A Potassium Thiocyanate Extract Vaccine Prepared from Pasteurella multocida 3:A Protects Rabbits against Homologous Challenge

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Potassium thiocyanate extracts of a virulent Pasteurella multocida 3:A rabbit isolate were prepared and used as a vaccine in rabbits. The extract contained protein, carbohydrate, hyaluronic acid, lipopolysaccharide, DNA, and RNA. The protein and lipopolysaccharide profiles of the extract were similar to those of the P. multocida cell membrane. Rabbits were vaccinated intranasally (i.n.) or intramuscularly (i.m.) four times at 1- or 3-week intervals and challenged i.n. with the homologous P. multocida 2 weeks after the last vaccination. Rabbits vaccinated with the extract by the i.n. route developed persisting serum immunoglobulin G (IgG) and nasal IgA antibodies, whereas rabbits immunized by the i.m. route produced persisting serum IgG and transient nasal IgA antibodies. The extract prevents the death of rabbits which were vaccinated by either route and challenged. Vaccination by the i.n. route in rabbits reduced the numbers of virulent P. multocida in nasal cavities and lungs and the prevalence and severity of rhinitis and pneumonia. These i.n.-vaccinated rabbits were also resistant to virulent P. multocida colonization in liver, spleen, uterus, and tympanic bullae. Similarly, i.m. vaccination in rabbits resulted in a reduction in the severity of rhinitis; the numbers of virulent P. multocida in lungs; and the prevalence of colonization in liver, spleen, uterus, and tympanic bullae. Vaccination by the i.n. route was superior to that by the i.m. route in that there was a significant reduction in the severity of pneumonia and numbers of virulent P. multocida in nasal cavities and lungs. Rabbits vaccinated with the extract without challenge showed no lesions.

Pasteurellosis caused by Pasteurella multocida is a common and serious disease of rabbits used for biomedical research. Specific studies involving respiratory, genital, and sensory systems in rabbits may be invalid because of the lesions caused by the bacteria. Furthermore, under various stresses the subclinically infected rabbits may develop severe clinical diseases and die.

Attempts to eliminate or control the disease have included use of antibiotics, the establishment of pasteurella-free colonies, development of genetically resistant strains of rabbits, and vaccines. The development of an effective vaccine is most attractive because of the failure of antibiotics to control rabbit pasteurellosis (14), the emergence of penicillin-resistant strains of P. multocida (34), and the infeasibility of establishing and maintaining pasteurella-free rabbits in most biomedical institutions. Vaccines should be useful in preventing transmission between conventional and pasteurella-free rabbits housed in the same facility.

Presently, there are no effective vaccines available commercially to control rabbit pasteurellosis. We (16) and others (6, 26) have reported that both streptomycin-dependent P. multocida 3:A and 12:A vaccines are potential candidates for controlling experimental rabbit pasteurellosis. The disadvantage of these live mutant vaccines is that the mutant may revert to the pathogenic wild type, causing disease and creating carriers. Some mutants, while conferring a degree of protection, cause mild to moderate pneumonia (16), although it is thought that this is a transient problem.

Promising alternatives to live P. multocida vaccines are subcellular vaccines. The potassium thiocyanate (KSCN) extract of P. multocida is a subcellular vaccine containing various subcellular components. The vaccine confers protection in chickens (10), mice (23), and cattle (22). Mice immunized with the KSCN extract of P. multocida survived challenge infections with homologous organisms, and the protection was superior to that of killed whole-cell vaccines. Cattle immunized with the KSCN extract of P. multocida developed an enhanced clearance of P. multocida and P. hemolytica (22). Furthermore, cross-protection was demonstrated against two serotypes of P. multocida in chickens vaccinated with the KSCN extract (10). All these data suggest that the KSCN extract contains a P. multocida antigen(s) which stimulates protection in mice and chickens and, possibly, in cattle. Results of a recent study (29) in rabbits, in which a toxigenic isolate of P. multocida (capsule D) was used, indicated that the KSCN extract of this isolate is protective against homologous challenge. The protection may have been directed to the P. multocida exotoxin, which is unique to the toxigenic strain of P. multocida as well as to the bacterium.

The present study was designed to evaluate the protective efficacy of the KSCN extract of a P. multocida 3:A isolate against homologous challenge in rabbits. This isolate was chosen because capsular type A P. multocida is the predominant P. multocida isolate in rabbits (4, 7, 17) and may provide broader protection overall. Intranasal (i.n.) and intramuscular (i.m.) immunization routes were used. Intranasal administration of antigens in mammals is known to produce local respiratory immune responses which are associated with protection against P. multocida (29) and other respiratory pathogens such as influenza virus (32) and Mycoplasma pneumoniae (11). The protection offered by i.n.
immunization of KSCN extracts of *P. multocida* was compared with that offered by the i.m. route, which is easier to perform in rabbits.

**MATERIALS AND METHODS**

**Animals.** Pasteurella-free New Zealand White female rabbits (weight, 2.7 to 3.6 kg) were purchased from Hazleton-Dutchland Laboratories, Denver, Pa. Before vaccination, nasal cavities of each rabbit were cultured twice for *P. multocida*, and the sera were tested for the presence of *P. multocida* immunoglobulin G (IgG) antibodies by using an enzyme-linked immunosorbent assay (ELISA). Rabbits free of *P. multocida* and *P. multocida* IgG antibodies were used in these experiments. Experimental rabbits were separated completely from other rabbits to avoid cross contamination.

BALB/c mice (weight, 20 to 25 g) were purchased from Jackson Laboratory, Bar Harbor, Maine. These mice were known to be free of mouse microbial pathogens including viruses, bacteria, mycoplasma, and parasites.

**Source and serotype of *P. multocida*.** Wild-type *P. multocida* UT-1 was used in these studies. It was isolated from a rabbit with supplicative rhinitis. The isolate belongs to serotype 3:A and was maintained by lyophilization.

**Experimental design.** A total of 46 New Zealand White rabbits were divided into six groups as follows: group 1 (14 rabbits) was vaccinated i.n. and challenged; group 2 (7 rabbits) was vaccinated i.m. and challenged; group 3 (16 rabbits) was not vaccinated and was challenged; group 4 (3 rabbits) was vaccinated i.n. and was not challenged; group 5 (3 rabbits) was vaccinated i.m. and was not challenged; group 6 (3 rabbits) was not vaccinated and not challenged. Results of a preliminary study showed that rabbits immunized i.n. with KSCN extracts of *P. multocida* 3:A were better protected than rabbits immunized i.m. Therefore, fewer rabbits were used in group 2. Each rabbit in groups 1 and 4 was inoculated i.n. with 1 mg of protein of the KSCN extract in 1 ml of phosphate-buffered saline (PBS) on days 0, 7, 14, and 35. Rabbits were vaccinated four times, because in a previous study (16) it was shown that rabbits immunized i.n. three times with a *P. multocida* 3:A mutant were protected against homologous challenge; the fourth immunization was added to compensate for the nonreplicating nature of the KSCN extract antigen. Similarly, each of the rabbits in groups 2 and 5 were immunized i.m. in the semimembranous-semitendinous muscles with 1 mg of protein of the KSCN extract mixed with complete Freund adjuvant on day 0 and 1 mg of protein of KSCN mixed with incomplete Freund adjuvant on days 7, 14, and 35. Results of a previous study in rabbits (33) indicated that a similar immunization protocol produces good antibody responses against *P. multocida*. Rabbits in groups 1, 2, and 3 were challenged i.n. with virulent *P. multocida* 3:A 2 weeks after the last vaccination and humanly killed 2 weeks later. Rabbits in groups 4 and 5 served as vaccine controls, and rabbits in group 6 served as normal controls.

**Preparation of protective antigen(s) from *P. multocida* 3:A.** The protective antigen of *P. multocida* was prepared by extraction with KSCN, as described previously (20). Briefly, *P. multocida* was grown on sheep blood agar plates, washed twice with PBS (pH 7.2), suspended in a solution of 0.5 M KSCN–0.8 M NaCl (pH 6.3), and incubated in a rotary shaker at 37°C for 5 h. The cells were removed by centrifugation at 27,000 × g for 30 min. The supernatant fluid was filtered (0.80 μm pore size; AA; Millipore Corp., Bedford, Mass.), concentrated with an Amicon apparatus (Amicon Corp., Lexington, Mass.) with a membrane (YM-10), and dialyzed extensively against 0.01 M PBS (pH 7.2). A portion of the extracts was inoculated onto blood agar plates to test for sterility.

**Chemical analyses of the KSCN extract.** The KSCN extract was analyzed chemically to determine the concentration of protein, carbohydrate, hyaluronic acid, lipopolysaccharide (LPS), DNA, and RNA.

(i) **Protein determination.** Total protein of the extract used for vaccine studies was determined by the method described by Bradford (3) by using a Bio-Rad reagent (Bio-Rad Laboratories, Richmond, Calif.).

(ii) **Carbohydrate determination.** Total carbohydrate was determined by the phenol-sulfuric acid reaction (9).

(iii) **Hyaluronic acid determination.** Hyaluronic acid levels were determined by the procedure described by Dische (8) by using purified human umbilical cord hyaluronic acid (Sigma Chemical Co., St. Louis, Mo.) as a standard.

(iv) **Estimation of LPS content.** The amount of LPS present in the KSCN extract was determined with 2-keto-3-deoxyoctonate as a marker by the method described by Karkhanis et al. (15). However, hydrolysis was carried out at 100°C for 20 min, which are the optimum conditions. An average of 0.57% 2-keto-3-deoxyoctonate in the LPS preparations of *P. multocida* isolated from rabbits (18) was used to calculate the LPS content in the extract.

(v) **DNA content estimation.** The colorimetric reaction of DNA with diphenylamine (5) was used to estimate the DNA content. Calf thymus DNA was used as the standard.

(vi) **RNA content estimation.** The reaction of RNA with acidified orcinol (13) was used to estimate the RNA content. Yeast RNA served as the standard.

**SDS-PAGE analysis of the KSCN extract.** The characteristics of the protein and LPS in the KSCN extract were further determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and compared with the cell membrane of *P. multocida* prepared by lithium chloride extraction (12).

**Preparation of membrane vesicles of *P. multocida*.** The lithium chloride extract of *P. multocida* was prepared by using the methods for membrane vesicle extraction of *Haemophilus influenzae* (12).

**Preparation of LPSs of *P. multocida*.** LPS was extracted from *P. multocida* and *P. multocida* by using a hot phenol-water procedure described previously for the extraction of *P. multocida* LPS (27).

**SDS-PAGE analysis for proteins.** An adaptation of the procedure described by Ames (2) was used for SDS-PAGE analysis for proteins. The samples were submitted to 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 carried out at 30 mA of constant current per gel at 4°C until the pyronin Y staining dye entered the separating gel, and then the constant current was increased to 45 mA per gel. Protein contents of samples were determined by the method described by Bradford (3) with the Bio-Rad reagent. Samples were boiled with digestion buffer (62.5 mM Tris [pH 6.8], 2% [wt/vol] SDS, 50% [vol/vol] glycerol) containing 5% (vol/vol) 2-mercaptoethanol before they were loaded onto the gel. Proteins on the gel were stained with Coomassie blue.

**SDS-PAGE analysis for LPS.** The method for SDS-PAGE analysis for LPS described by Manning et al. (19), using a 15% separating gel containing 4 M urea, was used to analyze the LPS preparations obtained from *P. multocida* and KSCN.
extracts of *P. multocida*. The gel was stained with a silver stain preparation (31).

**Inoculation of virulent *P. multocida*.** Wild-type *P. multocida* 3:A strain UT-1 organisms were grown from a lyophilized stock and inoculated into mice. The *P. multocida* cells were reisolated from the heart blood of infected mice and grown in brain heart infusion broth with shaking at 37°C for 6 h. The *P. multocida* cells were then washed three times with 0.01 M PBS (pH 7.2), serially diluted, and plated onto tryptic soy agar with 0.3% yeast extract. The inoculum contained 5.0 x 10^8 organisms per ml. Rabbits in groups 1, 2, and 3 were inoculated i.n. with the challenge organisms (1 ml per rabbit) by using Pasteur pipettes.

**Isolation of *P. multocida* from challenged rabbits.** To determine the effect of the KSCN vaccine on the nasal colonization by virulent *P. multocida*, nasal swabs were collected from rabbits in groups 1, 2, and 3 at 2- or 3-day intervals immediately after challenge until the animals died or were killed. Samples collected from both nostrils of each rabbit by using 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 titrated onto plates containing tryptic soy agar with 0.3% yeast extract in triplicate at each dilution. Plates were then incubated at 37°C for 24 to 48 h. The frequency and number of virulent *P. multocida* in nasal cavities were compared among the three groups of rabbits. At necropsy, bacterial cultures were collected from nasal cavities, left and right tympanic bullae, lungs, liver, spleen, uterus, and tissues with gross lesions. Each sample was inoculated onto a blood agar plate, and the plate was incubated at 37°C for 24 to 48 h. Parts (1.22 to 1.32 g) of the left diaphragmatic lobe of the lungs in each rabbit were collected aseptically and homogenized in PBS to make a 10% (w/vol) suspension. The lung suspensions were diluted 10-fold and plated onto blood agar plates to determine the number of CFU per gram of lung tissue.

**Collection of nasal secretions.** Nasal secretions from rabbits immunized i.m. or i.n. with the KSCN extract were collected prior to and at 10, 17, 24, 31, 40, 46, and 49 days after the first vaccination to determine the presence of IgA antibody by the ELISA. Nasal secretions were collected by a procedure established previously (29).

**ELISA.** The antigen used for ELISA was the KSCN extract of *P. multocida* 3:A. Alkaline phosphatase-conjugated goat anti-rabbit IgG (gamma chain specific; Miles Laboratories, Inc., Elkhart, Ind.) or goat anti-rabbit secretory IgA (alpha chain and secretory component specific; Cooper Biomedical, Westchester, Pa.) was diluted in 0.05 M Tris buffer with 1% bovine serum albumin—0.02% NaCl—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 titrations. Sera diluted 1:20 in PBS-Tween 20 (PBST; 0.01 M PBS, 0.05% Tween 20 [pH 7.2]) were used for the IgG ELISA antibody determination, and undiluted nasal secretions were used for the IgA ELISA determination.

A 0.15-ml volume of antigen in coating buffer (0.015 M Na_2CO_3, 0.035 M NaHCO_3, 0.02% NaCl [pH 9.60]) was added to either the rigid polystyrene U plate (catalog no. 1-220-24x; Dynatech Laboratories, Inc., Alexandria, Va.) for the IgG ELISA or the Immuno I U plate (catalog no. 011-010-3550; Dynatech) for the IgA ELISA. The plates were incubated overnight at 4°C. The plates were washed four times with PBST, whereas IgA plates were washed five times with PBST. A 0.15-ml volume of the test material (nasal secretion or serum) was added in duplicate to each well. The plates were covered, incubated at room temperature for 2 h, and washed four or five times with PBST. A 0.15-ml fraction of diluted conjugate was added, and the plate was incubated at room temperature for 3 h. The plates were washed with PBST and 0.15 ml of p-nitrophenyl phosphate substrate was added. 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 A405 of each well was read on a Microelisa reader (Dynatech). Each plate contained appropriate controls in duplicate, including a known positive serum sample; a known negative serum sample; and wells without antigen, antibody, or conjugate.

The optical density (OD) reading of the uncoated wells was subtracted from 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 (29). These were determined by using the log 10 of each value for each rabbit to calculate the mean and standard error of the mean. A log of the mean log of the OD reading, 1 standard error of the mean above and 1 standard error of the mean below the mean log of the OD reading, represent the geometric mean with an upper and lower limit of variance about that mean.

**Pathological evaluation.** Rabbits that died spontaneously or that 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 turbinate, lungs (all lobes), tympanic bullae, liver, spleen, and uterus were fixed in 10% neutral buffered Formalin, embedded in paraffin, divided into 5-μm-thick sections, stained with hematoxylin-eosin, and examined for microscopic lesions. Nasal turbinate and tympanic bullae were decalcified before they were embedded. Microscopic lesions in the lungs, nasal turbinates, and tympanic bullae were scored according to the following schema (16): for lungs, −, no lesions recognized; +, mild lesions characterized by thickening of alveolar septa with heterophils, mononuclear inflammatory cells, or both; ++, 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; for 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; for 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 the mucosa and a few into the tympanic cavity; ++++, severe lesions characterized by mucosal erosion and numerous inflammatory cells extending into tympanic cavity. To compare results among the various groups of rabbits, the sum of lesion scores for each organ from individual rabbits within a group was divided by the minimum possible scores to arrive at the group lesion index. A group lesion index of 1.0 was the most severe change possible for a group.

**Toxicity of the extract.** Toxicity of the sterile KSCN
RESULTS

Characterization of KSCN extract of P. multocida 3:A. The chemical characteristics of the extract were determined by chemical analysis and SDS-PAGE analysis.

Chemical analyses of the extract. The KSCN extract of the P. multocida 3:A used for i.n. or i.m. vaccinations was composed of protein (2 mg/ml), carbohydrate (462.5 µg/mg of protein), hyaluronic acid (1,700 µg/mg of protein), DNA (105 µg/mg of protein), and RNA (100 µg/mg of protein). In addition, the extract also contained LPS (2.51 mg of LPS per mg of protein) by using 2-keto-3-deoxyoctonate as a marker.

SDS-PAGE analysis. The extract was characterized further by SDS-PAGE analysis for the presence of membrane proteins and LPS. The protein profile of the KSCN extract was similar to that of membrane vesicles of the same organism prepared by lithium chloride extraction (Fig. 1). The KSCN extract contained the 37,500-molecular-weight outer membrane protein (Fig. 1, arrow) which is the predominant outer membrane protein of P. multocida 3:A.

Purified LPS obtained from P. multocida and the KSCN extracts of P. multocida were analyzed by SDS-PAGE. The presence of LPS in the KSCN extract (Fig. 2, lane A, arrow) as compared with the purified LPS obtained from P. multocida whole cells (Fig. 2, lane B) was observed. The purified P. multocida LPS has characteristics similar to those of rough mutants of Salmonella minnesota (Fig. 2, lanes D and E). Our LPS purified from P. multocida whole cells also showed a similar gel pattern, as reported previously (19).

Clinical observations. None of the 14 group 1 rabbits (i.n. vaccinated and challenged) developed clinical signs of dyspnea and anorexia, whereas 1 of the 7 group 2 rabbits (i.m. vaccinated and challenged) became dyspneic and anorexic 3 days prior to death. In contrast, 10 of the 16 group 3 rabbits (not vaccinated and challenged) were dyspneic and anorexic 2 to 4 days before death. None of the rabbits in groups 4, 5, or 6 became dyspneic or anorexic.

Mortality was used to evaluate the efficacy of the vaccine. Mortality was 0% in group 1 rabbits, 14% in group 2 rabbits, and 63% in group 3 rabbits (Table 1). There was a significant difference (P < 0.01) between groups 1 and 3 and between groups 1 and 2 combined (5% mortality) and group 3 (P < 0.001). None of the rabbits in groups 4, 5, or 6 died. These data indicate that the KSCN extract of P. multocida 3:A
TABLE 1. Prevalence and group lesion indices and mortality of rabbits immunized with the KSCN extract and challenged with the virulent *P. multocida* 3:A
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<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of rabbits</th>
<th>Pneumonia</th>
<th>Rhinitis</th>
<th>Typanitis</th>
<th>Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Prevalence</td>
<td>Lesion index</td>
<td>Prevalence</td>
<td>Lesion index</td>
</tr>
<tr>
<td>1</td>
<td>i.n. vaccinated; challenged</td>
<td>14</td>
<td>4/14 (28)</td>
<td>0.09</td>
<td>1/14 (7)</td>
<td>0.02</td>
</tr>
<tr>
<td>2</td>
<td>i.m. vaccinated; challenged</td>
<td>7</td>
<td>5/7 (71)</td>
<td>0.47</td>
<td>1/7 (14)</td>
<td>0.05</td>
</tr>
<tr>
<td>3</td>
<td>Not vaccinated; challenged</td>
<td>16</td>
<td>15/16 (94)</td>
<td>0.83</td>
<td>11/16 (69)</td>
<td>0.34</td>
</tr>
<tr>
<td>4</td>
<td>i.m. vaccinated; not challenged</td>
<td>3</td>
<td>0/3</td>
<td>0</td>
<td>0/3</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>i.m. vaccinated; not challenged</td>
<td>3</td>
<td>0/3</td>
<td>0</td>
<td>0/3</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>Not vaccinated; not challenged</td>
<td>3</td>
<td>0/3</td>
<td>0</td>
<td>0/3</td>
<td>0</td>
</tr>
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</table>

* Each of the rabbits in groups 1, 2, and 3 were inoculated with 5.0 × 10⁶ cells of the virulent *P. multocida* 3:a strain UT-1.

* Number positive/number sampled: values in parentheses are percentages.

** Represents the severity of the lesion of the group. A lesion index of 1.0 is the most severe change possible for a group.

Protects rabbits from the lethal effects of *P. multocida* challenge in all i.n. vaccinated and challenged rabbits and in most i.m. vaccinated and challenged rabbits.

**Serum IgG antibody response.** Rabbits vaccinated with KSCN extracts by the i.m. or the i.n. route developed IgG antibodies against the KSCN extract of *P. multocida* (Fig. 3). Significantly (P < 0.05) elevated IgG antibody activity was noted in i.m. and i.n. vaccinated rabbits at day 17 after the first vaccination. The elevated IgG antibody activity persisted through day 49, when the animals were killed. Although IgG antibody activity was detected in i.m. and i.n. vaccinated rabbits, the immune response was more profound in i.m. vaccinated rabbits (P < 0.05).

**Nasal IgA antibody response.** Rabbits immunized with KSCN extract by the i.m. or the i.n. route also developed nasal IgA antibodies (Fig. 4). However, nasal IgA antibody activity was much lower than serum IgG antibody activity. Significantly (P < 0.05) elevated IgA antibody activity was evident in i.n. vaccinated rabbits at day 31 after the first vaccination. Kinetic studies revealed that the IgA antibody activity in i.m. vaccinated rabbits peaked at day 24 after the first immunization and then gradually returned to the prevaccination level. In contrast, the elevated IgA antibody activity in i.n. vaccinated rabbits persisted throughout the experiment, with dramatic increases at days 46 and 49 after the first vaccination.

**Pathology.** The KSCN extract was evaluated for protection against development of pneumonia, rhinitis, and typanitis.

![FIG. 3. Geometric mean serum IgG antibody directed to the KSCN extract of *P. multocida*. Eight rabbits each were immunized with the KSCN extract of *P. multocida* 3:A by the i.m. (C) or the i.n. (●) route at days 0, 7, 14, and 35 (arrows). Four rabbits were not immunized and served as controls (■). The upper limits of variance are indicated.](http://iai.asm.org/)

![FIG. 4. Geometric mean nasal IgA antibody directed to the KSCN extract of *P. multocida*. Eight rabbits each were immunized with the KSCN extract of *P. multocida* 3:A by the i.m. (C) or the i.n. (●) route at days 0, 7, 14, and 35 (arrows). Four rabbits were not immunized and served as controls (■). The upper limits of variance are indicated.](http://iai.asm.org/)
rabbits of pneumonia showing rabbit cells mononuclear extracts KSCN 2972 LU/um.

FIG. 5. Section of lung from an i.n. vaccinated and challenged rabbit showing a mild lesion, characterized by accumulation of mononuclear cells in blood vessels and alveolar spaces. Bar, 100 µm.

Pneumonia. The i.m. or the i.n. vaccination of rabbits with KSCN extracts reduced the prevalence and group lesion index of pneumonia as compared with that in nonvaccinated animals (Table 1). Of the 14 group 1 rabbits, 4 had very mild (+) lung lesions (Fig. 5). The lungs of the remaining 10 rabbits of the group 1 animals were normal. Five of the seven group 2 rabbits developed microscopic lung lesions; two mild (+), one moderate (++), and two severe (+++). The lungs of the remaining two group 2 rabbits were normal. Of the 16 group 3 rabbits, 15 had microscopic lung lesions: 1 was mild (+), 3 were moderate (++), and 11 were severe (+++)(Fig. 6). A significant difference in the prevalence of pneumonia was observed between rabbits in groups 1 and 3 (P < 0.001), but not between other groups. Overall, i.n. vaccinated rabbits (group 1) had a tendency to develop lung lesions less frequently than did the i.m. vaccinated rabbits (group 2). Similarly, a significant difference in the group lesion index was observed between groups 1 and 3 but not between the other groups. However, the lesion indices of 0.09 and 0.47 (groups 1 and 2, respectively) were different at P < 0.06. These data indicate that the KSCN extract protects against the development of pneumonia and that the i.n. route is more effective than the i.m. route of immunization. No lung lesions were noted in rabbits in groups 4, 5, or 6. Figure 7 illustrates the normal lung of a group 4 rabbit which was i.n. vaccinated without challenge.

Rhininitis. Vaccination of rabbits with KSCN extracts by the i.m. or the i.n. route reduced the prevalence of rhinitis caused by P. multocida. One rabbit each in groups 1 and 2 developed mild (+) rhinitis. The remaining 13 and 6 rabbits were normal for groups 1 and 2, respectively. Of the 16 group 3 rabbits, 11 showed microscopic lesions: 8 were mild (+), 1 was moderate (++), and 2 were severe (+++). The remaining five group 3 rabbits were normal. The prevalence of rhinitis was significantly different (P < 0.005) between

FIG. 6. Section of lung from a nonvaccinated and challenged rabbit showing severe, necrotizing bronchopneumonia. Bar, 50 µm.
groups 1 and 3 but not between groups 2 and 3 ($P < 0.10$). Similarly, the group lesion indices were significantly different ($P < 0.05$) between groups 1 and 3 and between groups 2 and 3 but not between groups 1 and 2. These data indicate that the i.m. or the i.n. route of immunization with a KSCN extract is effective in reducing the prevalence and severity of rhinitis. None of the rabbits in groups 4, 5, or 6 developed rhinitis.

Tympanitis. Only 1 of the 16 rabbits in group 3 and none of the rabbits in groups 1 and 2 developed tympanitis (Table 1); thus, the efficacy of vaccination in preventing Pasteurella tympanitis could not be evaluated. None of the rabbits in groups 4, 5, or 6 developed tympanitis.

Nasal colonization of virulent *P. multocida* in vaccinated and nonvaccinated rabbits. One criterion that is used to evaluate the efficacy of a vaccine is whether the vaccine can prevent or reduce colonization by challenge organisms in the nasal cavity of vaccinated rabbits. Sequential nasal swabs were collected at 2, 3, 4, 5, 7, 9, 11, and 14 days after i.n. challenge; and the log CFU of the group was calculated and compared.

The results indicate that the challenge organisms colonized the nasal cavities of all the tested rabbits in groups 1, 2, and 3, beginning on day 2 after challenge and persisting until the rabbits died or were killed at 14 days postchallenge. These data indicate that the KSCN extract administered i.m. or i.n. to rabbits does not prevent the colonization of virulent *P. multocida* in nasal cavities of vaccinated rabbits. However, quantitative analyses of the numbers of organisms recovered from various groups of rabbits show that i.n. vaccinated rabbits (group 1) had significantly lower numbers of *P. multocida* in the nasal cavities as compared with the nonvaccinated and challenged group 3 rabbits at day 3 ($P < 0.001$), day 5 ($P < 0.005$), and day 7 ($P < 0.05$) postchallenge (Table 2). Generally, vaccination of rabbits with a KSCN extract by the i.m. route resulted in a reduction of 96-fold (day 3), 105-fold (day 5), and 12-fold (day 7) of the organisms in their nasal cavities as compared with the corresponding group 3 rabbits. Rabbits vaccinated with a KSCN extract by the i.m. route (group 2) also had lower numbers of virulent challenge *P. multocida* in their nasal cavities but were not significantly different from the corresponding group 3 rabbits.

![FIG. 7. Section of lung from an i.n. vaccinated and nonchallenged rabbit showing normal lung morphology. Bar, 100 μm.](image)

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

**Vaccination.** Virulent *P. multocida* organisms were isolated at necropsy from the nasal cavity (14 of 14) and lungs (2 of 14) of the group 1 rabbits (Table 3) and from the nasal cavity (7 of 7), lungs (4 of 7), and right tympanic bullae (1 of 7) of the group 2 rabbits. In contrast, virulent *P. multocida* organisms were recovered from the nasal cavity (16 of 16), lungs (13 of 16), left (3 of 16) and right (4 of 16) tympanic bullae, liver (5 of 16), spleen (1 of 16), and uterus (3 of 16) of the group 3 rabbits. The combined colonization prevalence of *P. multocida* in liver, spleen, uterus, and left and right tympanic bullae was 0% (0 of 70), 2.8% (1 of 35), and 20% (16 of 80) for rabbits in groups 1, 2, and 3, respectively, and was significantly different between groups 1 and 3 ($P < 0.001$) and between groups 2 and 3 ($P < 0.05$). Similarly, the prevalence of 1% (1 of 105) of the vaccinated group (groups 1 and 2) was significantly different ($P < 0.001$) from the 20% prevalence of the nonvaccinated group (group 3). Rabbits vaccinated with a KSCN extract by the i.m. or the i.n. route were protected from colonization of virulent *P. multocida* in lungs, tympanic bullae, liver, spleen, and uterus but not in the nasal cavity.

Vaccination, especially i.n., significantly reduced the prevalence of *P. multocida* colonization in lungs. The prevalences (Table 3) were significantly different ($P < 0.001$) between groups 1 and 3. Furthermore, quantitative analysis of lung homogenates showed that the geometric mean of *P. multocida* per gram of lungs was significantly different among the three groups of rabbits. The geometric mean (CFU per gram) was reduced from $1.11 \times 10^6$ in group 3 rabbits to 2.77 in group 1 rabbits; i.e., i.n. vaccination resulted in a 400,722-fold reduction ($P < 0.001$) in the number of *P. multocida* in lungs. Similarly, i.m. vaccination resulted in an 81-fold reduction in the number of *P. multocida* in lungs ($P < 0.001$) as compared with that in group 3 rabbits. Furthermore, i.n. vaccination was approximately 5,000-fold more effective that i.m. vaccination in reducing the number of *P. multocida* in lungs ($P < 0.001$). Results of these experiments indicate that the KSCN extract vaccine effectively reduces the prevalence of colonization and the number of virulent *P. multocida* in lungs. Furthermore, i.n. vaccination of the rabbits is superior to i.m. vaccination in reducing the prevalence of colonization and CFU in lungs.

**DISCUSSION**

Results of this study demonstrate that a KSCN extract vaccine prepared from a *P. multocida* 3:A isolate is safe and effective against experimental rabbit pasteurellosis. The protective efficacy of the extract was superior to that of other *Pasteurella* vaccine preparations. Of the rabbits vaccinated with a killed whole-cell *P. multocida* vaccine, 59%...
TABLE 2. Number of *P. multocida* in nasal cavities of rabbits vaccinated with KSCN extracts and challenged with virulent *P. multocida* 3:A, strain UT-1<sup>a</sup>

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>CFU/rabbit on the following days after intranasal inoculation of <em>P. multocida</em>&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>1</td>
<td>i.n. vaccinated; challenged</td>
<td>4.87 ± 0.28</td>
</tr>
<tr>
<td>2</td>
<td>i.m. vaccinated; challenged</td>
<td>5.07 ± 0.19</td>
</tr>
<tr>
<td>3</td>
<td>Not vaccinated; challenged</td>
<td>5.10 ± 0.31</td>
</tr>
<tr>
<td>4</td>
<td>i.n. vaccinated; not challenged</td>
<td>NT</td>
</tr>
<tr>
<td>5</td>
<td>i.m. vaccinated; not challenged</td>
<td>NT</td>
</tr>
<tr>
<td>6</td>
<td>Not vaccinated; not challenged</td>
<td>NT</td>
</tr>
</tbody>
</table>

<sup>a</sup> Each of the rabbits in groups 1, 2, and 3 were inoculated intranasally with 5.0 × 10<sup>8</sup> cells of the virulent *P. multocida* 3:A strain UT-1.

<sup>b</sup> Values are means ± standard error of the mean of the log of the geometric mean CFU per rabbit. The geometric mean CFU in each rabbit (two nasal swabs per rabbit) was calculated and converted to the logarithmic number, and the mean of the log of the geometric mean of the group was then calculated. Values in parentheses are the numbers of samples tested.

<sup>c</sup> NT, Not tested.

subsequently died after being naturally infected with *P. multocida* (1). In our studies, the mortality rates were 0 and 14% in rabbits vaccinated i.n. and i.m., respectively, with the KSCN vaccine and challenged. A live streptomycin-dependent mutant vaccine (16) was found to be highly protective against lethal homologous challenge. Neither the KSCN extract vaccine nor the streptomycin-dependent mutant vaccine caused lung lesions in rabbits. However, the KSCN preparation appeared to be superior to the streptomycin-dependent mutant vaccine in protecting against homologous challenge. In the present study, 28% (4 of 14) of the vaccinated and challenged rabbits had lung lesions, and the group lesion index was 0.09. In studies with the streptomycin-dependent mutant vaccine, 67% (six of nine) of the vaccinated and challenged rabbits had lung lesions, and the group lesion index was 0.26. Furthermore, it appeared that the KSCN extract prevented lung colonization by virulent *P. multocida* to a greater extent than did the streptomycin-dependent mutant. No doubt, the greatest advantage of the KSCN extract is its nonviable antigenic nature. While the streptomycin-dependent mutant vaccine shows great promise, the possibility of reversion to its virulent wild type exists.

Results of a previous study (29) showed that a KSCN extract of a capsular type D, exotoxin-producing rabbit *P. multocida* isolate conferred protection against experimental homologous challenge. Whether the protection is a result of neutralization of the exotoxin, immunological interference with the organism, or both is not known. Exotoxin-producing *P. multocida* is not commonly associated with naturally occurring rabbit pasteurellosis (28). Our isolate (serotype 3:A) was negative for exotoxin in mice (30), and the KSCN extract of the isolate was not toxic to rabbits at a level of 4 mg of protein. Inoculation of the KSCN preparation from the exotoxin-producing isolate by the i.n. route caused a mild pneumonia in rabbits, characterized by alveolar thickening due primarily to mononuclear inflammatory cell infiltration. No lesions were noted in the i.n. vaccinated and nonchallenged rabbits in our study.

Chemical analyses indicated that our KSCN extracts had

TABLE 3. Isolation of *P. multocida* from nasal cavities and lungs of rabbits vaccinated with the KSCN extract and challenged with virulent *P. multocida* 3:A, strain UT-1<sup>a</sup>

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Prevalence in nasal cavity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Lungs</th>
<th>Group comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Prevalence&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Mean ± SEM&lt;sup&gt;c&lt;/sup&gt;</td>
<td>CFU/g&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>1</td>
<td>i.n. vaccinated; challenged</td>
<td>14/14 (100)</td>
<td>2/4 (14)</td>
<td>0.442 ± 0.320</td>
</tr>
<tr>
<td>2</td>
<td>i.m. vaccinated; challenged</td>
<td>7/7 (100)</td>
<td>4/7 (57)</td>
<td>4.136 ± 1.477</td>
</tr>
<tr>
<td>3</td>
<td>Not vaccinated; challenged</td>
<td>16/16 (100)</td>
<td>13/16 (81)</td>
<td>6.044 ± 0.810</td>
</tr>
<tr>
<td>4</td>
<td>i.n. vaccinated; not challenged</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>i.m. vaccinated; not challenged</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>Not vaccinated; not challenged</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Each of the rabbits in groups 1, 2, and 3 were inoculated i.n. with 5.0 × 10<sup>8</sup> cells of the virulent *P. multocida* 3:A strain UT-1.

<sup>b</sup> Number of positive/number sampled: values in parentheses are percentages.

<sup>c</sup> Mean of the log of geometric mean CFU per gram of lung tissue. The geometric mean CFU per gram of lung in each rabbit was calculated and converted to the logarithmic number. The mean of the log of the geometric mean of the group was then calculated.

<sup>d</sup> The group mean in logarithmic units was converted to the geometric mean CFU per gram of lung tissue.
large amounts of LPS and hyaluronic acid and approximately equal amounts of carbohydrate, DNA, and RNA compared with those obtained previously (24). Since we used a different strain of *P. multocida* and a different growth medium (dextrose starch agar), it was expected that the extracts would differ in chemical composition. The high content of hyaluronic acid in our extracts may have been related to the growth of *P. multocida* in dextrose starch agar. The KSCN extract protein profile determined by SDS-PAGE analysis was similar to that of *P. multocida* membrane vesicles, indicating that the KSCN extract contains cell membrane vesicles. Whether protein or LPS of the extract plays the most important role in protection remains uncertain. Results of one study in mice indicate that the major protective immunogen is protein, whereas the LPS component plays a very minor role (25). However, other investigators (21) reported that the KSCN extracts containing LPS are more immunogenic in mice and chickens than are those without LPS.

Data from this study showed that protection is significantly better in rabbits vaccinated i.n. than in rabbits vaccinated i.m., indicating that the route of antigen administration is important in controlling the invading *P. multocida*. We suggest that the i.n. route be used for immunization with KSCN extracts of *P. multocida* to maximize protection against rabbit pasteurellosis.

The mechanism(s) of protection observed in this study is not yet known. Immunological results indicate that i.m. vaccinated rabbits develop persisting serum IgG antibody and transient nasal IgA antibody, whereas i.n. vaccinated rabbits elicit persisting serum IgG and nasal IgA antibodies. These results suggest that the persisting nasal IgA antibody correlates with the better protection observed in i.n. vaccinated rabbits. The significant reduction of *P. multocida* in respiratory tracts of i.n. vaccinated and challenged rabbits is probably related to the production of nasal IgA antibody. An antigen administered i.n. is known to produce local immunity, including secretory IgA, which is capable of preventing colonization of bacteria in the respiratory tract (35). Similar to our results, the KSCN extracts of a toxigenic strain of *P. multocida* inoculated i.n. into rabbits induced IgA antibody in nasal washings (29). Results of recent studies in our laboratory (Y.-S. Lu, L. Massey, and S. P. Pakes, Annu. Meet. Am. Assoc. Lab. Anim. Sci., abstr. no. 18, 1985) indicate that rabbit immune serum directed to a KSCN extract of *P. multocida* is protective against homologous i.n. challenge, strongly suggesting the protective role that serum antibodies play against rabbit pasteurellosis. It appears that serum IgG and nasal IgA antibodies both play some role in protection against rabbit pasteurellosis.

In conclusion, a KSCN extract of *P. multocida* 3:A elicits significant protection against homologous experimental challenge. The protection is manifested by a reduction in colonization by virulent *P. multocida*, a reduction of lesion development, and abrogation of the lethal effects of virulent *P. multocida*. The KSCN extract vaccine appears to be safe when inoculated by the i.n. or the i.m. route.

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LITERATURE CITED


