Polyphosphazenes Enhance Mucosal and Systemic Immune Responses in Mice Immunized Intranasally with Influenza Antigens

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Abstract: Polyphosphazenes are synthetic, biodegradable, water-soluble polymers with a great versatility for vaccine and drug delivery applications. We previously observed that polyphosphazenes enhance immune responses in mice when injected subcutaneously against X:31 influenza antigens. Here, we investigated the potential of polyphosphazenes as mucosal adjuvants for enhancing influenza-specific immune responses. Vaccine combinations with soluble polyphosphazenes (PCPP or PCEP) and influenza X:31 antigen were administered to BALB/c mice intranasally. Antigen-specific antibody responses were assayed in serum and mucosal washes by ELISA, while antigen-specific cytokine production was assayed in spleen cells by ELISPOT assay. We observed that the formulation of either of the two polyphosphazenes with X:31 induced significant and prolonged serum IgG1 antibody responses as early as 2 weeks after primary immunization. Interestingly, only PCEP + X:31 induced a significant and prolonged serum IgG2a response. These results implied that the PCEP+X:31 formulation induced better Th1 immune responses, suggesting increased cell-mediated immunity. To confirm this, we determined that IFN-γ, a Th1 cytokine, was produced at significantly higher levels in spleens from mice that were vaccinated with the PCEP+X:31 formulation, while IL-4, a Th2 cytokine, was produced at higher levels from both vaccinated groups. Finally, we determined that these polyphosphazenes were indeed effective as adjuvants in inducing mucosal immune responses, as IgA and IgG antibodies were detected in lung and vaginal washes. We conclude that polyphosphazenes increase mucosal immune responses effectively, and can be used to modulate the quality of Th1 and Th2 immune responses.

INTRODUCTION

Advances in biomedical research have allowed the rapid identification of antigens from pathogens. The major challenge in developing a vaccine against such pathogens is the formulation and delivery so that the vaccine will induce protective immunity. Nonetheless, vaccination remains the best scientific and economical approach to control infectious diseases.

It is estimated that over 90% of pathogens invade the body by traversing the mucosal barrier. Stimulation of immunity at mucosal sites would be the best approach to the control of mucosal infections, which could be achieved through mucosal vaccination [1]. However, administration of antigen alone at mucosal sites often fails to induce immunity and leads to induction of tolerance.

Instead, administration of antigen with adjuvants can break tolerance and lead to enhanced immune responses. While many trial and error approaches have been used to study various adjuvants, two criteria are paramount in the clinical use of adjuvants: efficacy and safety. There are many adjuvants that can be used for systemically administered vaccines. However, there are relatively few mucosal adjuvants. Microbial components remain the most potent mucosal adjuvants. Cholera toxin (CT), perhaps the most potent mucosal adjuvant known, is powerful at enhancing mucosal Th2 type immune responses [2]. Although less toxic forms of CT have been developed, safety remains a major concern and whether mutant versions of CT will ever be used clinically remains to be seen. Bacterial DNA as well as synthetic oligonucleotides containing CpG motifs have been shown to be potent mucosal adjuvants when given with antigens by intranasal, oral, and interestingly, vaginal routes [3-5]. Nonmicrobial substances have also been shown to have mucosal adjuvant activity. Immunostimulatory complexes (ISCOMs) primarily consists of cholesterol, saponin, phospholipids, and an immunogen that normally complex into 40 nm cage-like colloidal structures [6]. ISCOMs have been shown to induce protective and mucosal immunity against such diseases such as diphtheria in murine models and equine influenza [7,8].

Polyphosphazenes are a class of synthetic polymers consisting of a backbone with alternating phosphorus and nitrogen atoms with organic side groups attached to each phosphorus [9]. These structures can be modified by chemical synthesis to incorporate ionic moieties, permitting water-solubility. Polyphosphazenes are hydrolytically degradable, an important and attractive characteristic for vaccine applications.

One of the most investigated polyphosphazene polyelectrolytes, poly[di(carboxylatophenoxy)phosphazene] (PCPP),

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was previously shown to have adjuvant activity in mice with a variety of viral and bacterial antigens [10-12]. In early studies, an aqueous formulation of PCPP, in conjunction with influenza antigens, enhanced antibody titers by at least 10 fold compared to a formulation without PCPP [10]. PCPP has also shown to be extremely versatile; it was proven to be a potent adjuvant with a variety of antigens including tetanus toxoid, hepatitis B surface antigen (HBsAg), herpes simplex virus type 2 glycoprotein D [13], HIV env [14], cholera and non-microbial antigens such as bovine and porcine serum albumin (BSA, PSA, respectively). Finally, evidence has shown that PCPP formulations can promote long-lasting immune effects; immunogen mixed with PCPP resulted in high antibody titers which were sustained for a 6-month period [10,15]. In particular, rhesus monkeys immunized intramuscularly with PCPP and recombinant HIV-1 showed persistent antibodies for up to 43 weeks [14]. Clearly, this polyphosphazene has shown to have great promise as a potent vaccine adjuvant, as it demonstrated increased immune responses, can be used with more than one antigen, and can confer long-term immunization, all ideal properties of an adjuvant that is cost-effective to the vaccine industry.

Recent advances in the synthesis of polyphosphazene polyelectrolytes resulted in the generation of new, structurally diverse polyacids with a phosphorous-nitrogen backbone. One of these in particular, poly[di(sodiumcarboxylatoethylphenoxy)phosphazene] (PCEP) has been the focus of a recent investigation and was shown to induce significant adjuvant activity that far surpasses that of PCPP in magnitude and quality of immune responses induced in mice that were immunized subcutaneously [9]. Recent studies have shown that intranasal administration of PCPP and bovine respiratory syncytial virus can confer better protection than subcutaneous delivery [16]. However, a direct comparison between PCPP and PCEP, the number of immunizations, and a mucosal delivery of PCEP has not been investigated. Thus, we investigated the mucosal adjuvant activity of PCPP and PCEP with influenza X:31 antigen. Here, we show that, while both polyphosphazenes are able to enhance immune responses, PCEP was more effective than PCPP in promoting a mixed Th1/Th2 response, mucosally and systemically. These results suggest that polyphosphazenes, particularly PCEP, are potent mucosal adjuvants that can modulate the quality of Th1 and Th2 immune responses.

MATERIALS AND METHODOLOGY

Polymer Synthesis and Characterization

Polyphosphazene adjuvants PCPP and PCEP were synthesized by Idaho National Laboratory (Idaho Falls, ID, USA) using approaches described previously [9,15,17,18, 26]. Unit structures of PCPP and PCEP have been previously published [9]. Batches of polyphosphazenes were tested and found to have endotoxin levels that were below 0.034 ng/ml as assessed by Limulus Amebocyte Lysate assay (Biowhittaker, Walkersville, MD, USA). The synthesized polyphosphazenes were in a solid salt state and dissolved in Dulbecco's PBS (Sigma, St. Louis, MO, USA) at a concentration of 5 mg/ml and used appropriately in vaccine formulations.

Preparation of Influenza Virus X:31 Antigen

Purified influenza virus X:31 (A/Aichi/68 H3:N2) was purchased from Charles River Laboratories (North Franklin, CT, USA) as 1 mg/ml concentration. Briefly, the X:31 antigen was processed by first diluting X:31 with an equal volume of PBS, and then solubilised by adding and mixing Tween-80 to a final concentration of 0.25% at room temperature for 30 min. Subsequently, an equal volume of ether was added to the solution, and following another 30 min incubation with mixing, the solution was centrifuged to separate the non-soluble phases. The water-soluble phase was then collected and dried in a fume hood to evaporate residual ether for 1-2 days. The "split antigen" was then quantitated by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA).

Animals and Immunization

BALB/c mice were obtained from Charles River Laboratories (North Franklin, CT, USA). In all experiments, PCPP and PCEP were used at 50 μg per animal, and the experimental vaccine was formulated by simply mixing the X:31 split antigen with an aqueous solution of polymer. Mice were sedated before immunization. In the first experiment, we performed a dose titration study to determine the optimal dose of X:31 influenza antigen to use that would elicit appropriate immune responses for subsequent studies. Three doses (2.5 μg, 0.5 μg, and 0.1 μg) of X:31 antigen were studied where each dose was administered intranasally (IN) to four groups of 5 mice. Two groups were given one intranasal immunization of PCPP + X:31 or X:31 alone, and the other two groups were given a primary (IN) and a secondary subcutaneous (SC) immunization at 4 weeks. Serum was then collected at 8 weeks post IN immunization. It was determined that 2.5 μg/mouse was the optimal dose to elicit immune responses.

In the second experiment, mice (n = 6 mice per group) were given a primary immunization IN of 20 μl (10 μl per nostril) of the following formulations: 1) PCPP alone; 2) PCPP + X:31; 3) PCEP alone; 4) PCEP + X:31; 5) X:31 alone; and 6) PBS. Another 6 groups of mice received the same IN treatment, but were also given a secondary SC immunization. The immunization schedule is summarised in Table **1**. Mice were bled prior to immunization (week 0) and subsequently at 2, 4, 6, and 8 weeks after the primary immunization. All mice were observed for any signs of adverse reaction to immunization. Vaginal washes were collected before immunization and at 4 and 8 weeks. After 8 weeks, lung washes were also collected and the spleen was dissected from all animals in order to assay the antigen-specific cytokines (IFN-γ and IL-4) in splenocytes. All animal work was conducted according to the Guidelines for the Care and Use of Laboratory Animals as indicated by the Canadian Council on Animal Care and the University of Saskatchewan.

IN, INTRANASAL; SC, SUBCUTANEOUS

Collection of Mucosal Washes

Lung and vaginal washes were collected using the protease inhibitor Pefabloc SC^{Plus} (Roche, Indianapolis, IN, USA).

Table 1. Immunization Schedule

Group	Vaccine Formulation	Primary Immunization	Secondary (Booster) Immunization (at 4 Weeks after Primary)
1	PCPP	IN	
2	PCPP	IN	SC
3	PCPP + X:31	IN	
4	PCPP + X:31	IN	SC
5	PCEP	IN	
6	PCEP	IN	SC
7	PCEP + X:31	IN	
8	PCEP + X:31	IN	SC
9	X:31	IN	
10	X:31	IN	SC
11	PBS	IN	
12	PBS	IN	SC

Pefabloc powder was dissolved in PBSA and PSC protector solution as outlined by the manufacturer's instructions to make the 0.4 mM Pefabloc solution. For each wash, 50 and 500 μl of Pefabloc solution was introduced into the vaginal and lung cavities, respectively, and subsequently withdrawn for collection. All samples were promptly centrifuged and the resulting supernatants were collected and analyzed for antigen-specific antibodies.

Detection of Influenza Virus X:31- Specific Antibodies by ELISA

Immunolon II microtiter plates (Dynex Technology INC, Chantilly, VA, USA) were coated overnight at 4ºC with X:31 influenza at 5.0 μg/ml in carbonate coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) and 100 μl of the antigen/coating buffer mixture was added to each well of the plate. Plates were washed 6X with Tris-buffered saline (pH 7.4) containing 0.05% Tween 20 (TBST). Diluted mouse serum samples were added to each plate at 100 μl/well and allowed to incubate at 4ºC overnight. Plates were washed again with TBST. Biotinylated goat-anti mouse IgG1 and IgG2a antibodies (Caltag Laboratories, CA, USA) were added to each plate at 100 μl/well and was subsequently incubated for 1 h at room temperature. IgG and IgA antibodies (Caltag) were used to analyze mucosal secretions. Plates were washed and alkaline phosphatase conjugated with streptavidin (Cedarlane Laboratories, Hornby, ON, CA) was diluted 1/5000, and 100 μl was added in each well followed by another 1 h incubation at room temperature. Plates were washed 8X times in double distilled water (ddH_2O). Di(Tris) p-nitrophenyl phosphate (PNPP) (Sigma) was diluted 1/100 in PNPP substrate buffer and 100 μl/well was added to each plate. The reaction was allowed to develop for 5-15 min, and optical density (OD) absorbance values at 405 nm in a Microplate Reader (BioRad laboratories, Hercules, CA, USA) were recorded. Results are reported as titers, which are the reciprocal of the highest dilution that gave a positive OD reading. A positive titer was defined as an OD reading that was at least two times greater than the values for a negative sample. Negative samples were sera from naïve, unimmunized mice.

Isolation of Splenocytes

At the end of the 8 week experiment, all of the animals were euthanized and spleens were removed and placed in cold, incomplete RPMI 1640 medium (Gibco, Carlesbad, CA, USA). Cells were disrupted by teasing spleen tissue with a syringe plunger through a 40 μm nylon cell strainer (BD Falcon, San Jose, CA, USA). Erythrocytes within the cell suspension were lysed for 5 seconds with sterile ddH_2O; subsequently, 10X PBS was promptly added to prevent lysis of splenocytes. The splenocytes were washed once with incomplete RPMI medium and resuspended in complete RPMI medium (incomplete RPMI, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 10 mM HEPES, 1X antibiotic pen strep, 1% FBS, 20 μm L-glutamine, 50 μm 2-mercaptoethanol) to a final concentration of 1 x 10^7 cells/ml. Cells were counted using a Multisizer™ 3 Coulter Counter (Beckman Coulter, Mississauga, ON, CA) according to the manufacturer's instructions. Cell concentrations were determined using software provided by the manufacturer.

Detection of X:31-specific Cytokine Producing Cells by ELISPOT

Nitrocellulose microtiter plates (Whatman, Florham Park, NJ, USA) were coated with 1.25 μg/ml purified rat anti-mouse IL-4 and IFN-γ capture monoclonal antibodies (BD Biosciences, Mississauga, ON, CA) in coating buffer for 16 h at 4ºC. Plates were then washed and blocked with complete RPMI medium in a 37ºC incubator. Splenocytes were added to these plates at 1 x 10^6 cells/well in complete RPMI medium in triplicate. X:31 antigen (1 μg/well) was added to appropriate wells containing the spleen cells and incubated at

37ºC for 18 h. Cell suspensions were then removed and biotinylated rat anti-mouse IL-4 and IFN-γ monoclonal antibodies (BD Biosciences) diluted in PBS and 0.1% Tween-20 (PBST) at 1.25 μg/ml were added to each plate and incubated for 16 h at 4ºC. Plates were washed with PBST and a streptavidin alkaline phophatase/glycerol solution was added to the plates at 1/500 dilution in PBST for 1.5 h at room temperature. The microtiter plates were then rinsed eight times with ddH_2O and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (NBT/BCIP) (Sigma) insoluble alkaline substrate solution was added to all plates for 5 min at room temperature. Plates were finally washed with ddH_2O and left to dry at room temperature. Spots were counted manually using a Stemo 2000 inverted light stereomicroscope (Zeiss, Toronto, ON, CA).

Statistical Analysis

All data on total IgG, IgG1, IgG2a, and IgA antibody titers in BALB/c mice were analyzed using GraphPad Prism version 5.01 for Windows, GraphPad Software, San Diego, CA, USA, www.graphpad.com and Statistix 7.0 software. The mean serum titers from ELISAs were examined for significance using repeated measures ANOVA with Tukey's Comparison of Rank Sum. Data from ELISPOT assays and ELISAs from the lung and vaginal washes were examined using the Kruskal-Wallis test. If the means were found to be significant, median ranks between pairs of groups were performed using two-tailed Mann-Whitney U tests. Mean comparisons were conducted to compare the magnitude of responses. Significant effects were declared at $p < 0.05$.

RESULTS

Dose Titration of Influenza X:31 Antigen

To determine an optimal dose of X:31 influenza antigen that would elicit appropriate immune responses for these studies, a dose titration study was conducted comparing 0.1, 0.5, and 2.5 μg/mouse of X:31 antigens being administered intranasally into BALB/c mice. In the serum of mice that were given a low dose (0.1 μg/mouse) of X:31, primary IN immunization did not induce any IgG1 response, even when PCPP was added to the formulation (Fig. **1A**). A secondary immunization was necessary to induce significant IgG1 antigen-specific titers. Mice immunized with the medium (0.5 μg/mouse) and high (2.5 μg/mouse) doses had significantly increased IgG1 specific titers, and these were generally similar, regardless of the number of immunizations (Fig. **1A**). Interestingly, at medium and high doses of X:31, one immunization with PCPP was similar to two immunizations with X:31 with no adjuvant. However, when assaying for X:31-specific IgG2a antibody titers, only the mice given the high dose of X:31 antigen showed detectable IgG2a levels in all 4 groups of mice (Fig. **1B**). With the medium and low doses of X:31 administered, a second immunization with PCPP+X:31 was required to observe any IgG2a titers, indicating that PCPP has adjuvant activity when administered intranasally, even for doses as low as 0.1 μg/mouse (Fig. **1B**). Based on these results, 2.5 μg/mouse of X:31 was used in subsequent experiments.

Antibody Responses in Serum of Mice Immunized with X:31 and PCPP or PCEP

The ability of PCEP and PCPP to enhance X:31-specific IgG, IgG1 and IgG2a antibodies was compared in serum collected before and every two weeks after primary IN immunization. In all samples, serum from mice that were administered PBS, PCEP or PCPP alone, did not show any significant antigen-specific titers (data not shown) and thus, were not included in Fig. (**2**). In groups of mice which received only a single immunization during the 8 week experiment (Fig. (**2A**), open symbols), serum from mice given PCPP+X:31 or PCEP+X:31 showed significantly enhanced

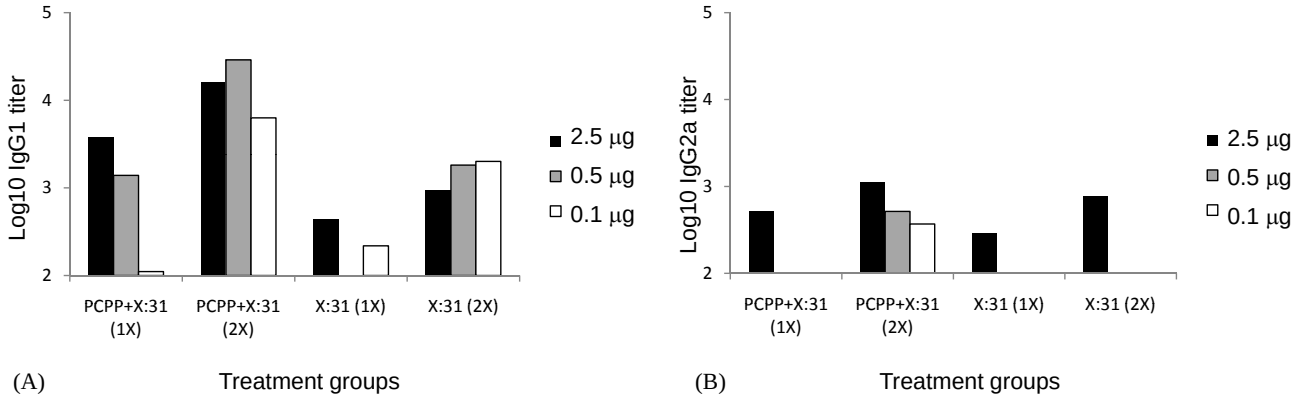

Fig. (1). Dose titration of X:31 and PCPP to optimize the dosage for studying murine immune responses. X:31-specific IgG1 (**A**) and IgG2a (**B**) antibody titers were assayed from serum collected 8 weeks post-immunization from mice that were given three doses (2.5 μg, black bars; 0.5 μg, grey bars; 0.1 μg/mouse, white bars) of X:31 antigen with and without PCPP. The median value from each group of 5 mice is plotted. Since 2.5 μg/mouse was the dose that gave effective IgG1 and IgG2a immune responses, this dose was used in subsequent experiments. Parentheses indicate the number of immunizations.

total IgG antibody titers as early as 2 weeks after the primary IN immunization, compared to mice given X:31 alone (Fig. (**2A**), open squares and circles, respectively, $p < 0.05$). The high antibody titers for both groups remained steady during the course of the 8-week experiment (Fig. **2A**). Mice vaccinated with X:31 alone did not show any significant increase in IgG antibody titers until week 4, when modest increases of IgG antibody responses were observed (Fig. (**2A**), open triangles). As observed previously [9], the pattern of antibody titers observed with IgG1 (Fig. **2B**) were similar to those seen with total IgG (Fig. **2A**). Interestingly, a single immunization with PCPP+X:31 or PCEP+X:31 induced IgG and IgG1 antibody titers at 2 weeks (Fig. **2A** and **2B**, open squares and circles, respectively) that were similar or higher than titers at week 8 in mice given X:31 antigen alone (open triangles).

Initially, the IgG1 response profile patterns seen in the serum of mice that were given a single IN immunization (open symbols) were similar to mice that were given a secondary SC immunization (solid symbols). As expected, at 2 and 4 weeks post-IN immunization, the PCPP+X:31 and PCEP+X:31 groups that were designated to receive a secondary immunization showed IgG1 antibody levels (Fig. **2B**, solid squares and circles, respectively) similar to mice that were not designated to be given the SC secondary immunization (open squares and circles). After the SC immunization,

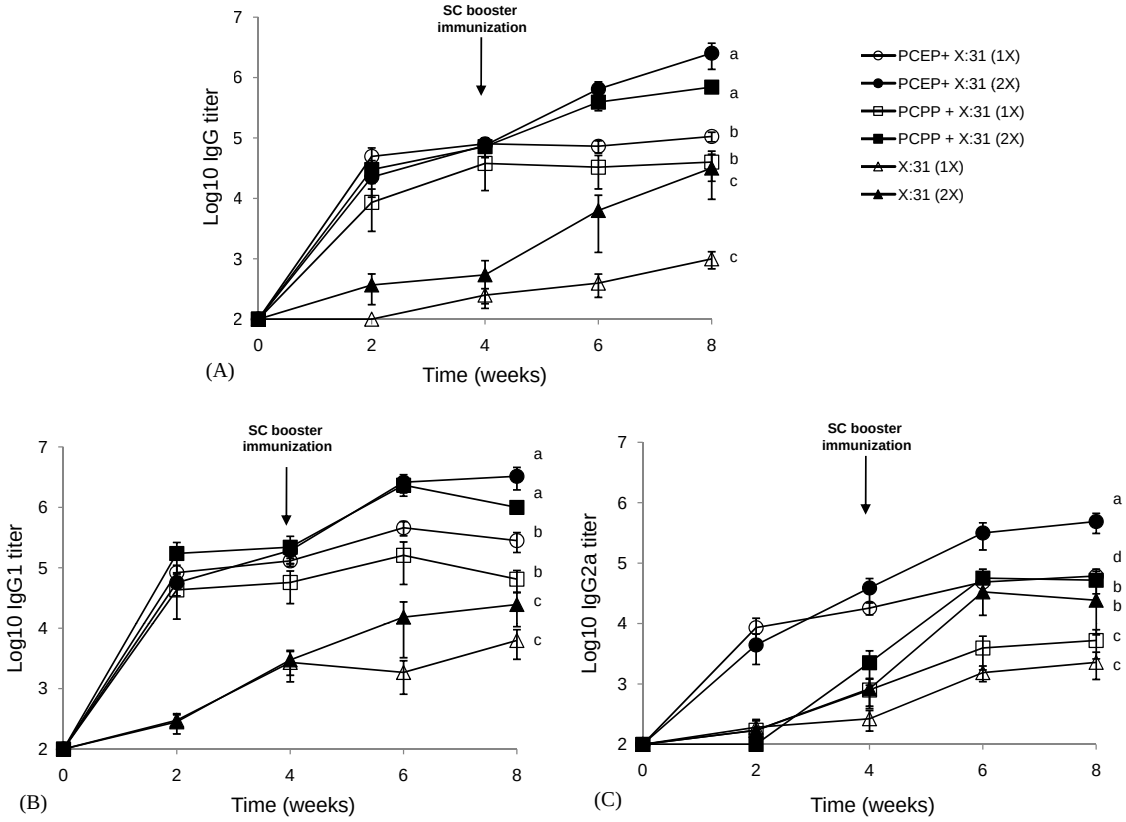

Fig. (2). X:31-specific IgG, IgG1 and IgG2a immune responses are strongly enhanced when PCPP or PCEP is combined with X:31. (**A**) At 2 weeks post immunization, mice given PCPP+X:31 or PCEP+X:31 similarly induced high IgG1 antibody titers significantly, while the X:31 alone group comparatively showed low IgG1 titers. After a secondary immunization was given at 4 weeks, mice that were given two immunizations (solid symbols) of PCPP+X:31 or PCEP+X:31 showed approximately a 10-fold significant ($p < 0.05$) increase of antigen-specific IgG1 titers in serum collected 8 weeks after IN immunization compared to mice that only received a single IN immunization (open symbols). (**B**) The immune response profiles when analyzing IgG1 titers were very similar to IgG. (**C**) As early as 2 weeks post IN immunization, PCEP+X:31 treated mice showed significant IgG2a antibody titers compared to mice given PCPP+X:31 or X:31 alone. The secondary immunization at 4 weeks further enhanced IgG2a titers from the PCPP+X:31, PCEP+X:31 and X:31 alone groups approximately 10-fold ($p < 0.05$, solid symbols). However, mice given PCEP+X:31 had significantly higher antigen-specific IgG2a titers compared to the PCPP+X:31 group. There were no significant differences in IgG2a titers between the PCPP+X:31 and X:31 alone groups, regardless of the number of immunizations. Groups with different letters are significantly different from each other ($p < 0.05$). Parentheses indicate the number of immunizations.

however, IgG1 antibody titers for PCPP+X:31 and PCEP+X:31 groups were significantly increased by about 10-fold (Fig. **2B**, solid squares and circles, $p < 0.05$), whereas in the X:31 group, there was only a moderate increase in IgG1 titers (Fig. **2B**, solid triangles). Furthermore, it was clear that one immunization with PCPP+X:31 or PCEP+X:31 (open squares and circles) was more effective in enhancing IgG1 antibody than two immunizations with only X:31 (solid triangles).

When IgG2a X:31-specific antibody titers were evaluated in mice given only one IN immunization (Fig. **2C**), PCEP+X:31 (open circles) induced significant IgG2a antibody titers, nearly 100-fold compared to the PCPP+X:31 (open squares) group as early as 2 weeks post immunization and 10-fold after 8 weeks (Fig. **2C**). Interestingly, there were no significant differences in IgG2a antibody titers between the mice immunized with PCPP+X:31 (Fig. **2C**, open squares) or X:31 antigen alone (Fig. **2C**, open triangles) during the 8 week experiment.

When randomly selected mice (Fig. **2C**, solid symbols) were given the secondary SC immunization, the PCEP+X:31 group showed a significant increase of serum IgG2a titers (Fig. **2C**, solid circles, $p < 0.05$). While the second immunization of PCPP+X:31 and X:31 further enhanced IgG2a titers at 8 weeks as well, there were no significant differences in titers between the 2 groups (Fig. **2C**, solid squares and triangles, respectively). The 10-fold difference in IgG2a titers between the PCEP+X:31 and PCPP+X:31 treatment groups was maintained after the second immunization (Fig. **2C**, solid circles and squares, respectively).

Mucosal Responses

Since the main aim of the present investigations was to determine if mucosal immunity was enhanced by polyphosphazenes, we assayed antigen-specific IgA and IgG antibody titers in secretions from broncheo-alveolar lavages in mice. Mice that received PCEP+X:31 showed four times more IgA antibody production in lung washes compared to mice primed with PCPP+X:31 (Fig. **3**). The administration of a second immunization further enhanced IgA levels for both groups of primed mice receiving the polyphosphazene/X:31 combination (Fig. **3**, $p < 0.05$). When IgG was assayed by ELISA, mice given two immunizations of PCPP+X:31 or PCEP+X:31 showed a significant 5-6 fold increase in X:31 specific IgG titers compared to mice given a single immunization (Fig. **3**). Clearly, polyphosphazenes greatly enhances mucosal immune responses which is indicative of their mucosal adjuvant activity.

In addition to lung washes, vaginal secretions were also analyzed for IgA and IgG. However, only a few samples from mice were observed to have any antibody production. Mice that did show increased IgA and IgG levels were from groups that were given PCPP+X:31 or PCEP+X:31, regardless of the number of immunizations (data not shown).

IFN-γ and IL-4 Cytokine Responses

To determine if the elevated levels of IgG1 and IgG2a were a result of appropriate Th2 and Th1 stimulation, respectively, mouse splenocytes were collected, cultured, and assayed for IL-4 and IFN-γ production, respectively, using

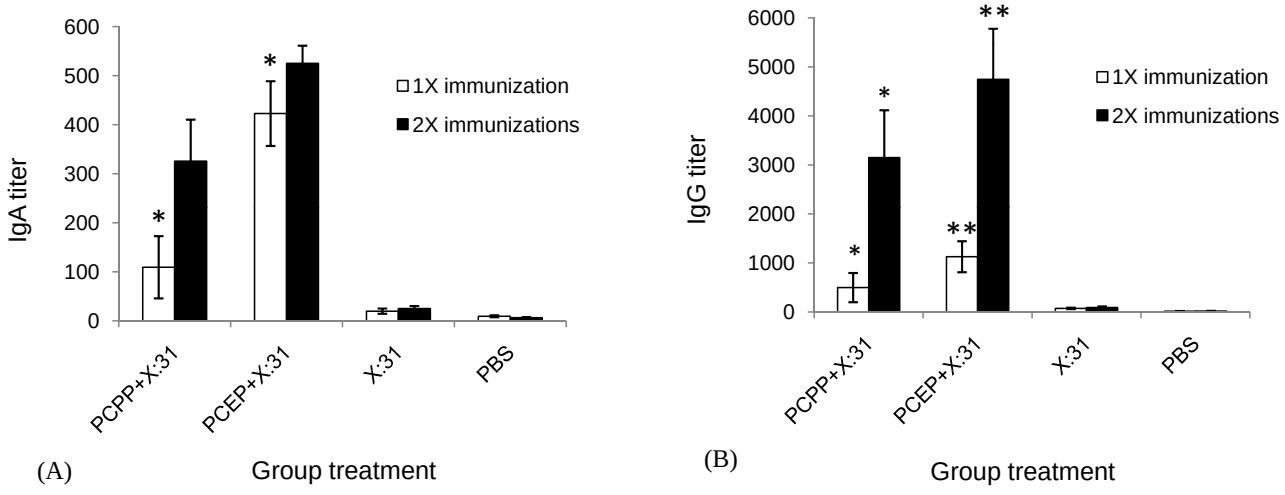

Fig. (3). IgA and IgG antigen-specific titers are elevated in lungs of mice immunized with polyphosphazenes and X:31. (**A**) To determine if mucosal immunity was enhanced by the intranasal administrations, IgA, an antibody commonly associated with mucosal immunity was analyzed in lung washes. In mice that did not receive the secondary immunization, the PCEP+X:31 group induced significantly higher levels of IgA antigen-specific titers compared to the PCPP+X:31 group. Regardless of the number of immunizations or the polyphosphazene used, mucosal immunity in the lungs was enhanced. Groups with asterisks indicate significant differences from each other ($p < 0.05$). (**B**) Similar to IgA antigen-specific titers, total IgG titers were enhanced by the X:31/polyphosphazene combinations. In particular, the secondary immunization showed significant increase in IgG antigen-specific titers compared to the group that only had one immunization. Noted groups with the same number of asterisks are significantly different from each other ($p < 0.05$).

X:31 split antigen as the immunogen. Groups of mice given a single immunization of PCPP+X:31 or PCEP+X:31 both showed enhanced IFN-γ production compared to controls ($p < 0.05$, Fig. **4**). While the second immunization of PCPP+X:31 further increased IFN-γ production approximately 3-fold, cytokine production was significantly enhanced nearly 8-fold in mice given the booster immunization of PCEP+X:31 ($p < 0.05$, Fig. **4**). The difference in IFN-γ production between PCPP+X:31 and PCEP+X:31 was significant as well ($p < 0.05$). Since the presence of IgG2a is an indicator of IFN-γ production and thus, a Th1 immune response, the enhanced IFN-γ production, as a result of the administration of PCEP+X:31, was consistent with the increased IgG2a production, and thus showed that PCEP+X:31 induced a stronger Th1 response compared to mice immunized with PCPP+X:31 (Fig. **2B**). Interestingly, one immunization with either PCPP+X:31 or PCEP+X:31 was equivalent in IFN-γ production to two immunizations of X:31 alone (Fig. **4**).

When IL-4 was assayed, cytokine production was clearly enhanced in spleen cells from mice that were immunized with the polyphosphazene/X:31 combination, regardless of the number of immunizations or which polyphosphazene was used compared to controls (Table **2**). These results confirmed that the similar increases of IgG1 titers (Fig **2A-B**) corresponded with the enhanced equivalent production of IL-4 (Table **2**). Enumerating the exact number of IL-4 producing cells per well was not possible with splenocytes from mice given the polyphosphazene/X:31 combinations because some wells had too many spots to count. This, in part, could be attributed to the genetic background of BALB/c mice.

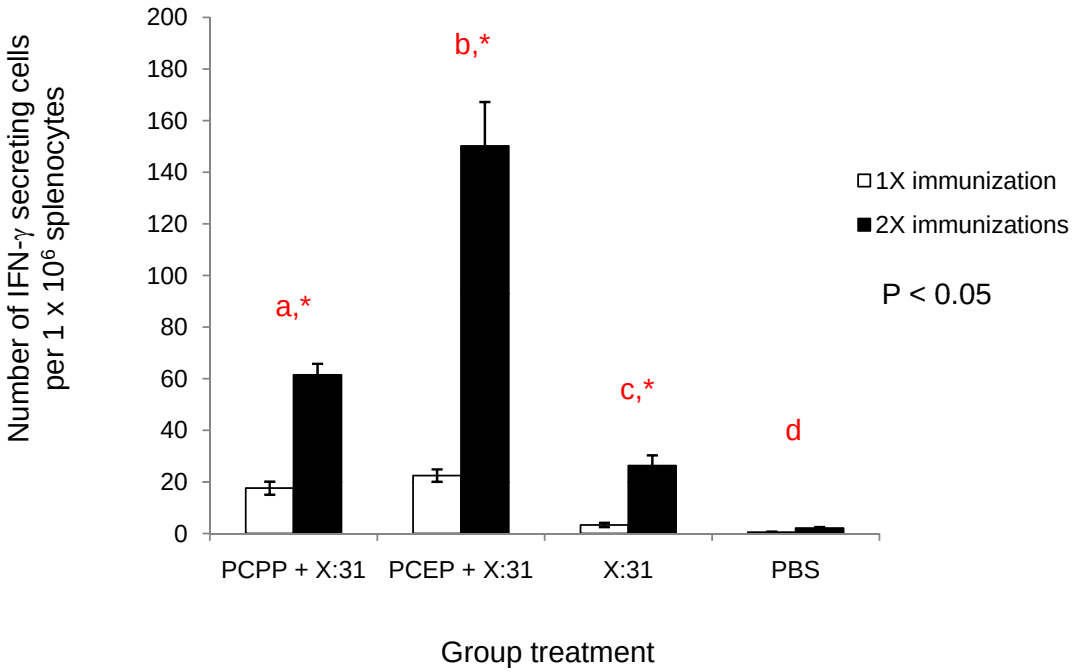

Fig. (4). Secondary immunization of X:31 and PCEP combination significantly enhances IFN-γ production. ELISPOT assays were performed with mice splenocytes that were stimulated with X:31 split antigen for 16 hours. Mice treated with PCPP+X:31 and PCEP+X:31 showed significant increase of IFN-γ production, particularly in the groups that received the second immunization. More importantly, mice that were given a booster immunization of PCEP+X:31 showed significantly higher IFN-γ production compared to the group that received PCPP+X:31, strongly suggesting that PCEP is better than PCPP in enhancing a stronger Th1 cell-mediated response. Groups with different letters are significantly different from each other ($p < 0.05$). Asterisks indicate a significant difference between one and two immunizations of the same formulation ($p < 0.05$).

Table 2. Relative Comparison of IL-4 Secreting Cells Stimulated by X:31 Influenza Antigen

Formulation	Number of IL-4 Secreting Cells Stimulated by X:31 after one Immunization	Number of IL-4 Secreting Cells Stimulated by X:31 after Two Immunizations
PCPP + X:31	81-200	> 200
PCEP + X:31	> 200	> 200
X:31	21-40	21-40
PBS	0-20	0-20

Previous experiments have shown that the BALB/c strain can have significantly higher production of IL-4 compared to other mice strains, such as C57 BL6 mice [19].

DISCUSSION

While polyphosphazenes have shown to be potent immunological adjuvants, this investigation reveals, for the first time, the potential of PCPP and PCEP to: 1) improve murine Th1 and Th2 immune responses following mucosal immunization, and 2) reduce the number of immunizations given. Both are particularly important in the vaccine industry. The ability of an adjuvant to change the quality of the immune responses is also important as this allows flexibility in designing vaccines appropriate for specific pathogens. Reducing the number of immunizations for a vaccine is an important factor in reducing the cost of vaccination and also in improving compliance with immunization schedules.

Biodegradable polyphosphazenes are becoming increasingly recognized for their safe and effective properties as adjuvants that enhance immune responses, not only in mice [9,16,19,20], but larger animals [21] as well. Since most infections invade the body through mucosal surfaces, it is only reasonable to ascertain that mucosal immunization is the best approach to target against infections. For example, intranasal immunizations have shown to be effective and superior to subcutaneous delivery in conferring protection against bovine respiratory syncytial virus (BRSV) following immunization with antigen formulated with PCPP and CpG oligodeoxynucleotides (ODN) [16]. While the addition of CpG to polyphosphazenes have shown to greatly enhance Th1 immune responses [16,19], our study showed that by merely using different polyphosphazenes, similar convincing results can be achieved. This is ideal since fewer components needed to formulate an effective vaccine would be less of a burden when cost of formulation is considered.

PCPP is one of the most investigated polyphosphazenes, where several studies have showed its adjuvant activity with many antigens [9,16,17,22-25]. Our results showed that PCEP is superior to PCPP, particularly increasing Th1 X:31-specific immune responses, which are consistent from previous results [9,26], regardless of the administration route that was used for immunization. Subcutaneous delivery of PCEP and X:31 antigen not only showed significant enhancement of IgG1 and IgG2a antigen-specific titers, but also a shift in the quality of Th1 versus Th2 immune responses from a solely Th2 response to a mixed Th1/Th2 type response, compared to formulations of PCPP and X:31 [9]. Similarly, our studies of intranasal delivery showed that PCEP+X:31 treatment enhanced IgG2a titers as early as 2 weeks, which is ideal for stimulating cell-mediated Th1 immune responses immediately to target and eliminate infectious agents. In fact, for one intranasal immunization, nearly 8 weeks was required for mice immunized with PCPP+X:31 to approach IgG2a titers obtained from the PCEP+X:31 in only 2 weeks post-immunization. In addition, since PCEP was equivalent to PCPP in enhancing X:31-specific IgG1 antibody and IL-4 production, yet PCEP significantly enhanced IFN-γ production, we conclude that PCEP is a better immunoadjuvant than PCPP in promoting both cell mediated and humoral immune responses.

Another important consideration in vaccine formulation is the number of immunizations needed to achieve a proper immune response. In our studies, a single IN immunization of PCPP+X:31 or PCEP+X:31 was sufficient to sustain IgG1 and IgG2a antibody titers over the course of the 8 week experiment without the need for the secondary booster immunization. Furthermore, a single immunization of either formulation was equivalent to two immunizations of X:31 alone in IFN-γ production. A similar study showed that PCEP-induced titers were sustained for at least 6 months from one immunization [9]. These observations have implications in the development of single dose vaccines, which are ideal in order to reduce booster immunizations. This is particularly significant since multiple immunizations negatively impacts on compliance with immunization schedules and also adds to the cost of the vaccines. Given that one immunization with polyphosphazenes was as effective as two immunizations of antigen alone, a second immunization of X:31/polyphosphazenes not only enhanced IgG1 and IgG2a titers approximately 10-fold, but also, the primed mice showed significant increases in IFN-γ production, particularly mice given PCEP+X:31, where there was nearly an 8-fold increase in IFN-γ production (Fig. **4**). As such, at least in murine models, the second immunization was important in further strengthening Th1 immune responses in primed mice.

Interestingly, regardless of the number of immunizations, IgG2a titers were practically similar between the PCPP+X:31 and X:31 groups. This strongly suggests that PCPP was ineffective at enhancing Th1 immune responses as delivery of X:31 alone could achieve similar results. This may have been one of the key reasons why some studies [16] included CpG to achieve a stronger Th1 immune response with regards to PCPP. However, ELISPOT assays showed that PCPP+X:31 was more effective than X:31 alone in IFN-γ production (Fig. **4**). As such, although examining IgG1 and IgG2a antibody titers can be an indicator of Th2 and Th1 immune responses, respectively [27], our observations suggest that cytokine analyses should be performed to properly study the quality of immune responses.

Due to the unexpected result of similar IgG2a immune response profiles between PCPP+X:31 and X:31 alone groups, it is conceivable that the dose of the antigen could have altered the magnitude and possibly quality of immune responses. Using five-fold less antigen, it was clear that IgG2a titers were significantly higher in mice given PCPP+X:31 compared to those given X:31 alone (Fig. **1B**); however, a second immunization was required to achieve this result. These results stress the need for dose titrations when evaluating adjuvant activity.

Ultimately, enhancing mucosal immune responses would be a key indicator in determining whether polyphosphazenes have adjuvant activity at mucosal surfaces. Consistent with the elevated IgG subtype titers, mice given PCEP+X:31 showed significantly higher IgA titers compared to mice given PCPP+X:31 in lung washes. This particular antibody was assayed because IgA is often associated with mucosal immunity [28,29]. With total IgG showing similar profiles, we conclude that polyphosphazenes clearly enhance mucosal immune responses more effectively than antigen alone. Moreover, one immunization was sufficient to significantly

increase IgA titers from mice given PCEP+X:31 by 4-fold. When vaginal washes were examined, only some of the mice responded in IgA and IgG ELISA assays. Only mice given X:31/polyphosphazene immunizations were among those that did show an immune response and none of the control groups did. It was noted that in many cases, the volume of vaginal wash collection was inconsistent, probably due to the fact that in some cases, washes were taken while the mice were alive and may have negatively responded to wash collection protocols. Nonetheless, the fact that enhanced levels of IgA and IgG were only observed from mice given X:31/polyphosphazene is encouraging since this would possibly indicate that the increase of mucosal immune responses as a result of intranasal administration can be systemic and be found at other mucosal surfaces.

Even though PCPP and PCEP are clearly able to enhance Th2 and Th1 immune responses, respectively, it is important to determine whether actual protection is being conferred. While our prior research has shown that functional anti-influenza antibodies, as evidenced by serum neutralizing titers, were induced by PCEP [9], immunized mice still need to be challenged with live influenza virus to determine if actual protection is conferred.

Despite the recent advances in polyphosphazene research and its promising potential, the majority of these studies have been performed using mice, and as such, do not necessarily reflect whether the adjuvant works effectively and safely in humans and in larger animals. This is the case with CpG; when used alone, it can induce systemic and mucosal activity in mice; however, in larger animals, CpG needs to be combined with other adjuvants or delivery systems to optimize CpG efficacy [30]. Fortunately, one study showed that PCPP was administered safely to a study of sheep [21]. In this experiment, the addition of PCPP to a model antigen, porcine serum albumin (PSA), increased IgG antibody titers by 100 fold compared to PSA antigen without PCPP. Also, the IgG levels remained persistent during the duration (10 weeks) of the experiment. The versatility of PCPP is prevalent as formulations of PCPP and truncated glycoprotein D (tgD) from bovine herpesvirus-1 also induced an increase of 10 fold tgD-specific antibody response in sheep [21]. More recent results suggest that PCEP is a powerful adjuvant in pigs (unpublished observation). Interestingly, PCEP significantly enhanced antigen-specific IFN-γ producing cells suggesting the potential of this adjuvant in pigs. As a result, the studies showed that the use of polyphosphazenes in larger animals is very promising as an effective and safe adjuvant.

CONCLUSION

Polyphosphazenes are a class of adjuvants that is generating great interest because of the many desirable features that are ideal in vaccine formulations. We have shown that polyphosphazenes not only enhances immune responses, but also can reduce the number of vaccinations, can induce mucosal immunity, and that PCEP is superior to PCPP in enhancing Th1 immune responses. While the mechanisms which mediate the adjuvant activity of polyphosphazenes are not fully understood, further research and development of polyphosphazene adjuvants is certainly warranted to fully explore their potential for application in human and animal vaccines.

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