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Immunization of rabbits against a bacterial pathogen with an alginate microparticle vaccine

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Abstract

Pasteurella multocida is an important bacterial pathogen of domestic rabbits. To evaluate the ability of a thiocyanate extract (PTE) of *P. multocida* to stimulate an immune response and protect against infection with *P. multocida*, rabbits were immunized subcutaneously or intranasally on Days 7, 21 and 35. Cholera toxin, a potent mucosal adjuvant, was included in one treatment group. Rabbits immunized subcutaneously (SC) or intranasally (IN) had significant increases in serum anti-PTE IgG but not IgA. In contrast, only rabbits immunized IN with PTE developed significant titers of nasal lavage anti-PTE IgA and cholera toxin significantly enhanced this response. In a second study rabbits were immunized via the drinking water with PTE incorporated into alginate microparticles on Days 7, 14 and 21. Mild increases in serum IgG were noted in rabbits immunized with PTE in microparticles, with or without cholera toxin, and this increase was significant ($P \leq 0.05$) on Day 21 for rabbits receiving PTE and cholera toxin. Nasal lavage anti-PTE IgA was significantly ($P \leq 0.05$) increased in rabbits immunized orally with PTE, with or without cholera toxin, in microparticles. This effect was not enhanced by cholera toxin. Rabbits orally immunized with PTE in microparticles had significantly fewer colony forming units of homologous *P. multocida* recovered from the lungs and nasopharynx following an intranasal challenge. These results demonstrate that PTE incorporated into alginate microparticles and administered orally is immunogenic and confers protective immunity.

Keywords: Microparticles; Alginate; *Pasteurella multocida*; Rabbit

1. Introduction

Pasteurella multocida colonizes the upper respiratory tract and is the most common bacterial pathogen of domestic rabbits. While infection is often subclinical, disease characterized by rhinitis, pneumonia, abscessation of viscera and subcutaneous sites, metritis, orchitis, septicemia and otitis interna is common [1].

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Chemotherapeutic measures are often effective for infectious diseases, including lapine Pasteurellosis [2,3], but treatment is often hampered by expense, limited supply of some antimicrobial compounds and non-compliance by patients or animal owners with respect to scheduled medication. In contrast, vaccination is widely viewed as a means to induce long lasting immunity in large numbers of individuals at relatively little expense and without the need for repeated self-medication.

Vaccination typically involves administering antigens parenterally, usually intramuscularly. This route of immunization stimulates the immune system to produce IgG antibody in the serum but fails to generate a mucosal antibody response. In contrast, vaccines that are delivered either orally or intranasally stimulate IgA antibody response along the mucosal surfaces of the gastrointestinal or respiratory tracts. IgG facilitates the phagocytosis of bacteria and activation of complement whereas IgA principally acts by preventing attachment of bacteria and colonization. For *P. multocida*, a number of preparations have been examined as antigens for immunization of rabbits, including lipopolysaccharide [4], outer membrane proteins [5] and potassium thiocyanate extracts [6,7] by intramuscular or subcutaneous routes of immunization.

Oral immunization presents the possibility of vaccinating large numbers of individuals quickly and cheaply. In addition, the hesitation some individuals may experience at the prospect of receiving an injection may be eliminated with oral vaccination. Orally administered vaccines must be presented in a way that critical antigenic epitopes are protected from enzymatic degradation in the digestive tract. Similar concerns exist with respect to maximizing antigen preservation and delivery to tissues of the immune system for vaccines delivered by the intramuscular, subcutaneous or intranasal routes.

Microencapsulation is a unique way to protect antigens and facilitate their uptake into lymphoid tissue such as gastrointestinal Peyer's patches in the case of orally administered vaccines [8]. One of the most common materials used to encapsulate antigens is poly(D,Llactide-co-glycolide) (DL-PLG) [9]. Although DL-PLG microparticles are taken up by Peyer's patches, where the antigen is then processed by the immune system, their production requires organic solvents and high temperatures which could threaten the integrity of fragile antigens. Furthermore, natural degradation of DL-PLG microparticles is accompanied by a concomitant decrease in pH which may adversely affect the immunogenicity of antigens.

Sodium alginate is a natural polysaccharide derived from seaweed and which forms a gel when mixed with divalent cations. Alginate has been used for incorporation of β-islet cells for the experimental treatment of diabetes [10]; reversal of hyperglycemia using encapsulated pancreatic islets in mice [11]; treatment of wounds by incorporation of antibiotics [12], and basic growth factor [13]; incorporation and inoculation of plasmid DNA to mice [14]; and regeneration of transected spinal cord [15] and amputated axons [16] in rats. The study described here was undertaken to evaluate the utility of alginate microspheres for the delivery of antigens as a vaccine to prevent disease in rabbits caused by *P. multocida*.

2. Materials and methods

2.1. Animals

Rabbits used in this study were *Pasteurella*-free New Zealand white males weighing 1.9–2.3 kg (Covance, Inc., Kalamazoo, MI). The *Pasteurella*-free status was confirmed by nasal lavage sample culture before experimentation was begun. Use of rabbits for this study was approved by the Institutional Animal Care and Use Committee. Rabbits were housed singly in stainless steel cages. The rooms were maintained at 15–16.7 °C and were illuminated on a 12-h light/dark cycle. Rabbits were given a daily ration of 110 g of commercial rabbit feed (Lab Rabbit Chow HF 5326; Purina Mills, Inc., Richmond, IN) and tap water was supplied through a water bottle and sipper tube.

2.2. Immunogens

Potassium thiocyanate extract (PTE) of *P. multocida* (serotype 3, 12, 15:D) was prepared as previously described [6]. Briefly, *P. multocida* was grown to confluence on tryptic soy agar containing

5% sheep blood and harvested in 6 ml of equal parts saline and 1.0 M KSCN. Following incubation at 37 °C for 6 h, whole cells were removed by centrifugation at 8000 *g* for 10 min, and the supernatant was dialyzed extensively against 0.01 M Tris–hydrochloride–0.32 M NaCl–0.01% NaN_3 buffer (pH 8.0). The extract was then concentrated with a Centriprep-10 concentrator (Amicon, Inc., Beverly, MA) and sterilized by a passage through a 0.22 μm filter.

Cholera toxin (CT) and aluminum hydroxide ($Al(OH)_3$) were obtained from Sigma Chemical Co. (St. Louis, MO).

2.3. Preparation of alginate microparticles

Alginate microparticles containing 5 mg of PTE protein/ml were prepared as previously described [17]. Briefly, sodium alginate, medium viscosity (Sigma Chemical Co., St. Louis, MO) was dissolved in distilled water at a 2% w/v concentration with constant stirring. PTE was added to the alginate solution to create a final alginate concentration of 1.2% w/v. Additional batches were similarly made to prepare microparticles which contained either no added protein or PTE with 200 μg/ml of CT. The mixture was placed into a syringe pump (Harvard Instruments, South Natick, MA) and infused into an atomizer (Turbotak, Inc., Ontario, Canada). The alginate/PTE mixture was then sprayed into a 1.5% $CaCl_2$ solution placed 50 cm from the tip of the atomizer. Particles were separated from the $CaCl_2$ by low-speed centrifugation and placed in a solution of 0.1% poly-L-lysine (mean MW 180 000; Sigma Chemical Co., St. Louis, MO) with stirring for 20 min. The size of the alginate microparticles ranged from less than 1 to 20 μm in diameter, with a mean size of approximately 15 μm as measured by a Microtrak particle analyzer (Northwales, PA). The particles were sonicated to reduce clumping, and washed twice with PBS to remove non-bound poly-L-lysine.

2.4. Immunization of rabbits and sample collection

Two studies were conducted; the first involved immunization of rabbits with PTE subcutaneously (SC) and intranasally (IN) to demonstrate that an immune response to PTE could be elicited by both parenteral and mucosal routes of immunization; the second involved immunization of rabbits orally through the drinking water to evaluate the ability of alginate microparticles to facilitate a protective immune response to PTE given in the drinking water. In both studies, rabbits were randomly assigned to treatment groups.

In the first study, groups of five rabbits were dosed with either PTE (IN), PTE with CT (IN), or PTE plus the adjuvant $Al(OH)_3$ (SC). In addition, rabbits dosed with only saline (IN) or $Al(OH)_3$ (SC) served as controls. IN doses were administered into the nares via a 22-gauge stainless steel ball-tipped rodent gavage needle in a volume of 1 ml. IN doses contained 1 mg of PTE protein with or without 20 μg of cholera toxin. SC doses were administered via a 22 gauge hypodermic needle into the nape of the neck in a volume of 1 ml. SC doses contained 1 mg of PTE protein either with or without $Al(OH)_3$. Rabbits were immunized on Days 7, 21 and 35.

In the second study, groups of five rabbits were immunized by placing 5 ml of alginate microparticles with either no added protein, 5 mg of PTE protein/ml, or 5 mg of PTE protein/ml plus 200 μg of cholera toxin/ml in the drinking water at Days 7, 14 and 21. Water was withheld for 12 h prior to immunization, and microparticles mixed in 20 ml of tap water to ensure that the full dose of the vaccine would be ingested. All rabbits ingested the full vaccine dose within 2 h and were then offered ad libitum access to additional fresh water. Other groups of rabbits were orally immunized with 5 mg of soluble PTE, with or without 1 mg of CT, in the drinking water, and one group was immunized IN with 1 mg of soluble PTE in a volume of 1 ml. In addition, one group was dosed with 20 ml of saline by ingestion as a nonimmunized control.

Serum was collected by aural venipuncture. Nasal lavage was performed by instilling 2.0 ml of sterile saline into one nostril using a ball-tipped rodent gavage needle. With the rabbit's head directed slightly downward, the effluent was collected from the contralateral nostril into a sterile petri dish. For the first study, samples were collected on Days 0, 14, 28 and 42. For the second study, samples were collected on Days, 0, 16 and 21.

2.5. Bacterial challenge of rabbits

To evaluate the efficacy of immunization against infection with *P. multocida*, rabbits were challenged with homologous bacteria. Groups of rabbits were immunized orally as before and inoculated IN with 1.0×10^6 colony forming units (CFUs) of homologous *P. multocida* in 1.0 ml of saline on Day 16. In addition, a group was immunized with PTE IN and challenged. A non-immunized, non-challenged group was included.

2.6. Bacterial culture of tissues

One week after inoculation with *P. multocida*, rabbits were humanely euthanized with an intravenous overdose of sodium pentobarbital, and the lungs and nasopharynx were cultured for *P. multocida*. Lung samples were collected and weighed, an equivalent amount (w/v) of sterile saline was added, and each organ macerated with a stomacher (Seward Medical, London, UK). One ml of the tissue supernatant was added to 9 ml of sterile saline and vortexed. Serial dilutions of the samples were plated on blood agar plates and grown overnight at 37 °C. The numbers of CFUs of *P. multocida* per gram of tissue were recorded. The identity of organisms recovered from cultures was confirmed with the API 20E system (Biomerieux, St. Louis, MO). The nasopharynx of each rabbit was vigorously swabbed, the swab vortexed at high speed for 15 s in 2.0 ml of sterile PBS, and 100 μl of 1:10 to 1:10 000 ten-fold dilutions of this suspension cultured. Colonies of *P. multocida* were enumerated following 24 h of growth.

2.7. Enzyme-linked immunosorbent assay (ELISA)

Serum and nasal lavage samples were assayed for anti-PTE IgG and IgA activity by ELISA as previously described [7]. Briefly, polystyrene microtiter wells were each coated with 10 μg of PTE protein. Immediately before testing the samples, the PTE was removed, and the wells were washed with phosphate buffered saline (PBS) containing 0.5% Tween 20. Nasal lavage samples were assayed undiluted, whereas serum samples were serially diluted from 1:100 to 1:6400 diluted in PBS–Tween 20 and assayed in duplicate. Samples were also assayed in uncoated wells to control for nonspecific absorption. Four hours after the samples were added, the wells were washed thoroughly with PBS–Tween 20. Solutions containing goat anti-rabbit IgG and IgA diluted to 1:20 000 with PBS–Tween 20 were added to the wells. After overnight incubation and thorough washing, rabbit anti-goat IgG conjugated to alkaline phosphatase, diluted to 1:1000 in PBS–Tween 20, was added to each well. Following further incubation and washing, substrate (*p*-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO) was added to the wells and the optical density at 405 nm measured 15 min later in a V_{max} microplate reader (Molecular Devices, Inc., Menlo Park, CA). Results are reported as end-point antibody titers. The titer was designated the reciprocal of the last dilution of sample more than two standard deviations from the mean of negative control samples. To normalize the data and permit valid statistical evaluation, each value was transformed to the geometric $\log_2$ value.

2.8. Statistical analysis

Multivariate analysis of variance (MANOVA) was used to analyze between- and within-group differences for all data. A P value of <0.05 was considered significant.

3. Results

3.1. Evaluation of PTE as an immunogen

ELISA analysis of serum samples demonstrated increased IgG anti-PTE titers in rabbits immunized IN or SC with PTE beginning with minimal increases at Day 14 and progressing to a strong response by Day 42 (Fig. 1). By Day 28 activity in these groups was significantly greater than non-immunized controls. On Day 42, anti-PTE IgG activity in the serum was significantly ($P\leq0.001$) greater in rabbits immunized SC compared to all other groups except those immunized IN with PTE/CT. No group had appreciable anti-PTE IgA activity in the serum (data not shown).

ELISA analysis of nasal lavage samples demonstrated that rabbits immunized IN with PTE/CT had

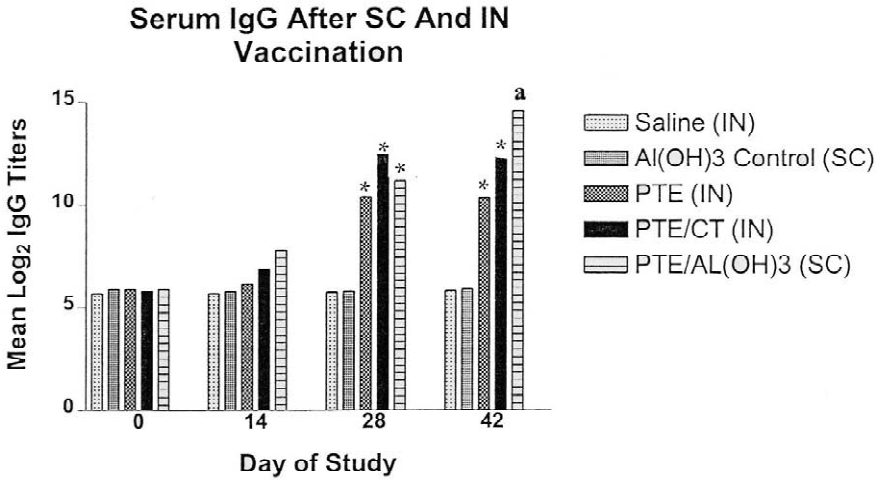

Fig. 1. Serum IgG response following IN or SC vaccination. Rabbits vaccinated IN or SC had significantly greater (designated by *) titers than other groups beginning at Day 28. Rabbits immunized with PTE SC had significantly greater titers than other groups (designated by 'a') only on Day 42.

significantly ($P \leq 0.001$) greater anti-PTE IgA titers than all other groups at Days 28 and 42 (Fig. 2). Rabbits immunized IN with PTE had significantly ($P \leq 0.001$) greater anti-PTE IgA titers on Day 42 than groups immunized with saline or those immunized subcutaneously. Other groups did not have appreciable anti-PTE IgA activity and no group had appreciable anti-PTE IgG activity in nasal lavage samples (data not shown).

3.2. Stimulation of antibody response by oral immunization

Rabbits immunized orally with PTE or PTE/CT

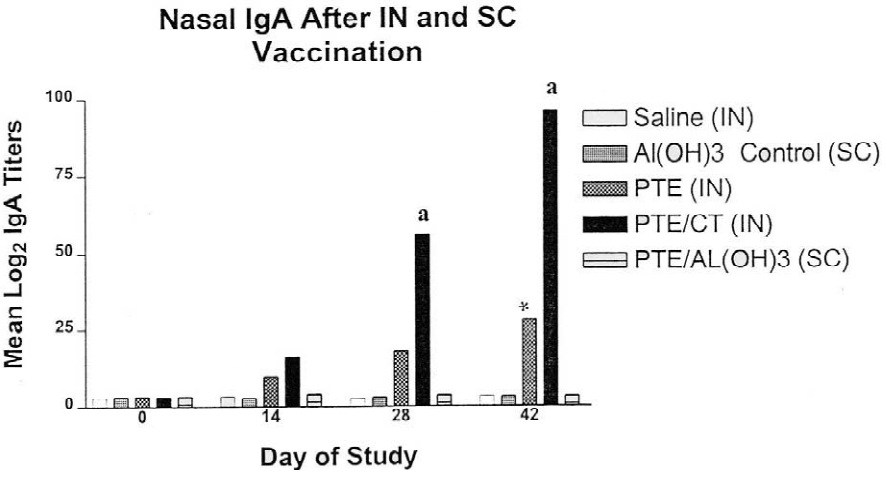

Fig. 2. Nasal IgA response following IN or SC vaccination. Rabbits immunized IN with PTE only had significantly greater (designated by *) titers than other groups on Day 42, except for rabbits immunized with PTE+CT. Rabbits immunized with PTE+CT had significantly greater titers than all other groups (designated by 'a') on Days 28 and 42.

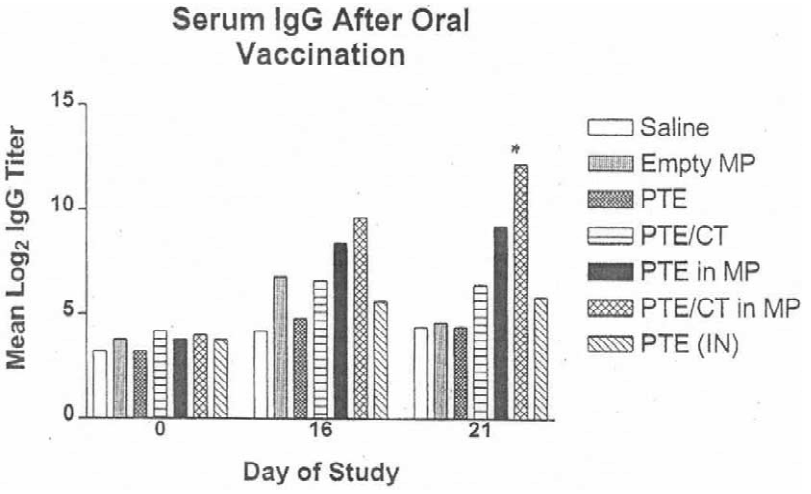

Fig. 3. Serum IgG response following oral vaccination. Rabbits immunized with PTE+CT in microparticles had significantly greater (designated by *) titers than all other groups on Day 21 only.

encapsulated in alginate microparticles had low anti-PTE IgG titers by Day 16 and continuing through Day 21 (Fig. 3). There was no significant difference in titer between groups except on Day 21, when rabbits immunized with PTE/CT in microparticles had an increased titer. There was no detectable serum anti-PTE IgA in any of the groups (data not shown). However, rabbits immunized orally with PTE or PTE/CT in microparticles or IN with PTE had a significant ($P \leq 0.05$) increase in anti-PTE IgA by Day 21 (Fig. 4). The greatest increase was in rabbits immunized orally with PTE/CT in microparticles. There was no significant difference in titers between groups immunized orally with PTE in

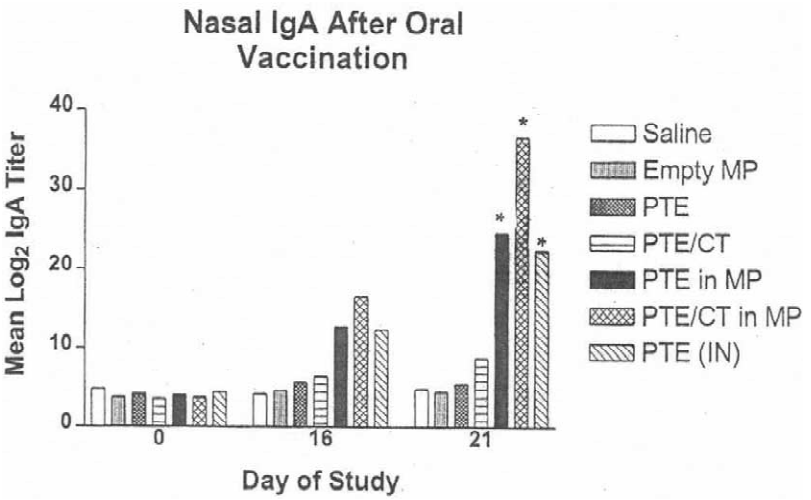

Fig. 4. Nasal IgA response following oral vaccination. Rabbit orally immunized with PTE in microparticles or IN with PTE had significantly greater (designated by *) titers than all other groups on Day 21 only.

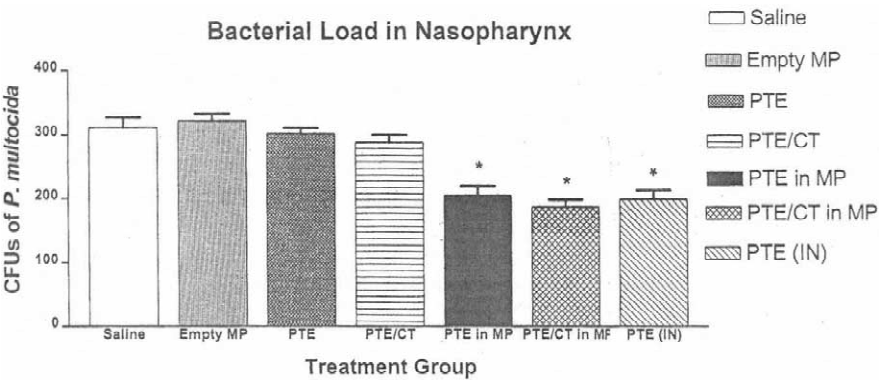

Fig. 5. Nasopharyngeal *P. multocida* burden after oral vaccination and challenge. Rabbits immunized orally with PTE in microparticles or IN with PTE had significantly fewer (designated by *) *P. multocida* colony forming units cultured from the nasopharynx than all other groups. Addition of CT to the vaccine did not yield additional significant protection.

microparticles or those immunized IN with PTE. None of the rabbits had nasal lavage anti-PTE IgG (data not shown).

3.3. Bacterial culture after oral immunization and intranasal challenge

The bacterial load of *P. multocida* was evaluated for all groups following challenge. In the nasopharynx, significantly ($P \leq 0.01$) fewer colony forming units of *P. multocida* were cultured from rabbits immunized orally with PTE or PTE/CT in microparticles or IN with PTE compared to all other groups (Fig. 5). Similarly, significantly ($P \leq 0.01$) fewer CFUs of *P. multocida* were cultured from the lungs of rabbits immunized orally with PTE or PTE/CT in microparticles or IN with PTE compared to all other groups (Fig. 6). These results demonstrate that oral immunization with PTE encapsulated in alginate microparticles can protect rabbits against a homologous bacterial challenge.

4. Discussion

Infectious pathogens remain an important cause of

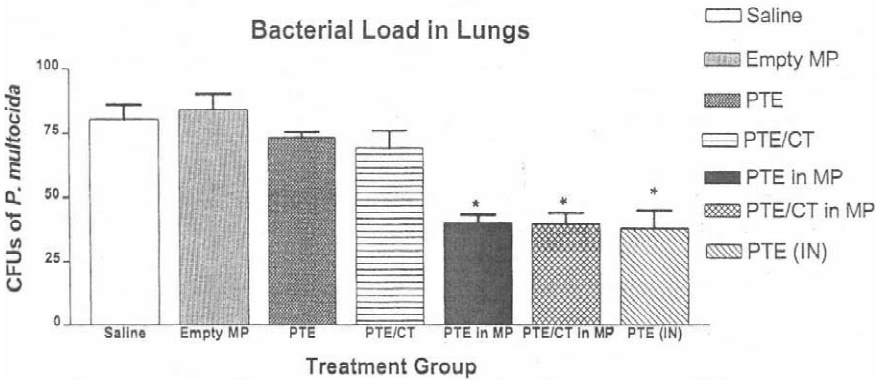

Fig. 6. Pulmonary *P. multocida* burden after oral vaccination and challenge. Rabbits immunized orally with PTE in microparticles or IN with PTE had significantly fewer (designated by *) *P. multocida* colony forming units cultured from the lungs than all other groups. Addition of CT to the vaccine did not yield additional significant protection.

morbidity in both humans and animals. While vaccination is a useful approach to the prevention of such disease, parenterally administered vaccines often fail to induce protective immunity to pathogens such as *P. multocida* which initially access the body at mucosal surfaces [1,18].

The mucosal immune system is functionally linked, so that exposure to an immunogen at one mucosal surface often results in an immune response to that immunogen at multiple mucosal sites [19]. This relationship facilitates oral vaccination against a respiratory pathogen such as *P. multocida*. Oral vaccination is advantageous in that it is a quick and painless means of immunizing a large number of individuals. Likely, compliance with vaccination will be greater for a vaccine that can be consumed orally as opposed to one that must be injected. A disadvantage of orally administered vaccines is the risk that immunogens will be destroyed in the harsh gastric milieu where low pH and enzymes degrade antigens. For this reason, approaches to protect immunogens during gastric transit would be valuable.

A unique approach to protect antigens from destruction within the gastrointestinal tract is to incorporate them into microparticles. Various polymers have been shown effective in delivery of antigens to intestinal Peyer's patches, resulting in strong local immunity in mice, monkeys and rabbits [20–23]. The study described here demonstrates the immunogenicity of an extract of *P. multocida* and the ability of this extract to induce protective immunity when administered via an alginate microparticle delivery system in the drinking water of rabbits.

Antibody activity was stimulated against PTE following SC and IN administration. Likewise, oral administration of PTE encapsulated in alginate microparticles resulted in strong nasal IgA anti-PTE activity. Oral vaccination with encapsulated PTE stimulated protective immunity to infection with *P. multocida* as evidenced by significantly lower bacterial loads in the lungs and nasopharynx. Taken together, these data show that PTE is an effective immunogen and can be used as an orally administered vaccine when encapsulated in alginate microparticles.

Cholera toxin and cholera toxin B-subunit adjuvancy has been described for a variety of gastrointestinal [24–28] and respiratory [29,30] tract immunogens, including *P. multocida* heat-labile toxin [31]. Coadministration of CT greatly enhanced the nasal IgA anti-PTE activity after IN vaccination. The increase in activity when CT was included as an adjuvant for PTE during oral immunization was less striking, possibly because the microparticles behaved as a strong adjuvant and any additional adjuvant property supplied by CT was not significant.

In summary, alginate microparticles were shown to be an effective oral delivery system for a vaccine to *P. multocida* in rabbits. Future studies will need to document the utility of this approach to other pathogens and in other species.

5. Conclusion

PTE is an immunogenic preparation that confers protective immunity to a bacterial pathogen when incorporated into alginate microparticles and administered through the drinking water.

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