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Intranasal immunization using biphasic lipid vesicles as delivery systems for OmlA bacterial protein antigen and CpG oligonucleotides adjuvant in a mouse model

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Abstract

The nasal mucosa is an important arm of the mucosal system since it is often the first point of contact for inhaled antigens. The ineffectiveness of the simple delivery of soluble antigens to mucosal membranes for immunization has stimulated extensive studies in appropriate delivery systems and adjuvants. We have evaluated biphasic lipid vesicles as a novel intranasal (i.n.) delivery system (designated as vaccine targeting adjuvant, VTA) containing bacterial antigens and CpG oligodeoxynucleotides (ODNs). Results show that administration of antigen and CpG ODNs in biphasic lipid vesicles resulted in greater induction of IgA levels in serum ($P < 0.05$) and mucosal antibody responses such as IgA in nasal secretions and lung ($P < 0.01$) after immunization with a combined subcutaneous (s.c.)/i.n. as compared to s.c./s.c. approach. Based on antibody responses, VTA formulations were found to be suitable as delivery systems for antigens and CpG ODNs by the intranasal route, resulting in a Th2-type of immune response, characterized by IgG1 and IL-4 production at the systemic level.

Introduction

The fact that most infectious agents use mucosal membranes as a frequent portal of entry into the host has led to the need for the development of vaccines and appropriate delivery systems that can efficiently induce mucosal immunity.

Administration of vaccines through mucosal sites is more effective in inducing mucosal immunity than parenteral immunization (McGhee et al 1992; Liu et al 1998; McCluskie et al 2002; Goonetilleke et al 2003). 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, more variable and short lived (Kaul & Ogra 1998). The nasal mucosa is an important arm of the mucosal system since it is often the first point of contact for inhaled antigens. There are several reasons why the nose is an attractive route for immunization: it is easily accessible and highly vascularized; the presence of numerous microvilli covering the nasal epithelium generates a large absorption surface; and mucosal and systemic immune responses can be induced (Partidos 2000). Mucosal immunization protocols frequently use large doses of antigen, live organisms (Liljeqvist & Stahl 1999) or bacterial toxins such as cholera toxin (CT) (Takahashi et al 1996; Rappuoli et al 1999; Williams et al 1999) as adjuvants. Furthermore, a recent study in mice suggested that formulation of vaccines with CT might direct nasally delivered antigens to neural tissue via the olfactory bulb, with potential harmful effects (van Ginkel et al 2000). Moreover, the ineffectiveness of simple delivery of soluble antigens to mucosal membranes for immunization has stimulated extensive studies of strategies for delivery systems that would: (i) increase antigen absorption, (ii) prevent its degradation and facilitate its uptake and transport to the lymphoid tissue for presentation to immunocompetent cells and (iii) induce the desired type of immune response (B- vs T-cell response; mucosal vs systemic; protective vs tolerance). CpG oligodeoxynucleotides (ODNs) have been shown to be potent mucosal and systemic adjuvants for inducing cellular and humoral responses to various antigens

given by the parenteral route or parenteral/intranasal prime/boost combinations (McCluskie et al 2001, 2002). However, administration by alternative, non-invasive routes would require further adjuvants or delivery systems to be more effective (McCluskie et al 2002; Alcón et al 2005).

Biphasic lipid vesicles are a novel type of lipid-based delivery system developed for the non-invasive delivery of macromolecules through the skin and mucosa (Foldvari et al 1999; Baca-Estrada et al 2000; Babiuk et al 2002; King et al 2002; and detailed description in Foldvari et al in preparation). The specific biphasic vesicle formulation compositions, designed for vaccine delivery, are designated as vaccine targeting adjuvants (VTAs). In previous studies we have shown that a biphasic vesicle VTA was necessary to achieve enhanced adjuvant activity for CpG ODN with a viral protein administered intranasally in mice (Babiuk et al 2004) and with a bacterial protein administered subcutaneously in pigs (Alcón et al 2003). In the present study, we evaluated whether these formulations could induce mucosal as well as systemic immune responses in mice against the bacterial protein OmlA using CpG ODN as an adjuvant and a combination of parenteral and mucosal delivery.

Materials and Methods

Antigen and delivery systems

OmlA from *Actinobacillus pleuropneumoniae* was prepared as previously described (Gerlach et al 1993). Biphasix VTAs (PharmaDerm Laboratories Ltd, Saskatoon, SK, Canada) were prepared as described previously (Foldvari 1998; Foldvari & Baca-Estrada 1999). Briefly, intranasal VTAs (code VTAM2) were 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 (hydrogenated soya phosphatidylcholine >90%, Phospholipon 90H (Natterman GmbH, Germany) 3.5%; cholesterol 1%; acylated amino acid derivative (compound code PDM19) 0.05%; propylene glycol 4%) to form lipid vesicles entrapping the emulsion droplets. For subcutaneous (s.c.) administration VTA formulation (code VTA2) was used. The submicron emulsion contained caprylic/capric triglycerides (Gattefosse, Mississauga, ON, Canada) 1%; glycerol monostearate 0.5%; linoleamidopropyl-PG-dimonium chloride phosphate (Mona, Paterson, NJ) 2%; methylparaben 0.15%; propylparaben 0.05%; phospholipid phase contained soya phosphatidylcholine (Phospholipon 90H) (Natterman GmbH, Germany) 4%; propylene glycol 2%. All ingredients were w/v, USP grade, from Spectrum (New Brunswick, NJ, USA) unless specified otherwise. Antigen in endotoxin-free saline (Baxter Corporation, Toronto, ON, Canada) was mixed with VTA formulations using a ratio of 1 part antigen to 9 parts VTA. CpG ODN 1826 (TCCATGACGTTCCTGACGTT) used in these experiments had a phosphorothioate backbone modification to increase resistance to nuclease degradation (QIAGEN GmbH, Hilden, Germany). Particle size and zeta potential were measured using a Malvern NanoZS particle sizer and microscopic observations were made on a Reichert Microstar IV optical microscope.

Animals

Six- to 8-week-old female BALB/c mice were purchased from the Animal Resource Center at the University of Saskatchewan. 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

Mice received two s.c. or one s.c. and one intranasal (i.n.) immunization 14 days apart. Subcutaneous administrations consisted of 100 μL of formulation containing 10 μg of antigen in combination with 10 μg of CpG ODN 1826 in VTA2. Intranasal administration consisted of 20 μL of formulation containing 10 μg of OmlA with 10 μg of CpG ODN 1826 in VTAM2. Naïve animals received saline to be used as negative controls.

Collection of mucosal secretions

Nasal and lung washes were obtained by making an incision in the trachea of euthanized mice. Nasal washes were obtained by injecting 0.3 mL of phosphate buffered saline (PBS) (pH 7.3) through the trachea towards the nose using a Teflon tube (16 TW, Cole-Palmer Instrument Co. Niles, IL). Fluid was collected in a microfuge tube as it exited the nares. Lung washes were obtained by injecting 0.5 mL PBS into the trachea with the Teflon tube inserted towards the lungs. The lavage was repeated by aspirating fluid back into the syringe and re-injecting once before the final withdrawal of fluid from the lungs. Samples were kept at $-20\,^{\circ}$C until analysed.

Cell preparation

Spleens were aseptically removed from naïve and immunized mice, and teased through a nylon mesh (Beckton Dickinson, Franklin Lakes, NJ, USA). Erythrocytes were lysed by 1 min of incubation with Tris-buffered ammonium chloride (0.14 M; pH 7.2). Cells were washed twice with minimal essential medium (MEM) (Gibco Life Technologies, Burlington, ON, Canada) and resuspended in AIM-V medium supplemented with 100 μM non-essential amino acids (Gibco), 1 mM sodium pyruvate (Gibco), 10 mM HEPES (Gibco) and 50 μM 2-mercaptoethanol (Sigma).

Lung cells were isolated by cutting lungs into small pieces and digesting the tissue in AIM-V medium containing 50 U mL^{-1} Dnase and 250 U mL^{-1} collagenase (Sigma) for 45 min at 37 °C. Lung tissue was pooled from each group. Cells were resuspended in 44% Percoll (Amersham Pharmacia Biotech, Piscataway, NJ, USA), layered over 67% Percoll and centrifuged at 800 g for 20 min. Cells at the interface were collected and washed with MEM. To avoid non-specific background in the

enzyme-linked immunospot assay (ELISPOT), adherent cells were removed by 1 h incubation in a tissue flask.

Detection of antigen-specific antibodies

Antibodies specific for OmlA were determined by an enzyme-linked immunosorbent assay. Ninety-six-well Immulon 2 plates (Dynatech Laboratories Inc., Alexandria, VA, USA) were coated with 100 μL of 1 μg mL^{-1} solution of antigen in 50 mM sodium carbonate buffer (pH 9.6). After overnight incubation at 4 °C different dilutions of serum in PBS 0.05% Tween 20 0.5% gelatin (PBS-TG, Sigma) were added (100 μL well^{-1}) and incubated for 2 h at room temperature. One hundred microlitres per well of biotinylated goat anti-mouse IgG (H + L), IgA, IgG1 or IgG2a 1/10 000 (Caltag, Toronto, ON, Canada) was added and incubated for 1 h at room temperature followed by incubation with streptavidin–alkaline phosphatase 1/10 000 (Jackson Immuno Research, Mississauga, ON,Canada). Di(Tris)-*p*-nitrophenyl phosphate (Sigma, Oakville, ON, Canada) substrate was added and absorbance was measured at 405 nm. Titres were expressed as the reciprocal of the dilution that intercepted the baseline (mean of negative control wells plus three s.d.).

Detection of cytokine-secreting cells

ELISPOT assays for cytokine-secreting cells were performed using spleen and lung cells as previously described (Baca-Estrada et al 1997). Briefly, 1×10^6 spleen or 0.3×10^6 lung cells well^{-1} were incubated in 96-well culture plates in the presence or absence of antigen (1 μg mL^{-1}) in AIM-V media and incubated at 37 °C and 5% CO_2 for 24 h. ConA (0.5 μg mL^{-1}) was included as a positive control. After incubation, cells were resuspended in fresh media and seeded on nitrocellulose plates (Millipore, Mississauga, ON, Canada) coated with either IFN-γ or IL-4 specific capture antibody (2 μg mL^{-1}) (Pharmingen, San Diego, CA, USA). Biotinylated anti-mouse IFN-γ or IL-4 specific antibodies (2 μg mL^{-1}) (Pharmingen) were used to detect secreted cytokines, followed by streptavidin–alkaline phosphatase 1/1000 (Jackson Immuno Research) and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate tablets (Sigma). After 10–30 min plates were washed in distilled water and air-dried. Spots representing individual cytokine-secreting cells were enumerated with a dissecting microscope. Values are expressed as the number of spots per 1×10^6 cells.

Statistical analysis

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

Results and Discussion

The nasal mucosa represents an attractive, non-invasive route for the delivery of antigens. However, for mucosal immunization, adjuvants are particularly important since most antigens are poorly immunogenic when given via this route. Currently some of the most potent mucosal adjuvants are toxins that are not practical or safe for human application. We have evaluated a novel delivery vehicle, biphasic lipid vesicles, as a potential intranasal delivery system for the co-administration of OmlA bacterial protein antigen and CpG ODN adjuvant in mice. Table 1 shows the physicochemical properties of VTA2 and VTAM2 biphasic vesicles used for s.c. or i.n. administration, respectively. The s.c. formulation contained cationic vesicles with a bimodal size distribution, whereas the i.n. formulation contained anionic vesicles (the negative charge is due to the enhancer compound PDM 19) with trimodal size distribution characteristics. These measurements are consistent with a topical formulation method where the biphasic vesicle components (submicron emulsion droplets and phospholipids vesicles trapping submicron emulsion droplets, from smaller to larger sizes in Table 1) are present. In addition, the small, about 40 nm, particles in VTAM2 probably represent surfactant micelles that are not part of the vesicle population.

Previously, we have shown enhanced adjuvant activity of CpG ODN and induction of protective immune responses

Table 1 Physicochemical characterization of biphasic vesicles

Biphasic vesicle formulation	Mean size (nm) Vesicles with antigen and CpG ODN	Microscopic observations Vesicles with antigen and CpG ODN	ζ potential (mV)
VTA2 s.c. formula	171 ± 11 (33%)[a] (range 80–300) 587 ± 11 (67%) (range 300–1500)	Very small vesicles, less than 1 μm, fairly homogeneous distribution	+31 ± 2.0
VTAM2 i.n. formula	39 ± 2 (11%) (range 20–60) 177 ± 5 (56%) (range 60–350) 1042 ± 84 (32%) (range 350–2300)	Mostly small vesicles (around 1–2 μm)	−35 ± 1.3

[a]Proportion of vesicles in the size range.

in pigs after systemic immunizations using biphasic vesicles (Alcon et al 2003). Moreover, different bacterial and viral protein antigens administered alone or with CpG ODN in saline by different routes of delivery and in different animal models were shown to be less effective than when combined with biphasic vesicles (Alcon et al submitted for publication; Babiuk et al 2004). To evaluate the efficiency of these formulations as delivery systems for antigens and adjuvants for induction of systemic and mucosal immunity, a combined systemic/mucosal protocol of immunization was used. Mice were first immunized by the s.c. route and 2 weeks later were boosted by the i.n. route (s.c./i.n.). Responses induced by the mucosal protocol were compared to systemic immunization (s.c./s.c.).

Both s.c./s.c. and s.c./i.n. immunization protocols with antigen and CpG ODN in biphasic vesicles induced significantly higher OmlA-specific IgG in the serum of mice compared to naïve animals (both protocols: $P < 0.01$; Figure 1A). In contrast, only the s.c./i.n. protocol induced significantly higher IgA levels in serum ($P < 0.05$; Figure 1B). Additional analysis showed that mucosal immunization (s.c./i.n.) also induced IgA in nasal secretions and lung ($P < 0.01$; Figure 2).

To further characterize the type of immune response induced, antibody isotype levels were determined. While s.c./s.c. immunization with biphasic vesicles containing CpG ODN induced a balanced response, with production of both IgG1 and IgG2a, s.c./i.n. administration induced predominantly IgG1 (Figure 3).

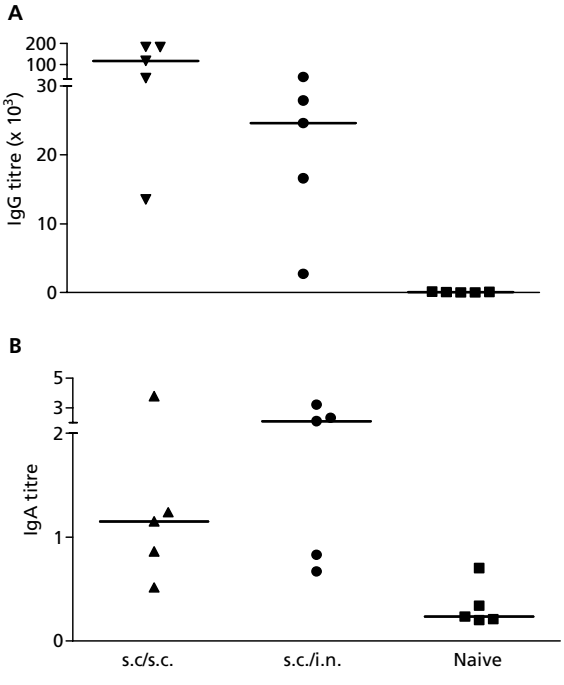

Figure 1 Induction of OmlA-specific antibodies in mouse serum after systemic or systemic/mucosal immunization with biphasic delivery systems containing CpG ODN. Mice were immunized twice s.c. (s.c./s.c. group) or once s.c. followed by i.n. boost (s.c./i.n. group), 14 days apart. IgG (A) and IgA (B) were determined in serum 1 week after the last immunization. Results are expressed as the mean titre ± s.e.m. of five mice.

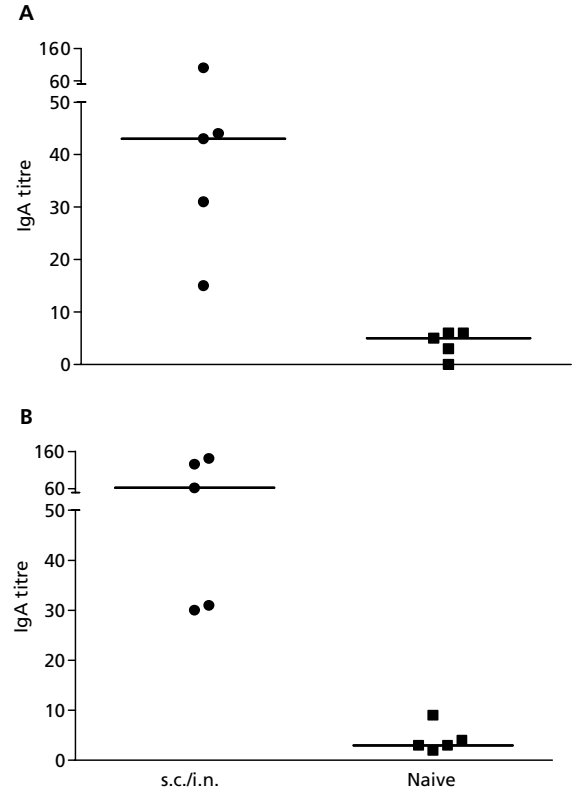

Figure 2 Induction of OmlA-specific antibodies in mucosa by biphasic lipid delivery systems containing CpG ODN. Mice were immunized s.c. and boosted i.n. 14 days later. Antigen-specific IgA was determined in nasal secretions (A) and lung wash (B) 10 days after the last immunization. Results are expressed as the mean titre ± s.e.m. of five mice.

Antigen-specific cellular immune responses induced by biphasic formulations administered intranasally were assessed by determining the frequency of antigen-specific secreting cells in spleen and lungs from immunized mice after the intranasal boost. Administration of CpG ODN in VTAM2 induced predominantly IL-4 ($P < 0.01$) and a lower but significant amount of IFN-γ ($P < 0.01$) in spleen compared to naïve animals (Figure 4A). A high frequency of IFN-γ secreting cells was detected in the lungs of immunized animals (Figure 4B).

Results presented here confirm previous observations that CpG ODN can be an effective mucosal adjuvant (McCluskie & Davis 1999; McCluskie et al 2000, 2001; Gallichan et al 2001) and further indicate that VTA formulations are suitable delivery systems for antigens and CpG ODNs by the intranasal route.

Although several reports have suggested that CpG induces a Th1-like or balanced immune response (Krieg 2000; Ioannou et al 2002), our results show an increase in serum IgA and a Th2-type of immune response, characterized by IgG1 and IL-4 production at the systemic level

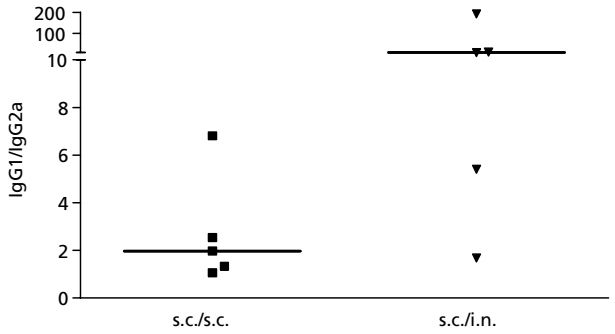

Figure 3 OmlA-specific IgG1 and IgG2a ratios induced by biphasic delivery systems containing CpG ODN. Mice were immunized twice s.c. (s.c./s.c. group) or once s.c. followed by i.n. boost (s.c./i.n. group), 14 days apart. IgG1 and IgG2a titres were determined in serum 1 week after the last immunization. Each point represents an animal, with bars indicating the median for each group.

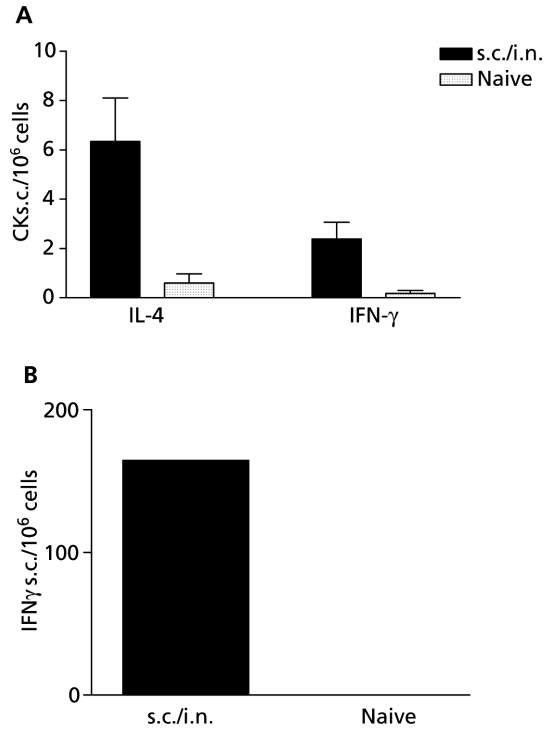

Figure 4 OmlA-specific cytokine (CK) secretion in splenocytes (A) and IFN-γ secretion in lungs from mice immunized intranasally with antigen and CpG ODN formulated in biphasic lipid formulations (B). Mice were immunized s.c. and boosted i.n. 14 days later. Antigen-specific IL-4- and IFN-γ-secreting cells were determined 10 days after the last immunization. Results are expressed as the mean $\pm$ s.e.m. of five mice.

following intranasal administration of CpG ODN in lipid vesicles, while systemic immunization induced a more balanced response. This could be due to the fact that the degree of polarization of cytokine responses is in part a reflection of the antigenic and environmental stimuli. The nature and the magnitude of the immune response elicited by a particular antigen are the result of multiple factors such as the biochemical properties of the antigen (Lee & Sung 1998; Joseph et al 2002), the type of adjuvant used (Ioannou et al 2002), the age of the animals (Brazolot Millan et al 1998) and the method of delivery (Joseph et al 2002). A lipid/ISS-ODN formulation was shown to induce a Th1-dominant response systemically and a more balanced response after i.n. immunization with influenza antigens. Furthermore, the same formulations induced different types of responses following i.m. administration of different antigens (Joseph et al 2002). Moreover, several experimental observations in mice and humans appear difficult to rationalize with the dichotomy of a cross-regulatory Th1/Th2 paradigm (reviewed by Muraille & Leo 1998) that seems to oversimplify the complexity of cytokine patterns.

Clearance of CpG ODN in vivo occurs rapidly (Klimuk et al 2000), and this limits its uptake and subsequently its effectiveness. Formulation of CpG ODN in appropriate delivery systems constitutes a way to potentiate the adjuvant effects of these immunostimulatory molecules by protecting them from degradation while increasing their uptake by cells of the immune system (Gursel et al 1999). At the same time, delivery systems are a critical factor in mucosal immunization (Olszewska et al 2000). Formulating antigen and CpG ODN in VTA formulations was found to be essential for nasal administration to elicit a specific local IgA response in pigs (Alcón et al 2005). The effects of formulating OmlA in VTA may be due to an increase in antigen presentation within the mucosal-associated lymphoid tissue of the respiratory tract and the draining lymph nodes. Hence, VTAM2 may increase the uptake of antigen at mucosal surfaces and CpG ODN by antigen-presenting cells at mucosal surfaces, which in turn migrate to the regional lymph nodes. Alternatively, from the highly vascularized tissue of the nasopharynx, antigen may directly enter the circulation and disseminate to the spleen, priming for the systemic immune response. Further work will provide more understanding of the mechanisms of action by which these formulations induce antigen-specific immune responses, which is critical for the rational design of vaccine delivery systems that produce effective immune responses.

Conclusions

The development of less-invasive and more readily administered vaccines that can be effectively administered by simple, economical and practical immunization procedures has become a priority in the prevention of human and animal diseases. VTA lipid vesicles have been shown to be effective mucosal delivery systems for a bacterial protein antigen and CpG ODNs, inducing both systemic and mucosal immune responses.

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