus, and tissues with gross lesions. Samples prepared from all tissues except lungs were inoculated onto blood agar plates, and the plates were incubated at 37°C for 24 to 48 h. Parts of the lungs from each rabbit were homogenized in PBS to make a 10% (wt/vol) suspension. The lung suspensions were diluted 10-fold and plated on blood agar plates to determine the number of CFU per gram of lung tissue.

Collection of nasal secretions and lung lavage. Nasal secretions were collected at day 14 p.i. to determine IgA antibody by the ELISA method. The secretions from each naris were collected immediately prior to necropsy by inserting a curved 20-gauge blunt intubation needle, injecting 1 ml of sterile 0.01 M PBS, allowing the animal to sneeze the secretion into a petri dish, and repeating the process (24). Collected secretions were frozen at -20°C until tested by ELISA. Lung lavage was done at necropsy by inserting an infant feeding tube (8 French, 15 in.; Seamless Hospital Products Co., Wallingford, Conn.) into bronchial trees and injecting 20 ml of sterile 0.01 M PBS. The lungs were gently squeezed while the PBS lavage was aspirated. This process was repeated twice. Bovine serum albumin was added to the lung lavages to obtain a concentration of 0.1% (vol/vol), and the lavage samples were frozen until tested for IgA and IgG antibodies. Unconcentrated nasal secretions were tested for IgA antibody by ELISA. Lung lavage samples were concentrated prior to ELISA by straining through three pieces of sterile gauze and reducing to 1/10 the original volume with an Amicon apparatus and a Y10 membrane (10,000- M_r cutoff; Amicon Corp., Danvers, Mass.).

ELISA. The antigen used for ELISA was the KSCN extract of *P. multocida* 3:A prepared as described previously (22). Alkaline phosphatase-conjugated goat anti-rabbit IgG (gamma-chain specific; Miles Laboratories, Elkhart, Ind.) or goat anti-rabbit secretory IgA (alpha-chain and secretory-component specific; Cappel Laboratories, Cochranville, Pa.) was diluted in 0.05 M Tris buffer with 1% bovine serum albumin, 0.02% NaN_3 , and 9.5% MgCl_2 . *p*-Nitrophenyl phosphate was prepared as a 0.1% solution in 10% diethanolamine buffer (pH 9.8) containing 0.01% MgCl_2 . Optimal concentrations of antigens and IgG and IgA conjugates were determined by checkerboard titration. IgG ELISA antibodies were measured in serum samples diluted 1:20 in PBS/T and in lung lavage samples diluted 1:10 in PBS/T. Undiluted nasal secretion and lung lavage samples were used for IgA ELISA antibody determinations.

A 0.15-ml volume of antigen in coating buffer (0.015 M Na_2CO_3 , 0.035 M NaHCO_3 , 0.02% NaN_3 , pH 9.60) was added to a rigid polystyrene U-bottomed plate (catalog no. 1-220-24X; Dynatech Laboratories, Alexandria, Va.) to determine IgG level or to an Immulon I U-bottomed plate (catalog no. 011-010-3550) for IgA. Plates were covered and incubated overnight at 4°C. IgG test plates were washed four times with PBS/T, and IgA test plates were washed five times with PBS/T. A 0.15-ml volume of the testing material (nasal secretion, serum, or lung lavage) was added to each

well in duplicate. Plates were covered, incubated at room temperature for 2 h, and washed four or five times with PBS/T. A 0.15-ml amount of diluted conjugate was added, and the plates were incubated at room temperature for 3 h and washed again with PBS/T. A 0.15-ml amount of *p*-nitrophenyl phosphate substrate was added, and the plates were incubated uncovered in a humidified incubator at 37°C for 30 min (IgG) or 50 min (IgA). The reaction was stopped by the addition of 0.02 ml of 3 M NaOH. The absorbance of each well was read at 410 nm on a Microelisa reader (Dynatech Laboratories). Plates contained appropriate controls in duplicate, including known-positive sera, known-negative sera, and wells without antigen, antibody, or conjugate.

The optical density (OD) reading of the uncoated wells was subtracted from that of all other sample readings. The known-positive OD readings were averaged and normalized to a predetermined value. All sample readings were then normalized and extrapolated to 100 min. The OD readings are presented as geometric means (24). These were determined by using the $\log_{10}$ of each value for each rabbit to calculate the mean and standard error of the mean (SEM). Antilogs of the mean log of OD reading, 1 SEM above and 1 SEM below the mean log OD reading, represent the geometric mean with an upper and lower limit of variance about that mean.

To determine antibody titers of pooled hyperimmune sera used in passive protection experiments, the concentrated hyperimmune sera against the KSCN extract of 3:A *P. multocida* and normal rabbit sera were tested undiluted and diluted 1:10, 1:100, and twofold dilutions thereafter. The titer is defined as the highest dilution of serum with an OD reading 2 standard deviations above the mean of the negative serum.

Pathological evaluation. Rabbits that died spontaneously or were killed at the end of experiments were necropsied and examined for gross lesions. Rabbits were killed at the end of experiments by intravenous injection of an overdose of pentobarbital sodium. The nasal turbinates, lungs (all lobes), and tympanic bullae were fixed in 10% neutral buffered Formalin, embedded in paraffin, sectioned at 5 μm , stained with hematoxylin-eosin, and examined for microscopic lesions. Nasal turbinates and tympanic bullae were decalcified before embedding. Microscopic lesions in the lungs, nasal turbinates, and tympanic bullae were scored according to the following schema (19). Lungs: -, no lesions recognized; +, mild lesions characterized by thickening of alveolar septa with heterophils and/or mononuclear inflammatory cells; ++, moderate lesions characterized by inflammatory cell infiltration of alveolar and bronchiolar spaces and interstitium, with or without pleuritis; +++, severe lesions characterized by extensive necrosis, infiltration of inflammatory cells, predominately heterophils, and accumulation of inflammatory cells and fibrin in bronchi and alveoli, with or without pleuritis. Nasal turbinates: -, no lesions recognized; +, mild lesions characterized by submucosal infiltration of inflammatory cells; ++, moderate lesions characterized by migration of inflammatory cells into mucosal epithelium and nasal cavities; +++, severe lesions characterized by mucosal necrosis and accumulation of inflammatory cells, predominately heterophils. Tympanic bullae: -, no lesions recognized; +, mild lesions characterized by slight infiltration of submucosa with inflammatory cells; ++, moderate lesions characterized by extension of inflammatory cells into mucosa and a few into the tympanic cavity; +++, severe lesions characterized by mucosal erosion and

FIG. 1. RIP of ^{125}I -labeled *P. multocida* proteins with hyperimmune and normal rabbit sera. Lanes: A, ^{125}I -labeled *P. multocida* proteins precipitated by PBS; B, ^{125}I -labeled *P. multocida* proteins precipitated by concentrated pooled normal rabbit serum; C, ^{125}I -labeled *P. multocida* proteins precipitated by concentrated pooled hyperimmune rabbit serum; D, molecular weight markers (10^5); E, ^{125}I -labeled *P. multocida* whole cells. Numbers 1 to 10 identify different antigens; antigens 2, 6, and 8, the three major antigens, were 27K, 37.5K, and 64.4K, respectively.

numerous inflammatory cells extending into the tympanic cavity. To compare scores, the sum of lesion scores for each organ from individual rabbits within a group was divided by the sum of the maximum possible scores to determine the group lesion index. A group lesion index of 1.0 indicates the most severe lesions possible for a group.

Statistical analyses. Analysis of variance and Student-Newman-Keuls multiple-comparison procedure were applied to determine significant differences in lesion indices, CFU of *P. multocida* in lungs, nasal IgA, lung lavage IgG and IgA, and serum IgG among the groups. Fisher's exact test was employed to determine significant differences in mortality, prevalence of lesions, and prevalence of *P. multocida* colonization in lungs and nonrespiratory organs among the groups (28).

RESULTS

Characterization of the hyperimmune serum. The rabbit hyperimmune serum directed to the KSCN extract of 3:A *P. multocida* was characterized by measuring the ELISA antibody titers against the KSCN extract of the homologous organism. The titers for IgG and IgA were 1:409,600 and 1:10, respectively. Normal rabbit serum had an IgG antibody titer less than 1:10 and a titer of zero for IgA antibody.

An RIP and an immunoblotting method were used to characterize the *P. multocida* antigens to which the IgG antibodies in the hyperimmune serum are directed. In the

RIP assay, all labeled antigens which have corresponding antibodies in the hyperimmune serum can be detected. As shown in Fig. 1, immune serum (lane C) detected at least 10 different antigens with various molecular weights. The three major antigens had molecular weights of 27,000 (27K antigen) 37.5K, and 64.4K. Lane E represents the labeled antigens that did not react with immune serum. All 10 antigens detected by the hyperimmune serum (lane C) were also detected in the solubilized labeled *P. multocida* cells (lane E), indicating that the hyperimmune serum detected most of the labeled antigens. Lane B shows one faint 64.4K antigen detected by the concentrated normal rabbit serum. However, this antigen was not detected with nonconcentrated normal rabbit serum (data not shown). Lane A represents the results of using PBS to precipitate the labeled *P. multocida* antigens. Labeled antigens were not detected, indicating that the reaction was specific.

Since it is possible that the antigens detected by RIP may not represent all antigens which have corresponding antibodies in the hyperimmune serum, we used the immunoblotting assay to complement the RIP method for detection of antigens. In this assay, antigens are detected by separating the unlabeled antigen mixtures in SDS-PAGE, transferring to NCP, and reacting to hyperimmune serum. We used the outer membrane and the outer membrane vesicles (lithium chloride extract) of the 3:A *P. multocida* as antigens. Identical antigen profiles were observed when the outer membrane (lane A) and outer membrane vesicles (lane C) were used as antigen sources (Fig. 2). The hyperimmune serum (lanes A and C) detected 10 different antigens, with four major antigens of 29.9K, 38.5K, 40.9K, and 71K. No antigens were detected by the pooled normal rabbit serum (lanes B and D). The major outer membrane antigen (38K) was detected by the RIP and immunoblotting methods.

Clinical observations. Animals were observed daily for clinical signs of pasteurellosis. Three of the 12 group 1 rabbits became dyspneic and died during the experiments. Most of the animals in this group showed transient decreases in food and water consumption. Eight of the 12 group 2 and six of the eight group 3 rabbits died, most showing severe degrees of dyspnea, anorexia, adipsia, and lethargy. Milder signs were observed in rabbits that survived challenge. The four rabbits in group 4 showed no clinical signs of illness throughout the experimental period.

Mortality rates (Table 1) were 25, 67, and 75% for group 1 (hyperimmune serum-challenge), 2 (PBS-challenge), and 3 (normal serum-challenge) rabbits, respectively. A significant difference ($P < 0.03$) was observed between group 1 and combined groups 2 and 3, indicating that rabbit hyperimmune serum significantly protects rabbits from lethal challenge with *P. multocida*. Rabbits (group 4) given normal rabbit serum only did not die.

Pathology. The prevalence and severity of lesions in lungs, nasal cavities, tympanic bullae, and other lesions were compared among all experimental groups.

Pneumonia. The prevalences of pneumonia were 33, 83, and 88% for groups 1, 2, and 3, respectively. Since the results for groups 2 and 3 were similar, they were combined and compared with group 1. A significant reduction ($P < 0.006$) in the prevalence of pneumonia was observed in group 1 compared with groups 2 and 3.

Group lesion indices (GLI), which measure lesion severity, were also used to evaluate the protective efficacy of the hyperimmune serum. The GLI was 0.25, 0.72, and 0.75 for groups 1, 2, and 3, respectively, and significant differences were observed between groups 1 and 2 ($P < 0.05$) and groups

FIG. 2. Immunoblotting analysis of *P. multocida* membrane antigens with hyperimmune and normal rabbit sera. Outer membranes of *P. multocida* prepared by sucrose gradient centrifugation and outer membrane vesicles extracted by lithium chloride buffer were used as antigen sources. Lanes: A, outer membrane antigens revealed by incubating outer membrane of *P. multocida* with pooled hyperimmune rabbit serum; B, outer membrane antigens revealed by incubating outer membrane of *P. multocida* with pooled normal rabbit serum; C, outer membrane antigens revealed by incubating outer membrane vesicles with pooled hyperimmune rabbit serum; D, outer membrane antigens revealed by incubating outer membrane vesicles with pooled normal rabbit serum; E, molecular weight markers (10^3). Numbers 1 to 10 identify different antigens; the four major antigens (nos. 1, 3, 4, and 8) were 29.9K, 38.5K, 40.9K, and 71K, respectively.

1 and 3 ($P < 0.05$). Hyperimmune serum dramatically reduced the prevalence and severity of pneumonia in rabbits exposed to homologous challenge.

Lung lesions were graded as outlined in Materials and Methods. Ten of 12 rabbits in group 2 developed microscopic lung lesions: 1 mild (+), 2 moderate (++), and 7 severe (+++). Lung lesions were noted in seven of eight rabbits in group 3 and were essentially the same in extent: one mild (+), one moderate (++), and five severe (+++). In contrast, only 4 of 12 group 1 rabbits had lung lesions: 1 mild (+), 1 moderate (++), and 2 severe (+++). None of the group 4 rabbits had lung lesions.

Rhinitis. The prevalence and GLI of rhinitis in groups 1, 2, and 3 are shown in Table 1. Statistic analyses indicate no significant differences among the groups, indicating that the hyperimmune serum had no protective effect against *P. multocida*-induced rhinitis in rabbits.

Tympanitis. Since the group 2 and 3 rabbits had a low prevalence (10%) and GLI (0.08) of tympanitis, the efficacy

of the hyperimmune serum in preventing tympanitis cannot be evaluated.

Gross evaluation of other tissues indicated that no lesions were observed in any of the experimental groups.

Nasal colonization of virulent *P. multocida* in rabbits treated with hyperimmune serum. The efficacy of the hyperimmune serum in preventing and reducing nasal colonization of *P. multocida* was evaluated. The number of rabbits cultured at various days ranged from 1 to 12, as indicated in Fig. 3. The geometric mean CFU for an individual rabbit was calculated and logarithmically converted. The mean and SEM of the log CFU of the group at various days were then calculated and compared among the groups.

The challenge organisms colonized the nasal cavities of all rabbits in groups 1, 2, and 3 beginning on day 4 after challenge and persisting until the rabbits died or were killed at 14 days postchallenge. These data indicate that the hyperimmune serum did not prevent colonization of the virulent *P. multocida* in nasal cavities when given in large challenge doses. However, quantitative analysis of the number of organisms recovered from the nasal cavities of various groups of rabbits indicates that hyperimmune serum-treated rabbits had lower numbers of *P. multocida* than those treated with PBS or normal rabbit serum (Fig. 3), particularly at days 4 and 7 postchallenge. An approximately 10-fold reduction of *P. multocida* was observed in group 1 rabbits at days 4 and 7 compared with the corresponding PBS and normal serum-treated animals. No apparent differences ($P > 0.05$) were evident at days 8, 10, 12, and 14 postchallenge, indicating transient reduction of *P. multocida* colonization of nasal cavities.

Isolation of *P. multocida* from other tissues at necropsy. *P. multocida* is known to colonize the respiratory tract (nasal cavity and lungs) and nonrespiratory organs such as tympanic bullae, liver, spleen, and uterus of infected rabbits. The efficacy of the immune serum was evaluated further by determining the prevalence of *P. multocida* colonization in these organs at the time of necropsy.

Rabbits treated with hyperimmune serum had significantly lower prevalence and numbers of virulent *P. multocida* in the lungs (Table 2). The prevalence of colonization in lungs was 25% (3 of 12), 67% (8 of 12), and 88% (7 of 8) for groups 1, 2, and 3, respectively, a significant difference ($P < 0.05$) between groups 1 and 2 and groups 1 and 3. Quantitative analysis of lung homogenates showed that the geometric mean was reduced from 8.52×10^6 CFU/g in group 3 rabbits to 1.44×10^2 CFU/g in group 1 rabbits, i.e., treatment of rabbits with hyperimmune serum resulted in a 59,166-fold reduction ($P < 0.05$) in the number of *P. multocida* in the lungs.

As shown in Table 3, 23% (14 of 60) of the nonrespiratory tissue samples collected from group 1 rabbits were positive for virulent *P. multocida*, whereas 55% (22 of 40) of the samples collected from group 3 rabbits were positive for *P.*

TABLE 1. Effects of rabbit immune serum on the development of lesions and mortality in rabbits inoculated with virulent *P. multocida*^a

Group (treatment)	No. of rabbits in group	Pneumonia		Rhinitis		No. dead (%)
		No. of rabbits (%)	Mean GLI ^b $\pm$ SEM	No. of rabbits (%)	Mean GLI $\pm$ SEM	
1 (immune rabbit serum)	12	4 (33)	0.25 $\pm$ 0.12	3 (25)	0.14 $\pm$ 0.07	3 (25)
2 (PBS)	12	10 (83)	0.72 $\pm$ 0.11	7 (58)	0.31 $\pm$ 0.10	8 (67)
3 (normal rabbit serum)	8	7 (88)	0.75 $\pm$ 0.13	3 (38)	0.13 $\pm$ 0.06	6 (75)
4 (normal rabbit serum)	4	0 (0)		0 (0)		0 (0)

^a Each rabbit in groups 1, 2, and 3 was inoculated i.n. with 4.0×10^9 cells of virulent 3:A *P. multocida* (UT-1 strain). Rabbits in group 4 were not challenged.

^b The GLI represents the severity of the lesion of the group. A GLI of 1.0 is the most severe change possible for a group.

FIG. 3. Geometric mean CFU of *P. multocida* in nasal cavities of rabbits inoculated with hyperimmune rabbit serum (●), PBS (▲), or normal rabbit serum (○) and challenged. Nasal swab samples collected from these three groups of rabbits at days 4, 7, 8, 10, 12, and 14 p.i. were cultured on tryptic soy agar with yeast extract. Data represent geometric mean CFU and SEM; numbers in parentheses indicate numbers of rabbits tested.

multocida. The difference was significant ($P < 0.01$). Hyperimmune serum protected against the colonization of virulent *P. multocida* in nonrespiratory organs in most rabbits. *P. multocida* organisms were not isolated from group 4 rabbits, further indicating that control animals were free of preexisting *P. multocida* infection.

ELISA antibody response of rabbits to challenge. One potential effect of passive immunization with hyperimmune serum is interference with the host humoral immune response to the infecting organism. IgA ELISA antibodies in nasal secretion and lung lavage samples and IgG ELISA antibodies in lung lavage and serum samples of group 1, 2, and 3 rabbits were determined and compared to address this issue.

IgA antibody in the nasal secretions of challenged rabbits (groups 1, 2, and 3) were increased at day 14 p.i. compared with the group 4 rabbits, which were not challenged. Significantly increased ($P < 0.05$) nasal IgA antibody activity was observed between groups 1 and 4 but not between other groups. The small number of animals in groups 2 and 3 may account for the insignificant increase in nasal IgA antibody in

these two groups. Similarly, IgA antibody activities in lung lavage samples of challenged rabbits collected at day 14 p.i. were increased significantly ($P < 0.05$) compared with group 4 rabbits. The IgG antibody activities in lung lavage samples of challenged rabbits were also increased, with significant elevation ($P < 0.05$) between groups 2 and 4 and between groups 3 and 4, but not between groups 1 and 4. With regard to serum IgG antibody, significantly elevated IgG antibody activities ($P < 0.05$) were observed in sera collected at day 14 but not day 7 p.i. in group 1, 2, and 3 rabbits compared with the corresponding preexperiment samples. These results indicate that rabbits inoculated with hyperimmune serum directed to *P. multocida* and challenged homologously can mount specific immune responses to the challenge homologous *P. multocida* by production of nasal IgA, pulmonary (lung lavage) IgG and IgA, and serum IgG.

DISCUSSION

In this experiment, we demonstrated that rabbit hyperimmune serum directed to the KSCN extract of 3:A *P. multocida* protects against homologous challenge with virulent *P. multocida*. The protection is expressed at several levels. The hyperimmune serum-treated rabbits showed a reduced number of virulent challenge organisms in the nasal cavity and significant reductions in prevalence and number of virulent *P. multocida* in the lungs. The paucity of organisms in the lungs of passively immunized rabbits probably accounts for the observed significant reductions in the prevalence and severity of pneumonia. The significant reduction in the prevalence and number of *P. multocida* in lungs is probably due to the i.n.-inoculated hyperimmune serum entering lung alveolar spaces. This was confirmed by tracing the movement of hyperimmune serum in a few rabbits by using trypan blue dye. It is believed that antibodies delivered to the lungs are involved in clearance of challenge organisms.

The mechanism of action of rabbit antibody on *P. multocida* is mediated through opsonic activity by enhanced polymorphonuclear cell phagocytosis as demonstrated in vitro (1, 16, 25). Bactericidal action may also be involved in vivo, although in vitro studies (16, 25) suggest that rabbit antibody against *P. multocida* is not bactericidal. Since rabbit pasteurellosis is characterized by the accumulation of a large number of polymorphonuclear cells in the lungs, it is possible that the i.n.-inoculated hyperimmune serum is capable of enhancing killing activity of recruited polymorphonuclear cells in lungs or enhancing the phagocytosis of *P. multocida* by alveolar macrophages (2, 10).

The hyperimmune serum was administered before and after challenge to maximize the protective potential of the immune serum by eliminating or reducing the initial load of the virulent challenge *P. multocida* and any surviving organism postchallenge. Hyperimmune serum was administered

TABLE 2. Effects of rabbit immune serum on the prevalence of isolation and CFU of virulent *P. multocida* in rabbit lungs at necropsy

Group	Treatment	No. of rabbits in group	<i>P. multocida</i> in lungs		Mean ratio		
			No. of rabbits (%)	Mean CFU/g of lung $\pm$ SEM ^a	Groups compared	Ratio	P
1	Immune rabbit serum	12	3 (25)	$1.44 \times 10^2 \pm 1.38 \times 10^1$	1 and 3	59,166	$P < 0.05$
2	PBS	12	8 (67)	$3.14 \times 10^5 \pm 1.51 \times 10^1$	1 and 2	2,180	$P < 0.05$
3	Normal rabbit serum	8	7 (88)	$8.52 \times 10^6 \pm 1.26 \times 10^1$	2 and 3	27	$P < 0.2$
4	Normal rabbit serum	4	0				

^a The geometric mean CFU per gram of lung in each rabbit was calculated and converted to a logarithmic number to obtain the mean of the log geometric mean of the group. The group mean in log was then converted to geometric mean CFU per gram of lungs.

TABLE 3. Isolation of virulent *P. multocida* from nonrespiratory organs of rabbits inoculated with rabbit immune serum and challenged

P. multocida isolated (no. of rabbits)							
Group	No. of rabbits in group	Tympanic bullae		Liver	Spleen	Uterus	No. positive/total no. of samples (%)
		Left	Right				
1	12	3	4	3	3	1	14/60 (23)
2	12	4	3	3	2 ^a	1	13/59 (22)
3	8	4	2	6	6	4	22/40 (55)
4	4	0	0	0	0	0	0/20

^a Only 11 rabbits tested.

i.n. to concentrate humoral factors (antibody) in the lungs, the major target organ of *P. multocida*. It is believed that intravenous or intraperitoneal administration of hyperimmune serum may not result in concentrations of antibody in the lungs high enough to protect against infection.

It is known that specific antibody suppresses the host humoral immune response. Maternal antibodies to measles and polio viruses block the induction of antibody synthesis by the corresponding viral antigens in the young infant (5). Since the hyperimmune serum inoculated into experimental rabbits contained a high titer of IgG antibody and a negligible amount of IgA antibody, it was expected that nasal and pulmonary IgA antibody in rabbits receiving hyperimmune serum and challenged would be elevated, as in the controls. Conversely, pulmonary IgG and serum IgG antibody in rabbits receiving hyperimmune serum and challenged would be inhibited. As shown in Table 4, elevated nasal IgA antibody was observed in the hyperimmune serum-treated and challenged rabbits (group 1). This significant elevation of nasal IgA antibody was not due to the inoculated residual hyperimmune serum, since the hyperimmune serum had negligible IgA antibody. Similarly, significant elevations of pulmonary IgA antibody (lung lavage) were noted in all challenged rabbits whether they received hyperimmune serum or not. Since hyperimmune serum has a negligible amount of IgA, the elevated pulmonary IgA antibody level reflects the immune response of rabbits to the challenge virulent *P. multocida*. However, in pulmonary IgG antibody, significantly elevated IgG antibody was noted in rabbits receiving PBS or normal rabbit serum and challenged but not in rabbits receiving hyperimmune serum and challenged (group 1). The latter may result from blockage of local production of IgG in the respiratory tract by hyperimmune serum because the inoculated IgG antibody acts as an anti-immunogen (5). Since the hyperimmune serum is rich in IgG antibody and the hyperimmune serum was inoculated

intranasally, the passively administered IgG antibody must be concentrated in the lungs. Similar to the results in mice inoculated i.n. with live influenza virus (17, 20), the production of serum IgG antibody in challenged rabbits (groups 1, 2, and 3) at day 14 p.i. is most likely due to the spillover of *P. multocida* organisms replicating in the lungs to the spleen. Because passive IgG antibody was inoculated i.n., it is reasonable to predict that this passive IgG antibody is low in the serum and has no suppressive effect on the host serum IgG antibody response in group 1 rabbits after challenge.

As mentioned previously, the role of humoral antibody in protection against *P. multocida* infection in rabbits remained unknown until we performed the passive transfer experiment. These passive transfer experiments showed that hyperimmune serum containing a high level of IgG antibody protects rabbits against homologous challenge. This indicates that serum IgG antibody to *P. multocida* is involved in protection. Although IgA antibody has been demonstrated in rabbits inoculated i.n. with a streptomycin-dependent *P. multocida* vaccine (Y. S. Lu and S. P. Pakes, Abstr. Annu. Meet. Am. Assoc. Lab. Anim. Sci., 1983, abstr. 31, p. 17) and KSCN extracts of *P. multocida* (24; Lu et al., in press), its involvement in protection has not been studied. Based on our previous observation (Lu et al., in press) that rabbits vaccinated i.n. with a KSCN extract vaccine had high levels of nasal IgA antibody and survived challenge better than rabbits vaccinated intramuscularly, which had low levels of nasal IgA antibody, we believe that IgA antibody may in fact have a positive protective effect. Additional studies are needed to determine the function of nasal IgA and pulmonary IgA antibody in protecting against *P. multocida* infection in rabbits. The involvement of cell-mediated immunity in protection against *P. multocida* infection in rabbits is also unknown. Experimental studies with mice (11) indicate that cell-mediated immunity does not confer significant protection. However, in chickens, cell-mediated immunity appar-

TABLE 4. ELISA antibody responses of rabbits inoculated with rabbit immune serum, PBS, or rabbit normal serum and challenged with virulent homologous 3:A *P. multocida*

Group	Geometric mean ^a ELISA reading [no. of samples] ^b					
	Nasal IgA	Lung lavage		Serum IgG		
		IgA	IgG	Preexperiment	Day 7 p.i.	Day 14 p.i.
1	0.192 (0.22–0.168) [9]	0.199 (0.22–0.18) [4]	0.034 (0.043–0.027) [4]	0.081 (0.088–0.074) [9]	0.091 (0.108–0.076) [9]	0.231 (0.311–0.172) [9]
2	0.096 (0.126–0.072) [4]	0.215 (0.260–0.177) [6]	0.118 (0.202–0.07) [6]	0.093 (0.097–0.090) [3]	0.083 (0.124–0.056) [3]	0.280 (0.427–0.183) [3]
3	0.120 [1]	0.134 (0.168–0.108) [4]	0.094 (0.168–0.052) [4]	0.098 (0.125–0.076) [3]	0.091 (0.125–0.066) [3]	0.181 (0.258–0.127) [3]
4	0.052 (0.067–0.041) [4]	0.05 (0.006–0.004) [4]	0.015 (0.02–0.012) [4]	0.059 (0.076–0.047) [4]	0.068 (0.084–0.055) [4]	0.089 (0.128–0.062) [4]

^a The value shown is the geometric mean reading for the group. The numbers in parentheses represent the geometric mean plus 1 SEM and the geometric mean minus 1 SEM. Nasal and lung samples were obtained at necropsy (day 14 p.i.).

^b IgA was determined from undiluted nasal and lung samples, whereas IgG was determined from 1:10 dilutions of lung samples and 1:20 dilutions of serum samples.

ently confers significant protection (3). The role of cell-mediated immunity in protecting rabbits against *P. multocida* infection will be difficult to define because inbred strains of rabbits are not available.

In this study, hyperimmune serum was used to demonstrate that a humoral factor (IgG antibody) plays a role in protection against homologous challenge. However, the characteristics of the antigens directed to these protective immune sera are unknown. Therefore, identification of the immunogens which are reactive to the hyperimmune serum will be necessary to define the immunogens and possible protective immunogens of *P. multocida* in rabbits.

In summary, hyperimmune rabbit serum containing a high titer of IgG antibody directed to the KSCN extract of *P. multocida* protects against homologous challenge. It is evident that serum IgG antibody to *P. multocida* plays a role in protection.

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