Mucosal Delivery of Bacterial Antigens and CpG Oligonucleotides Formulated in Biphasic Lipid Vesicles in Pigs

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ABSTRACT

The ineffectiveness of simple delivery of soluble antigens to mucosal membranes for immunization has stimulated extensive studies of strategies for appropriate delivery systems and adjuvants. Biphasic lipid vesicles are formulations suitable for the delivery of proteins, peptides, and oligo/polynucleotides. The purpose of these studies was to investigate the ability of biphasic lipid vesicles (as vaccine-targeting adjuvants) containing a bacterial antigen and unmethylated oligonucleotides containing CGdinucleotides - CpG motifs (CpG ODNs) to induce systemic and mucosal immune responses in pigs. Results showed that while the protein, either alone or with CpG ODNs, did not induce mucosal immune responses, administration of antigen and CpG ODNs in biphasic lipid vesicles resulted in induction of both systemic and local antibody responses after immunization using a combined mucosal/systemic approach.

KEYWORDS: Mucosal immunity, biphasic lipid delivery systems, CpG ODNs, porcine, Actinobacillus pleuropneumoniae

INTRODUCTION

Development of less invasive and more readily administered vaccines has become a priority for public health agencies, and new vaccine formulation and delivery technologies have emerged. There is great interest in the use of mucosal membranes for noninvasive vaccine administration. However, most vaccines licensed for use in humans and animals are injected intramuscularly or subcutaneously, and mucosal immune responses induced by parenteral immunization are generally weaker, are more variable, and last for a briefer time than do mucosally induced responses.[1-5]

Increased awareness of the fact that most infectious agents use mucosal membranes as portals of entry has led to efforts to develop vaccines and antigen delivery systems that can efficiently induce mucosal immunity. Mucosal immunization offers many benefits, including reduced vaccine-associated side effects and the potential to overcome the known barriers of parenteral vaccination: preexisting systemic immunity from previous vaccination, or, in young animals, preexisting systemic immunity from maternal antibodies.[6] The nasal mucosa is an important arm of the mucosal system since it is often the first point of contact for inhaled antigens and represents an attractive, noninvasive route for the delivery of antigens.

For mucosal immunization, adjuvants are particularly important since most antigens are poorly immunogenic when given via this route. Many investigators have exploited the immunomodulatory effects of soluble holotoxins, such as cholera toxin (CT) and heat labile enterotoxin (LT), as adjuvants. Although CT and LT have been shown to be effective mucosal adjuvants for nasal delivery of numerous antigens, their use is restricted because of their toxicity.[7-9] The ineffectiveness of simple delivery of soluble antigens to mucosal membranes for immunization has stimulated extensive studies of strategies for delivery systems that would (1) increase the antigen absorption, (2) prevent its degradation and facilitate its uptake and transport to the lymphoid tissue for presentation to immunocompetent cells, and (3) induce the appropriate type of immune response (B vs T cell; mucosal vs systemic; protective vs tolerance). Mucosal immunization protocols frequently use large doses of antigen, live organisms,[6] or bacterial toxins such as CT[7-9] as adjuvants. A recent study in mice suggested that formulation of vaccines with CT might help to direct nasally delivered antigens to neural tissue via the olfactory bulb, with potential harmful effects.[10] Hence, current efforts to overcome obstacles to the development of effective mucosal vaccines are mainly directed toward finding a more efficient means of delivering appropriate antigens to the mucosal immune system and toward discovering effective, safe mucosal adjuvants or immunoregulatory agents that provide protective immunity against infections.

CpG oligonucleotides (ODNs) are potent adjuvants that significantly enhance cellular and humoral responses to coadministered antigens when given parenterally or by mucosal

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routes.[11,12] However, in vivo degradation of ODNs limits their uptake and their effectiveness as adjuvants. Biphasic lipid vesicles are a novel type of lipid-based formulation suitable for the delivery of proteins, peptides, and ODNs. These formulations, which are designed for vaccine application and are also called vaccine-targeting adjuvants (VTA), have been shown to enhance the adjuvant activity of CpG ODNs following intranasal delivery with a viral protein in mice[13] and after systemic administration in pigs.[14] In the present study, we evaluated whether these formulations enhance the adjuvanticity of CpG ODNs when administered mucosally in pigs.

Materials and Methods

Antigen and Delivery Systems

Outer membrane lipoprotein A (OmlA) from *Actinobacillus pleuropneumoniae* (*App)* was prepared as previously described.[15] Biphasix-VTA (PharmaDerm Laboratories Ltd, Saskatoon, SK) was prepared as described previously.[16,17] Briefly, for intranasal (i.n.) immunization, i.n. VTA (Code VTAM1) was prepared by mixing a submicron emulsion (olive oil, 2%; polyoxyl 40 hydrogenated castor oil, 2%; methylparaben, 0.15%; propylparaben, 0.05%) and the phospholipid phase (soya phosphatidylcholine [Phospholipon 90H, Phospholipia GmbH, Cologne, Germany], 3.5%; cholesterol, 1%; cetylpyridinium chloride, 0.05%; and propylene glycol, 4%) to form lipid vesicles entrapping the emulsion droplets. The cetylpyridinium chloride used in this formulation was selected because of its mucosal compatibility. All ingredients were % wt/vol, USP grade, from Spectrum (New Brunswick, NJ) unless specified otherwise. Antigen in endotoxin-free saline (Baxter Corporation, Toronto, ON) was mixed with VTA formulations using a ratio of 1 part antigen to 9 parts VTA. CpG ODN 2007 (TCGTCGTTGTCGTTTTGTCGTT) was chosen because its GTCGTT motif is optimal for stimulation of lymphocytes in several domestic species, including pigs.[18] The ODNs had a phosphorothioate backbone modification to increase resistance to nuclease degradation (QIAGEN GmbH, Hilden, Germany).

Animals

Four-week-old male and female pigs from an *App*-free herd were purchased from the Prairie Swine Center Inc, Saskatoon, SK). Animals were treated in compliance with the regulations of the Canadian Council for Animal Care under protocols approved by the University Committee on Animal Care and Supply.

Immunization

Pigs received 2 i.n. immunizations on days 0 and 30, consisting of 1 mL per nostril of the different formulations. OmlA, CpG ODN 2007, and CT doses were 250 μg, 50 μg, and 10 μg, respectively. On day 21, all the animals received a subcutaneous (s.c.) immunization, consisting of 500 μL of OmlA (50 μg) and CpG ODN 2007 (50 μg) in 30% (vol/vol) Emulsigen (MVP Laboratories Inc., Ralston, NE). A negative control group received saline only. One group received 2 s.c. immunizations with OmlA (50 μg) and CpG (50 μg) in VTA2, 3 weeks apart.

Collection of Nasal Secretions

Nasal secretions were collected with 2 absorptive swabs (Merocel Inc, Jacksonville, ON) after 150 μL of phosphate buffered saline (PBS) (pH 7.3) was sprayed into each nostril. The swabs were placed proximal to the external nares to absorb fluid without disrupting the nasal mucosa. Nasal swabs were placed in 1.5-mL Eppendorf tubes (Brinkmann Instruments, Mississauga, ON) and kept on ice. Tubes were pierced at the bottom, placed inside a second tube containing 10 μL of 0.1M Phenylmethylsulponylflouride (PMSF) (Sigma-Aldrich Canada Ltd, Oakville, ON), and centrifuged for 30 seconds at 15 850 × *g*.

Detection of Antigen-Specific Antibodies

The concentrations of OmlA- and CT-specific antibodies in serum and nasal secretions were determined by an enzyme-linked immunosorbent assay. Ninety-six well Immulon 2 plates (Dynatech Laboratories Inc, Alexandria, VA) were coated with 0.1 μg of antigen per well and incubated with serially diluted samples. Mouse antiporcine immunoglobulin A (IgA) 1/250, IgG1 1/200, or IgG2 1/500 (Serotec, Oxford, UK), and then biotinylated goat-antimouse IgG(H^+L) 1/5000 (Zymed, San Francisco, CA) or alkaline phosphatase goat antiporcine IgG(H^+L) 1/5000 (KPL, Gaithersburg, MD), were used as detecting antibodies. Di(Tris) p-nitrophenyl phosphate (PNPP; Sigma) was used as the chromogenic substrate. A porcine immunoglobulin reference serum in which 1 unit is equivalent to 1 μg (Bethyl Laboratories Inc, Montgomery, TX) was used as a reference standard from 1000 to 1.95 ng/mL and 100 to 0.78 ng/mL, for serum and nasal secretions, respectively, to determine IgG and IgA concentrations.

Statistical Analysis

Data were analyzed using the Graph Pad InStat program Version 3.0 (Graphpad Software, Inc, San Diego, CA). The significance of differences among groups was analyzed using Kruskal-Wallis 1-way analysis of variance followed by Dunn's test, or Mann-Whitney *t* test when comparing 2 groups. *P* values of 0.05 or less were considered significant.

Results and Discussion

We have previously shown that biphasic lipid vesicles enhance the adjuvant activity of CpG ODNs and induce protective immune responses in pigs after systemic immunizations.[14] To evaluate the efficiency of these formulations as mucosal delivery systems for antigens and adjuvants, a combined mucosal/systemic protocol of immunization was used in pigs. Pigs received 2 i.n. immunizations at days 0 and 30 and 1 s.c. at day 21 (Table 1). CT was included as a positive control for mucosal immunization. Negative control animals received saline. Antibody responses were assessed after each immunization and compared with responses induced by a biphasic lipid formulation containing antigen and CpG ODNs after 2 s.c. immunizations. The biphasic vesicles (VTAM1 formula) were characterized, and their main properties are described below. The i.n. formulation contained anionic vesicles with trimodal size distribution characteristics. Average size distributions for the 3 particle populations were 23 ± 1 nm (3% of particle population in this size range), 141 ± 18 (55%), and 784 ± 82 (42%) (n = 3). This is consistent with a topical formulation method where the biphasic vesicle components such as the submicron emulsion droplets (mean diameter 141 nm) and phospholipid vesicles trapping submicron emulsion droplets (mean diameter 784 nm) are present. In addition, the small particles with a mean diameter of 23 nm in VTAM1 probably represented surfactant micelles that were not part of the vesicle population. For administration into the nasal cavity, the antigen was extemporaneously mixed with the biphasic vesicles. The average zeta potential of the vesicles with antigen and CpG ODNs was –5.8 ± 0.5 (n = 3). Binding/association (not entrapment) of the antigen with the vesicles helps increase the residence time of the antigen in the nasal cavity and localizes the antigen in the required regions. The extemporaneous admixture of antigen with the vesicles also improves stability and versatility. It is envisaged that biphasic vesicles could be used as a "universal" adjuvant for many other antigens as well.

The main objective of these experiments was to assess the effectiveness of i.n. delivery. Therefore, groups of pigs received different formulations through the i.n. route but were immunized with the same formulation systemically.

After the i.n. prime and s.c. boost, animals that received the antigen and CpG ODNs formulated in VTAM1 presented higher IgG and IgA responses in serum compared with naïve animals ($P < .01$ and 0.001, respectively) (Figure 1). However, local immune responses were not detected, even in the group that received OmlA with CT (Figure 2).

To evaluate whether it was possible to induce mucosal immunity, animals received a second i.n. immunization and antibody responses were assessed 1 week later. After the i.n. boost, there was an increase in serum IgG and IgA levels, particularly in animals vaccinated with the antigen and CT ($P < 0.001$) or CpG ODNs in VTAM1 ($P < .01$), although animals receiving OmlA alone also showed increased serum antibodies ($P < .05$) (Figure 1). IgA in nasal secretions from positive control animals was higher than in naïve animals ($P < .05$; Figure 2). However, only animals immunized with the antigen and CpG ODNs in VTAM1 developed a higher local immunity consisting of both IgG ($P < .01$) and IgA ($P < .001$), indicating that biphasic lipid formulations combined with CpG ODNs were more effective at inducing a local immune response than CT, a well-known mucosal adjuvant,[7] at the administered dose. Low adjuvant activity of CT in pigs was also reported after oral administration.[19]

The presence of antigen-specific antibodies in the mucosa may be due to local production by antibody-secreting cells (IgA) or through transudation of circulating immunoglobulin from the sera (IgG or IgA). In general, mucosal IgG is thought to be transudative,[20] although Gockel et al[21] showed local production of IgG after topical administration of

Table 1. Evaluation of Immune Responses After a Mucosal/Systemic Protocol of Immunization*

Group†	i.n Immunization‡	s.c. Immunization§
1) Naïve	Saline	Saline
2) OmlA	OmlA	OmlA+CpG 30% Emulsigen (vol/vol)
3) CT	OmlA+CT (10 μg)	OmlA+CpG 30% Emulsigen (vol/vol)
4) CpG	OmlA+CpG (250 μg)	OmlA+CpG 30% Emulsigen (vol/vol)
5) VTAM1/CpG	OmlA+CpG/VTAM1 (1:9)	OmlA+CpG 30% Emulsigen (vol/vol)

*CT indicates cholera toxin; i.n., intranasal; OmlA, outer membrane lipoprotein A; s.c., subcutaneous; VTA, vaccine-targeting adjuvants.
†Each group consisted of 7 animals.
‡Animals received i.n. immunizations on days 0 and 30. Antigen dose was 250 μg.
§Animals received an s.c. immunization on day 21. OmlA and CpG doses were 50 μg each.

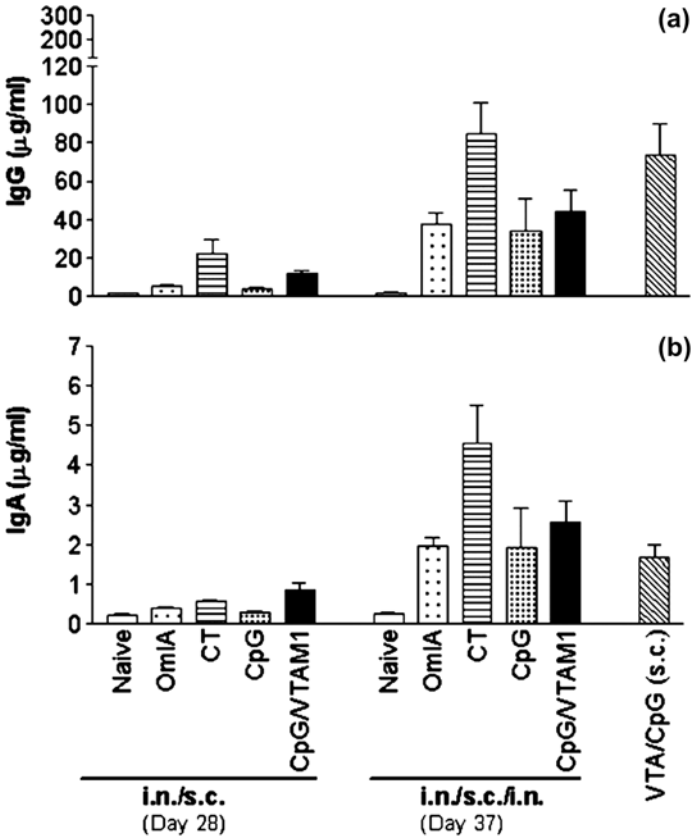

Figure 1. OmlA-specific serum antibodies after a mucosal/systemic immunization protocol. Animals received 2 i.n. immunizations with different formulations at days 0 and 30 and 1 s.c. immunization at day 21, as shown in Table 1. Anti-OmlA IgG (A) and IgA (B) concentrations in serum were determined 7 days after each boost and compared with the 2 s.c. immunizations. Results are expressed as the mean concentration ± standard error of the mean of 7 pigs. CT indicates cholera toxin; Ig, immunoglobulin; i.n., intranasal; OmlA, outer membrane lipoprotein A; s.c., subcutaneous; VTA, vaccine-targeting adjuvants.

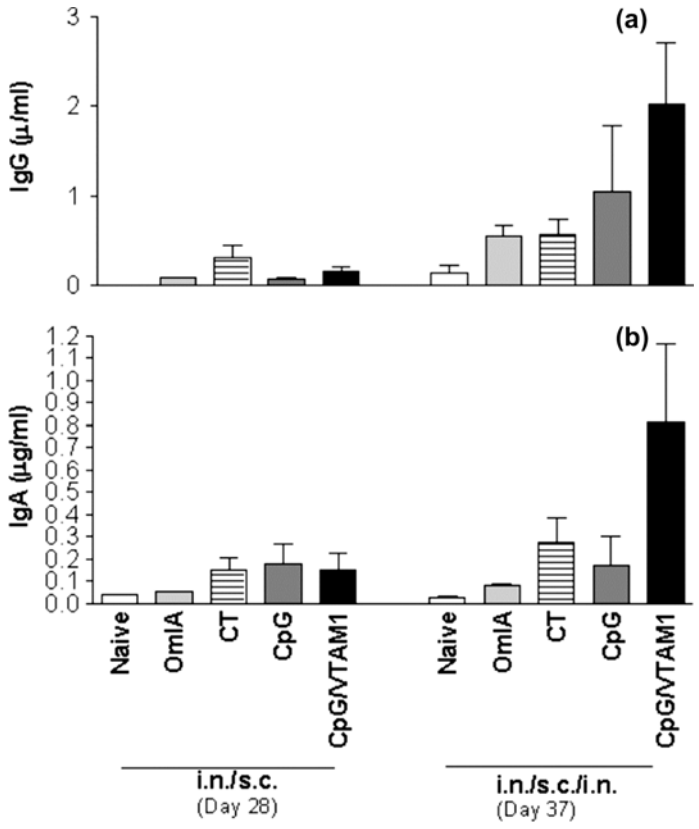

Figure 2. Antibody responses in nasal secretions after a mucosal/systemic immunization protocol. Animals were immunized as described in Figure 1. Anti-OmlA IgG (A) and IgA (B) concentrations in nasal secretions were determined 7 days after each boost. Results are expressed as the mean concentration ± standard error of the mean of 7 pigs. CT indicates cholera toxin; Ig, immunoglobulin; i.n., intranasal; OmlA, outer membrane lipoprotein A; s.c., subcutaneous; VTA, vaccine-targeting adjuvants.

tetanus toxin to mice. In contrast, IgA detected at the mucosal level is usually presumed to be of local origin,[22] but this remains to be determined for the anti-OmlA IgA detected in nasal secretions, since high levels of anti-OmlA IgA were also present in serum and the possibility of transudation cannot be discounted.[23] Further studies need to be done to exclude this possibility.

Our results confirmed previous observations that CpG ODNs are an effective mucosal adjuvant[12,19,24-26] and further indicate that VTA formulations are suitable delivery systems for antigens and CpG ODNs by the i.n. route in an outbred population. In addition, i.n. administration was shown to be an effective needle-free vaccine delivery route in pigs, inducing both systemic and local immune responses, when combined with an s.c. boost. Furthermore, antibody responses induced in serum by CpG/VTAM1 after the combined protocol were similar to those induced after s.c. administration (Figure 1).

There are clear advantages to using noninvasive vaccine delivery methods. Since many immunizations in the past were s.c., the boost injections could be avoided by i.n. administration. Results also demonstrated that CpG ODNs had stronger mucosal adjuvant activity when formulated in VTAM1 than when alone, confirming that the delivery system is a critical factor in mucosal immunization.[27] These results are also consistent with results obtained using the same biphasic formulations with plasmid DNA-lipid complexes[28] and viral proteins[13] in mice.

Clearance of CpG ODNs in vivo occurs rapidly because of degradation and rapid absorption into the systemic circulation.[29,30] This limits CpG ODNs' uptake and subsequently their ability to activate immune cells. The formulation of antigen and CpG ODNs in VTAM1 was found to be essential to elicit a local response, in addition to the systemic response. This capacity of lipid vesicles to enhance the activity of CpG ODNs may be due to several mechanisms. VTAM1 may protect the ODNs from degradation in the

nasal cavity, consequently increasing their half-life.[31] At the same time, VTAM1 may increase the uptake of both antigen and CpG ODNs by antigen presenting cells (APCs) at mucosal surfaces by facilitating intracellular delivery, leading to an increase in antigen presentation within the mucosal associated lymphoid tissue of the respiratory tract and the draining lymph nodes. Further work is needed to understand the mechanisms of action by which these formulations enhance the adjuvant activity of CpG ODNs in mucosal immunization.

CONCLUSIONS

Using noninvasive routes for vaccine delivery makes administration simple, painless, and economically practical. Formulation of antigens and adjuvants in appropriate delivery systems may be a critical factor in mucosal immunization. VTA lipid vesicles enhanced the adjuvant activity of CpG ODNs following i.n. administration and were as effective as CT at inducing local immunity. Hence, they may constitute an effective yet safe alternative to bacterial toxins.

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