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Oral vaccination with alginate microsphere systems

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Abstract

Oral vaccination is a simple, efficient way of inducing immunity at mucosal surfaces. The slow development of oral vaccines has been mainly due to the lack of suitable delivery systems. We have used hydrogel microspheres to deliver various vaccines to several animal species by oral administration. Oral delivery of vaccines using alginate microspheres elicited the production of secretory IgA (sIgA) at the mucosal surfaces in mice, rabbits, and cattle. Oral vaccination of chicken resulted in an increased delayed-type hypersensitivity, a cell-mediated immune response, indicating a positive response to the vaccine. Our studies have clearly shown that alginate microspheres are effective for the oral administration of vaccines.

Keywords: Oral vaccination; Alginate microsphere; Mucosal immunity; Secretory IgA

1. Introduction

Immunization is an efficient and cost-effective technology for the prevention of various diseases. While the benefits of immunization are invisible, immunization can be considered the best medicine for many diseases [1]. Historically, immunization has relied on the induction of humoral immunity by parenteral administration of vaccines. Antibodies induced in this manner, however, do not necessarily reach mucosal surfaces where most infectious agents enter the host. At mucosal sites, secretory IgA (sIgA) is the predominant antibody isotype present. sIgA prevents the attachment of bacteria and viruses to mucosa, and neutralizes viruses and toxins that can damage the host. Since mucosal immunity provides the first line of immunological defense, protective immunity should ideally consist of antibodies or cells that are active at mucosal sites. Induction of immunity at mucosal surfaces requires administration of antigen directly to the mucosal site. For example, to prevent a lower respiratory infection, a vaccine would need to be administered by aerosol into the lung. While mucosal immunization is highly

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desirable, it has not been widely used mainly due to a lack of understanding of the mechanisms of mucosal immunity.

Despite the advantages of oral vaccination, the development of oral vaccines has been slow. Oral administration of antigens must overcome several challenges. In the absence of suitable delivery systems, most oral vaccines, with the exception of cholera toxin (CT) and its nontoxic B-subunit pentamer moiety, undergo degradation by bacteria and enzymes present from the mouth to the lower gastrointestinal (GI) tract. Furthermore, dilution and loss of antigen in ingesta as well as poor diffusion through the mucus layer on intestinal villi prevent antigen from being taken up by the lymphoid tissue. These events result in limited absorption of antigens, which in turn results in insufficient immune responses [2]. Some animals offer further anatomic challenges. Ruminants have a 4-compartment stomach which antigens must traverse to reach the gut-associated lymphoid tissue (GALT) in the small intestine. Ruminal microflora (in the first stomach) can degrade protein before it has a chance to reach the GALT. In birds, the crop and ventriculus (gizzard) could also retain vaccines and break them down mechanically or enzymatically before they could reach the intestinal lymphoid tissue. To overcome these problems, various orally administered immunogenic delivery vehicles have been developed to deliver vaccine-relevant antigens to the GALT. Oral vaccines can then stimulate specific secretory immunoglobulin A (sIgA) for protection against pathogens at a variety of mucosal sites [3].

Antigen delivery systems can be divided into two classes: live, replicating vectors and nonreplicating antigen carriers. Antigen carriers are easier to handle and are expected to have fewer complications than live vectors. Commonly used nonreplicating antigen carriers are microparticles, liposomes, oils, polymeric matrices, proteosomes, immune-stimulating complexes, conjugates of antigens with cholera toxin and its B subunit, and lectins [4–8]. Each carrier has its own advantages and limitations. We have used hydrogels to overcome problems associated with oral delivery of vaccines. The effectiveness of the hydrogel vaccine delivery systems has been tested in four species of animals. Since oral vaccination is based on stimulation of GALT in the intestinal tract, it is worthwhile to briefly review how oral vaccines elicit mucosal as well as humoral immune responses.

2. Mechanism of oral vaccination

Mucosal immunity is stimulated by administering antigen directly to the mucosal site where an infection begins. Antigen is processed by mucosa-associated lymphoid tissue (MALT). Major concentrations of MALT are found in the upper respiratory (nasal associated lymphoid tissue or NALT), and perhaps the lower respiratory (bronchus associated lymphoid tissue or BALT), as well as in the gastrointestinal (gut associated lymphoid tissue or GALT) tracts [9,10]. Antigen is processed in MALT in inductive sites where plasma cell precursors are induced. Thus, administration of vaccines to MALT by either oral, intranasal, or intrabronchial administration results in protective immunity at other mucosal sites [2,11]. For the purpose of this paper, we will focus on uptake of antigen in MALT by the Peyer's. Peyer's patches are collections of lymphoid tissue containing B and T lymphocytes and macrophages, and are found in the duodenum, jejunum and ileum. Lymphoid tissue analogous to Peyer's patches can be found in the large intestine including the rectum. Specialized epithelial cells in the dome region of the patch, which are called follicle associated epithelial or microfold (M) cells, pick up antigen and transport it into the underlying lymphoid tissue. Antigen is processed and stimulates antigen-specific B lymphocytes (plasma cell precursors) in the germinal centers of follicles located beneath domes. These B lymphocytes are influenced by $CD3^+$, $CD4^+$, $CD8^-$ T helper to preferentially produce IgA [12–14].

The IgA plasma cell precursors do not mature and produce antibody in the Peyer's patches. Instead they leave the sites of antigen uptake and processing and traffic through local draining lymph nodes, such as the mesenteric lymph nodes (MLN) that drain the GI tract (Fig. 1). Further differentiation and maturation occurs in the lymph nodes before the lymphocytes enter the lymph, and eventually, the general circulation. Plasma cell precursors enter the blood and migrate to mucosal sites. Most plasma cells return to the organ where immune response was initiated. However, a significant number of plasma cells also

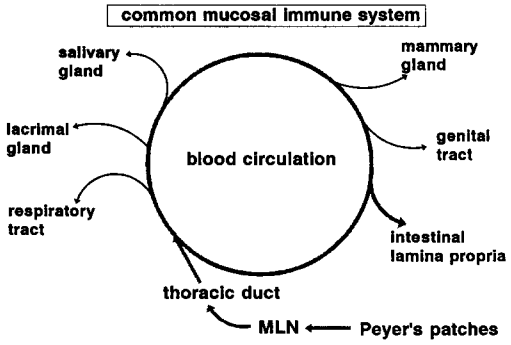

Fig. 1. Schematic description of common mucosal immune system. MLN, mesenteric lymph node.

migrate to other mucosal sites. The homing of lymphocytes to mucosal sites is not fully understood. However, special receptors on high endothelial venule cells and lymphocytes (selectins and integrins) are thought to interact to allow extravastion of lymphocytes selectively into the lamina propria of mucosal sites [15]. The lamina propria is the effector site of the mucosal immune system where plasma cells produce primarily IgA, and T cell-mediated responses occur.

Oral administration of vaccines generally requires large doses of antigen due to inefficient stimulation of GALT. Antigens are altered by the environment of the GI tract and diluted in the ingesta. Even if antigens reach the GALT in a form that can be recognized by the immune system, not all antigens stimulate the Peyer's patch equally well. This is due in part to the varying efficiency with which antigens bind to or are picked up (endocytosed) by the M cells. This makes administration of most antigens highly inefficient. The acid of the stomach and enzymatic activity of the GI tract can degrade or alter antigens. Mucus that covers the intestinal surface can prevent attachment and uptake of antigen by M cells. Yet there are certain viruses [16], bacteria [17], bacterial toxins [18], lectins [19], and protozoa [20] that have a great affinity for either intestinal epithelial cells or the glycocalyx or surface receptors of M cells. The uptake of antigen by M cells can be enhanced by binding antigen to microbiological products or other substances that promote attachment to M cells. The enhanced attachment and uptake of antigen will stimulate a greater immune response to the antigen [21]. However, the antigens are still susceptible to the pH and enzymatic environment of the GI tract. Protective encapsulation of antigens is a logical improvement in oral vaccine delivery.

Encapsulated microparticles protect antigens and provide a means of delivery to Peyer's patches. Particle size influences the uptake by M cells as well as the character of the immune response. Particles that are less than 1 μm in diameter appear to intercalate within the glycocalyx of M cells more easily than larger particles and are therefore more likely to be taken up [22]. Particles less than 5 μm may be transferred to the draining lymph nodes and spleen and stimulate both a mucosal and a systemic immune response. Particles that are 5–10 μm tend to remain in Peyer's patches to stimulate primarily a mucosal immune response [22]. Particles larger than 10 μm are not likely to be taken up at all [23]. The hydrophobicity of materials that constitute microparticles has been shown to directly correlate to the uptake Peyer's patches. The more hydrophobic, the better a material is taken up [24].

Poly(lactide-co-glycolide) microparticles have been used to encapsulate a variety of antigens for oral administration [23–25]. This material was used since it is biocompatible and used in suture materials for humans and animals. The lactide-glycolide copolymer system is not without disadvantages. Production of microparticles requires temperatures and reagents that could damage labile antigens. Modified live organisms cannot be encapsulated in this system. Therefore, the search for better polymers and delivery systems continues. We have shown that hydrogels can be used to orally deliver antigens in livestock. Polymethacrylic acid (PMA) hydrogels that are loaded with a bacterial culture supernatant induced pulmonary immunity in cattle that protected them from challenge with virulent organisms [26,27]. PMA hydrogels are effective, but cannot be used for incorporation of viable organisms. We are interested in developing a delivery system that can be used for multiple species of animals for delivery of any type of antigen, viable viruses, bacteria, as well as labile antigens. We have been using alginate microspheres for oral delivery of various types of antigens to different animals. Alginate microparticles are easy to prepare and antigens do not degrade during microparticle formation since it is done in aqueous

solution at room temperature. In this paper we describe results of the oral administration of antigens encapsulated in sodium alginate microspheres to a variety of animal species.

3. Experimental methods

3.1. Preparation of microspherical hydrogels

Sodium alginate was used to prepare microspherical hydrogels. Sodium alginate was mixed with vaccine and sprayed into water containing $CaCl_2$. Upon interaction with Ca^{2+}, alginate molecules crosslink to form hydrogels. The size of the alginate spheres ranged from less than 1 μm to larger than 30 μm in diameter, but the mean diameter of spheres was about 15 μm as measured by Microtrak Particle Analyzer (Northwales, PA). The formed alginate microspheres were further stabilized by poly(L-lysine). The alginate spheres were washed with buffer solution and stored in buffer containing $CaCl_2$ until use. Alginate microspheres were kept up to 6 months before use.

3.2. Vaccination of mice

BALB/c female mice bred at the Biological Science Animal Facility at Purdue University were used in these studies. Mice were housed in the Biological Science Facility with free choice feed and water for the duration of the studies. Three mice per group were administered two 100 μg doses of ovalbumin in microspheres 2 weeks apart either by subcutaneous or oral administration. One group of mice was given two oral doses of ovalbumin containing microspheres at 2 week intervals following a subcutaneous (SC) priming dose of ovalbumin. Groups were also included that had been inoculated by SC immunization two times 2 weeks apart with ovalbumin adjuvanted to either Freund's or aluminum hydroxide. Serum was collected at day 0 and 2 weeks after the final inoculation and assayed by ELISA for ovalbumin specific IgA and IgG antibodies. At 4 weeks the mice were killed, spleens were collected, and splenic lymphocytes were isolated. The splenic lymphocytes were tested by ELISPOT assay to determine the total of all antibody isotype (IgA, IgG, and IgM)-secreting cells to ovalbumin. Gastrointestinal contents were collected using a previously described technique [28]. Briefly, mice were orogastrically administered a hyperosmotic solution consisting of 0.025 M NaCl, 0.04 M Na_2SO_4, 0.01 M KCl, 0.002 M $NaHCO_3$, and 0.0485 M polyethylene glycol four times at 15 min intervals. 30 min after the last lavage, each mouse received 0.5 ml of lactated Ringer's solution subcutaneously and 1.0 ml of 0.1% (w/v) pilocarpine intraperitoneally. Intestinal secretions were collected over the next 60 min in 3 ml of soybean trypsin inhibitor (Sigma, St. Louis, MO). Intestinal secretions were diluted to 6 ml with phosphate-buffered saline (PBS) and centrifuged at $600 \times g$ for 10 min. to remove particulate debris. 30 μl of 17% (w/v) phenylmethylsulfonylflouride (PMSF, Sigma) in 95% (v/v) ethanol was added to the supernatant prior to centrifugation at $27\,000 \times g$ for 20 min at 4°C. PMSF and 1% sodium azide were added to the resulting supernatant. After incubation for 15 min at room temperature, 50 μl of fetal calf serum was added to each ml of supernatants which were then frozen at -20°C until assayed.

3.3. Vaccination of rabbits

Potassium thiocyanate (KSCN) extracts of *Pasteurella multocida* were used in a vaccine for rabbits [29]. This extract was mixed with alginate to form microspheres that contained 1 mg/ml of the KSCN extracted protein, or 1 mg/ml extract protein and 200 μg of cholera toxin, or no extract protein. Rabbits were immunized by placing 5 ml of microspheres in their drinking water at day 0, 7, and 14. Rabbits were given enough water to ensure that all the microspheres were ingested. Other groups of rabbits were inoculated with the protein extract which ws not incorporated into microspheres by oral administration with or without cholera toxin, and another group was inoculated by intranasal administration. A nonimmunized-nonchallenged group of rabbits was also included. Serum and nasal lavage were collected at day 0, 10, 16, and 21 of the study. Nasal lavages were performed by instilling 2.0 ml of sterile PBS into one nostril using a ball-tipped rodent gavage needle attached to a syringe. With the rabbit's

head directed in a slight downward direction, the effluent was collected from the contralateral nostril into a sterile petri dish. Rabbits were challenged at day 16 by an intranasal inoculation of viable *P. multocida*. Each rabbit was euthanized at day 21 and lung, liver, nasopharynx, and both tympanic bullae were cultured for *P. multocida*. The tympanic bullae and nasopharynx were exposed by aseptic dissection and a sterile swab inserted into the site. The swab was then used to inoculate a tryticase agar plate containing 5% sheep blood (TCA 5% blood, Difco, Madison, WI). A 1 g section of the right apical lung lobe was macerated in 1 ml of sterile saline. Serial 1:10 dilutions of this macerated tissue were made and used to inoculate TCA 5% blood plates. The number of bacteria per gram of tissue was determined by counting the number of bacteria for each dilution. Only plates with counts between 30 and 300 CFU/ml were counted. A similar method was followed for enumeration of bacteria in liver. Antibody activity to the KSCN extract was measured in serum and nasal lavage fluid by ELISA.

3.4. Vaccination of chickens

10-week-old white leghorn chickens (SPAFAS, Roanoke, IL) were equally divided into two groups. One group was administered three oral doses of 300 μg of flagellin of *Salmonella enteritidis* in microspheres at 2 week intervals. The other group was given 300 μg of bovine serum albumin (BSA) in microspheres instead of flagellin. 1 week after the last oral dose of antigen, serum and intestinal fluid were collected and assayed for flagellin specific antibodies by ELISA as previously described [30]. A cell-mediated immune response was measured as cutaneous delayed type hypersensitivity. 2 weeks after the last oral dose of microspheres all chickens were given an intradermal injection saline in the right lateral toe web of 0.4 μg of flagellin in 50 μl of phosphate-buffered saline. The left lateral toe web was inoculated with 50 μl of PBS to serve as a control. The net increase in palmar-plantar width of the right lateral toeweb at 48 h, which was the measure of the DTH response, was calculated by the following equation: (right toeweb width at 48 h – right toeweb width at 0 h) – (left toeweb width at 48 h – left toeweb width at 0 h).

3.5. Vaccination of cattle

Microspheres containing ovalbumin were incorporated within larger alginate hydrogels to give them a size (4–5 mm in diameter) and density (> 1.1 specific gravity) to bypass the rumen and drop into the reticulum. These in turn were placed into a gelatin bolus and given by balling gun to each calf. Four groups of calves were used in this study. One group received two inoculations of 3 mg of ovalbumin in incomplete Freund's adjuvant by subcutaneous injection as parenterally inoculated controls. A second group was inoculated with one parenteral injection of ovalbumin to prime the immune system followed by three intrabronchial inoculations containing 5 mg of ovalbumin in saline as positive respiratory immunized calves. A third group was inoculated with one parenteral dose of ovalbumin followed by two regimens of oral ovalbumin in microspheres. A fourth group was inoculated with two oral regimens of ovalbumin in microspheres. An oral regimen included five daily doses of 5 mg of ovalbumin in microspheres encapsulated within larger alginate hydrogels. Serum and bronchoalveolar lavages (BAL) were collected at 0, 21, 42, and 54 days and assayed for isotypic antibody responses to ovalbumin by ELISA. Intrabronchial inoculations and BALs were performed as previously described [30]. The lymphocytes were recovered from each BAL and subjected to ELISPOT assay to determine ovalbumin-specific antibody-secreting cells.

3.6. Immunological assays

3.6.1. ELISPOT

The ELISPOT assay was performed as described previously [31] with modifications. Briefly, spleens were harvested, disrupted, and mononuclear cells suspended at $1-2 \times 10^6$ cells/ml in complete tissue culture medium RPMI-1640 containing 10% FetalClone I serum (Hyclone, UT). For BAL cells from cattle, the BAL fluids were centrifuged to collect cells which were suspended in complete RPMI-1640 plus FetalClone I, distributed in 100 mm tissue culture petri dishes (Costar), and incubated at 37°C for 1 h. The non-adherent cells were washed from these plates, suspended at $1-2 \times 10^6$ cells/ml, and used in ELISPOT assays as lymphocyte enriched

mononuclear cells. For the assay, 96-well microtiter plates were coated overnight with 0.5 μg/well of ovalbumin. The plate was washed with phosphate-buffered saline containing 0.5% Tween 20 (PBS-tween), non-specific binding inhibited by coating the plate with 0.1% bovine serum albumin, and then washed with sterile PBS-tween, followed by sterile saline. The cells were placed in the wells and the plate incubated at 37°C and 5% CO_2 for 4 h. The plate was washed with PBS-tween to remove cells, and monoclonal antibody to bovine IgG1, IgG2, IgA, or IgM (Serotec), added to the wells at previously optimal dilutions and incubated for overnight at 4°C. The plate was washed and alkaline phosphatase conjugated goat anti-mouse IgG (Sigma) and incubated at room temperature for 1 hour. For mice, alkaline phosphatase conjugated goat anti-mouse IgA, IgG, or IgM were placed in the wells and incubated overnight. After the final wash, 100 μl of 5-bromo-4-chloro-3-indoyl phosphate (BCIP, Sigma) in 0.6% agarose was added to each well. Spots developed in 30 min. and were counted using an inverted microscope. The mean number of spots for six wells were converted to ASCs per 10^6 cells. Final results were reported as the number of isotype specific antibody secreting cells (ASC) per 10^6 cells.

3.6.2. ELISA

3.6.2.1. Mice. The ELISA assay used was a variation of the previously described protocol [32]. Polystyrene microtiter plates were coated with ovalbumin as described above for ELISPOT. Instead of adding cells to the wells, serum was added at a dilution of 1:25 in PBS-tween and incubated for 4 h. at room temperature. The plate was washed and alkaline phosphatase conjugated anti-mouse IgG, IgA, or IgM added to the wells and incubated overnight at 4°C. The plate was washed and the substrate *p*-nitrophenyl phosphate (NPP, Sigma) was then added to each well. The plate was incubated at room temperature for 45 min and read at a wavelength of 405 nm on a microtiter plate spectrophotometer (Molecular Devices, Menlo Park, CA).

3.6.2.2. Rabbits. The ELISA was performed for anti-KSCN antibodies using a previously described protocol [26]. Briefly, polystyrene microtiter plates were coated with 10 μg/well of protein of KSCN extract. Plates were incubated overnight at 4°C, washed, and serum diluted 1:25, or nasal lavage fluid diluted 1:2 in PBS-tween was added to wells and the plate incubated at room temperature for 4 h. After washing, horseradish peroxidase conjugated sheep antig-rabbit IgA (Accurate Chemical, Westbury, NY) or goat anti-rabbit IgG (Sigma) was added to each well and the plate incubated for 1 h at room temperature. After a final wash, the substrate *o*-phenylenediamine dihydrochloride (OPD, Sigma) was added, the reaction stopped by adding 2.5 M sulfuric acid, and the plate read at a wavelength of 490 nm in a microtiter plate reader.

3.6.2.3. Cattle. The ELISA assay was performed as described above for the mice with the reagents listed as described for the cattle ELISPOT. Instead of cells, undiluted BAL fluids and serum diluted at 1:10 in PBS-tween were tested. Samples were incubated at room temperature for 3 h. Monoclonal antibodies were incubated overnight at 4°C. For ELISA,

Table 1
Immune response of mice following oral or subcutaneous administration of ovalbumin in alginate microspheres

Inoculation	Serum IgG (ΔO.D.)	Serum IgA (ΔO.D.)	ASC/10^6 cells
Plain MS by oral	0	0	0
OVA in MS by oral	0.181 ± 0.520	0.113 ± 0.120	38 ± 10
OVA in MS by SC	0.424 ± 0.400	0.152 ± 0.047	52 ± 53
OVA/CFA by SC	0.131 ± 0.116	0.214 ± 0.112	272 ± 10
OVA/Alum by SC	0.288 ± 0.313	0.129 ± 0.146	117 ± 42

ΔO.D., change in optical density; ASC, antibody-secreting cells (all isotypes of antibodies); OVA, ovalbumin; MS, microspheres; SC, subcutaneous; CFA, complete Freund's adjuvant; Alum, aluminum hydroxide adjuvant.

horseradish peroxidase conjugated goat anti-mouse IgG was used and the plate incubated for 1 h at room temperature. After a final wash the substrate (OPD, Sigma) was added and the plate incubated for 10 min. The reaction was stopped when pre-determined absorbance of positive control wells was reached by adding 2.5 M sulfuric acid. The absorbance reading for each well was obtained at a wavelength of 490 nm using a microtiter plate reader.

4. Results

4.1. Mouse studies

Mice that were inoculated with ovalbumin in alginate microspheres by oral administration had a good immune response with increased serum IgG and IgA titers to ovalbumin and increased antibody-secreting cells in the compared to mice that received plain microspheres (Table 1). The level of the immune response by oral administration was about the same as that by subcutaneous administration of ovalbumin in microspheres. Microspheres apparently released antigen in vivo to stimulate an immune response. The immune responses seen in mice inoculated with ovalbumin in complete Freund's adjuvant (CFA) or aluminum hydroxide adjuvant were similar to those obtained using microspheres. This suggests that alginate microspheres act as an adjuvant. The mouse studies have shown that alginate microspheres are quite effective in inducing an immune response upon oral administration. It appears that there is little difference in the route of administration of ovalbumin in the induction of serum IgA.

4.2. Rabbit study

In the rabbit study, potassium thiocyanate (KSCN) extract of the bacterium *Pasteurella multocida* was used as an antigen, since it has shown promise in producing partial protection against rhinitis, pneumonia, metritis, otitis media, and septicemia when injected parenterally in rabbits [26]. Rabbits vaccinated orally with plain microspheres and KSCN extracts alone had minimal antigen-specific IgA antibodies in serum (data not shown) or nasal washes (Table 2). The use of cholera toxin in the oral administration of KSCN extracts helped only modestly. The optical density (O.D.) ELISA value increased about 3-fold to 0.145 at day 21. However, this was not a statistically significant increase over pre-inoculation values as determined by the Wilcoxon rank sum test. On the other hand, rabbits vaccinated orally with microspheres or intranasally with KSCN extracts had significantly increased anti-*P. multocida* antibodies in nasal washings (IgA) and serum (IgG: data not shown). The titers peaked at an O.D. of 0.600 by day 21. These results indicate that cholera toxin did not enhance the immune response to orally administered KSCN extracts whether administered within microspheres or not.

The ultimate test of a vaccine delivery system is whether vaccinated animals are protected following challenge with a pathogen. To test this system, rabbits were challenged with an intranasal instillation of viable *P. multocida* 7 days after the last dose of vaccine. The rabbits were euthanatized 3 days after challenge and serum and nasal washes were assayed for antibodies that were specific for the KSCN extracts of *P. multocida*. The number of bacteria iso-

Table 2
Change in optical density of IgA present in nasal lavage of rabbits inoculated with KSCN extracts of *P. multocida*

Group	Day 0	Day 10	Day 16	Day 21
Non-immunized	0.025 ± 0.012	0.070 ± 0.020	0.050 ± 0.010	0.050 ± 0.021
MS by oral	0.065 ± 0.020	0.050 ± 0.016	0.030 ± 0.015	0.050 ± 0.022
KSCN by oral	0.060 ± 0.016	0.090 ± 0.024	0.125 ± 0.040	0.140 ± 0.031
KSCN + CT by oral	0.050 ± 0.032	0.105 ± 0.025	0.135 ± 0.037	0.145 ± 0.029
KSCN by intranasal	0.058 ± 0.021	0.130 ± 0.014	0.500 ± 0.053	0.530 ± 0.047
KSCN in MS by oral	0.055 ± 0.008	0.140 ± 0.031	0.560 ± 0.047	0.600 ± 0.070

MS, microspheres; KSCN, potassium thiocyanate extracts of outer membrane proteins; CT, cholera toxin.

Table 3
Isolation of bacteria from tissues following intranasal challenge in immunized rabbits

Group	Positive cultures [a]	Number of bacteria in lung	Number of bacteria in nasopharynx
Nonimmunized and non-challenged	0/15	0	0
MS by oral	7/15	84 ± 6	321 ± 11
KSCN by oral	4/15	73 ± 2	302 ± 9
KSCN + CT by oral	4/15	69 ± 7	287 ± 13
KSCN by intranasal	1/15	38 ± 7	198 ± 14
KSCN in MS by oral	3/15	40 ± 3	205 ± 15
KSCN in MS + CT by oral	1/15	40 ± 4	188 ± 10

[a] Five rabbits per group.
Liver and right and left tympanic bullae were cultured from each rabbit. MS, microspheres; KSCN, potassium thiocyanate extracts of outer membrane proteins; CT, cholera toxin.

lated from nares, lungs, and liver and tympanic bullae is shown in Table 3. Lung bacterial counts were over 70 and nasopharynx bacterial counts were over 300 in rabbits that were inoculated orally with plain microspheres or KSCN extracts. The addition of cholera toxin to the KSCN extracts for oral administration did not decrease the numbers appreciably. On the other hand, bacterial counts were about 40 in the lung and 200 in the nasopharynx of rabbits that were inoculated orally with KSCN extracts in microspheres or inoculated intranasally with KSCN extracts. These values are the same as those obtained by intranasal administration of the KSCN extracts. Table 3 clearly demonstrates that the oral administration of KSCN extracts of *P. multocida* in microspheres stimulated protective immunity in other mucosal surfaces than the gut. This study demonstrates that protective immunity can be stimulated at distant mucosal sites in animals by oral administration of antigens in alginate microspheres [32].

4.3. Chicken study

Salmonella enteritidis is a major cause of disease in laying hens and can be passed to chicks through the egg infecting subsequent generations. Contaminated eggs are also the source of zoonotic infection in humans who consume eggs which are not thoroughly cooked. Since infection begins by this bacteria attaching and invading the intestinal mucosa, and long-term infection involves colonization of intestinal lymphoid tissues, stimulation of mucosal immunity is imperative to control this disease. A major immunogen of this organism is the flagella. Vaccines containing flagellar antigens have shown promise in inducing immunity and reducing shedding by vaccinated chickens [33]. Since *Salmonella* spp. infect the intestinal tract, protective immunity at the mucosal surface could prevent infection of laying hens and decrease the spread of this serious disease.

The results of ELISA showed no significant difference in antibody titers to flagellin between groups of birds. However, the flagellin vaccinated birds had a significantly increased delayed-type hypersensitivity (DTH) response than the BSA vaccinated controls. The flagellin-vaccinated chickens had a mean net increase in toe web width of 0.62 ± 0.06 mm and the BSA-vaccinated birds had a mean increase of 0.19 ± 0.02 mm ($p < 0.05$ by student's t-test). This is unexpected for immunization using a soluble antigen such as flagellin. Incorporation of the flagellin within the microspheres may change the presentation of the antigen and stimulate a cell-mediated immune response characterized by the increased DTH. This is an important immune response for this disease since *S. enteritidis* invades leukocytes and is retained for long-term infection within these cells. Only cell-mediated immunity such as DTH can help a bird clear the bacteria and avoid a long-term carrier state.

4.4. Cattle study

There is a need for effective vaccines to prevent respiratory diseases in cattle since this is one of the most economically important problems to the cattle industry. In this study, ovalbumin was used as a model vaccine. Four groups of calves were inoculated with ovalbumin by four different methods. The inoculation regimen for four groups of calves are described above in the experimental method section. Table 4 shows the number of ovalbumin-specific antibody-secreting cells (ASC) per million lymphocytes in bronchoalveolar lavages (BAL) obtained 42 days after the inoculation. The second highest number of ASC for IgG_1 was observed with group 3 which was inoculated with one subcutaneous (SC) dose of ovalbumin followed by two oral doses of ovalbumin in microspheres. Calves in group 2

Table 4
Number of ovalbumin specific antibody secreting cells per 10^6 lymphocytes from bronchoalveolar lavages of calves inoculated with ovalbumin in microspheres

Antibody	Inoculation group			
	1	2	3	4
Isotype	(SC + SC)	(SC + IB)	(SC + Oral)	(Oral + Oral)
IgG_1	2 ± 0.5	275 ± 60	247 ± 284	0
IgG_2	2 ± 0.5	35 ± 18	9 ± 10	0
IgM	0	20 ± 70	0	1 ± 0.7
IgA	7 ± 0.6	1770 ± 423	25 ± 15	7.5 ± 7.8

$n = 8$ for groups 1 (SC + SC) and 2 (SC + IB) and $n = 4$ for groups 3 (SC + Oral) and 4 (Oral + Oral); SC, subcutaneous; IB, intrabronchial; Oral, within alginate microspheres.

(primed with SC followed by three intrabronchial inoculations) had shown the largest number of IgG_1 ASC. Group 2 calves also had the greatest number of IgG_2-ASC. A small number of IgM-ASC were detected in group 2 calves. Group 2 calves showed the largest number of IgA-ASC while only a small number of IgA-secreting cells were observed in group 4 calves which had received two oral doses of ovalbumin in microspheres. Although group 4 did not have a large number of IgA-secreting cells at day 42, there was a marked increase in IgA in the bronchoalveolar lavage fluids (see Table 5). This suggests that although a large number of IgA-secreting cells were not detected, a significant amount of ovalbumin-specific IgA was produced in the lungs of these calves.

Table 5 shows the IgG_1 and IgA titers as detected by ELISA. Table 5 shows the data for IgG_1 and IgA for only groups 3 and 4, since they are most interesting in our study. IgG_1 is found primarily in blood as well as in alveoli, while IgA is mainly found in large airways like bronchi. In BAL fluids, IgG_1 increased in time in both groups 3 and 4. On the other hand, the IgA response was not significant at day 20 but was the predominant antibody isotype present at day 42. About the same level of IgA was maintained at Day 54. Although the level of IgA at day 42 in Group 4 (which was inoculated by 2 oral administrations of OVA in microspheres) was not as high as that in Group 3 (inoculated by SC followed by oral vaccination), a very high level of IgA indicates that oral vaccination using alginate microspheres was quite effective.

5. Discussion

Orally administered vaccines are being studied intensively using in mice, guinea pigs, and non-human primates as experimental models for human diseases such as cholera, tetanus, influenza, and HIV. Development of vaccines for animals has a distinct advantage in that delivery systems and antigens can be tested for use in the target animal species for which the vaccines are intended. Hopefully, this will lead to quicker use and approval of oral vaccines. Information gained from oral vaccines developed for one species can be used for more efficient develop-

Table 5
Increase in absorbance values for BAL IgG_1 and IgA compared to day 0

Group	Antibody isotype					
	IgG_1			IgA		
	Day 21	Day 42	Day 54	Day 21	Day 42	Day 54
Control	0.035 [a]		0.075	0		0
	(0.030) [b]		(0.021)			
SC + Oral	0.201		0.610	0	.106	.787
(Group 3)	(0.153)		(0.057)		(.085)	(.174)
Oral + Oral	0.103	0.214	0.273	0.050	.349	.366
(Group 4)	(0.047)	(0.027)	(0.030)	(0.010)	(.199)	(.319)

[a] Mean.
[b] Standard deviation.

ment of vaccines for other species. The development of respiratory syncytial virus vaccines is an example where success in cattle could benefit humans and vice versa.

Oral administration of vaccines offers several advantages. Vaccine can be administered to a large number of individuals via the food or water with minimal restraint and labor. Restraint of animals may induce stress that renders the vaccine less effective and increases the risk of infectious disease. Oral inoculation would be quick and efficient. Formulations that can be administered in one dose eliminate the repeated handling of animals that is required for booster inoculations [23,34]. This is also an issue in human vaccination programs where patients often fail to return to a medical center for second or third booster inoculations. Oral administration of vaccines is safe because adverse immune reactions are less likely to occur compared to parenteral methods. For meat-producing animals, oral administration of vaccine has another advantage in that injection site reactions are avoided. Broken needles, contamination of the injection site, or highly reactive adjuvants can induce abscesses that damage the carcass and the hide. These reactions decrease the value of the animal at slaughter.

We have explored the possibility of alginate microspheres in delivering vaccines by oral administration. Our studies have clearly shown that the vaccine-containing alginate microspheres are effective for oral vaccination in several animal species. There are several advantages of the alginate vaccine delivery system. First, the alginate vaccine delivery system eliminates the use of organic solvents or high temperatures which are often required for the preparation of microparticles by other methods. By maintaining an aqueous environment throughout the preparation of microspheres, our method can be used with live bacteria and viruses. Second, the alginate microspheres are able to protect antigens/vaccines against degradation in the GI tract. This allows the stimulation of the same immune response with a smaller amount of antigen/vaccine. Third, the alginate system was shown to act as an adjuvant in our study. Fourth, alginate microspheres provide a means to bypass the stomach(s) to deliver antigens to GALT in man as well as animals. These factors efficiently stimulate mucosal immunity. We have previously demonstrated for the first time that an oral delivery of vaccine using crosslinked poly(methacrylic acid) (PMA) hydrogels was effective in cattle [26]. The PMA hydrogels were highly effective in this application partially because they were made to have the right size and density to pass through the rumen thereby protecting the vaccine [27]. Alginate microspheres can also be formulated for the same purpose.

Our results show promise for the use of alginate microspheres in a host species with a complex stomach where delivery of oral vaccines is especially challenging. Alginate is readily available, inexpensive, requires mild conditions to incorporate antigens, and can be used to incorporate viable viruses or bacteria if desired. In addition, it is easy to make microspheres using alginate. The ability to produce small sized particles is important, since it has been reported that microparticles with a diameter between 1 and 10 μm are preferentially absorbed by the Peyer's patches [35,36]. Our studies have also shown that alginate microspheres adhered to the Peyer's patches when tested in rabbits [32]. This is an essential step in the uptake and processing of microspheres by the GALT of an animal. The diameter of alginate microspheres which resulted in excellent immune responses in our study ranged from 1 μm to more than 30 μm. Thus, it seems that the selective absorption to the Peyer's patches alone can not really explain the results of our study. One cannot assume that the microspheres with a diameter larger than 10 μm do not contribute to the immune response at all. Results of our study are similar to previous reports in which microparticles 20–125 μm in diameter enhanced the immune response to an antigen when administered with particles under 10 μm [23]. The release of vaccines from the hydrogels in the intestine and selective adsorption of the released vaccines cannot be ignored. Since various hydrogel delivery systems with different physical and physicochemical properties can be prepared to meet the specific requirements of each vaccine, one can optimize the delivery of vaccines by oral administration.

The challenge for use in humans is also tremendous. The hope of pediatricians has been expressed recently by Hall [37] who has expressed the hope that infants will someday be vaccinated for infectious diseases with one oral dose of vaccine. This is a huge challenge, but one worthy of the effort needed.

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