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Epidermal powder immunization of mice and monkeys with an influenza vaccine

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Abstract

Epidermal powder immunization (EPI) with an influenza vaccine and an adjuvant such as QS-21, LTR72, or cholera toxin elicited augmented serum and mucosal antibody responses in mice. Rhesus macaques, which have an immune system and skin structure similar to humans, were used to further evaluate the immunogenicity of the influenza vaccine following EPI. EPI of rhesus macaques with an influenza vaccine and QS-21 adjuvant elicited significantly higher serum hemagglutination inhibition (HI) titers than antigen alone administered by EPI or by intramuscular (IM) injection using a needle and syringe. In the absence of QS-21, EPI and IM injection elicited comparable HI titers in the monkeys. This study suggests that EPI is a promising technique for administering human vaccine and that QS-21 augments the immunogenicity of co-administered influenza vaccine.

Keywords: Vaccine; Adjuvant; Influenza; Mucosal antibody; Primate

1. Introduction

Influenza affects millions of people every year. It causes a high morbidity in people of all ages and a high mortality in the elderly and other high-risk populations [1,2]. Annual vaccination remains the most effective means of controlling the disease. The current influenza vaccine, which is normally administered by intramuscular (IM) injection using a needle and syringe [3–5], has an efficacy in the range of 50–80%, depending on the recipient's age and health status. One strategy to improve vaccine efficacy may be to alter the route of immunization and/or to use an adjuvant.

We previously reported that epidermal powder immunization (EPI), by targeting antigens to Langerhans cells, is potentially a more effective immunization method than traditional needle injection, which delivers vaccine to a deeper tissue, thus, bypassing the immunopotent skin [6]. EPI of mice was found to elicit augmented serum antibody responses to a number of antigens including influenza antigen, hepatitis B surface antigen, HIV gp120, and diphtheria toxoid [7–9]. Co-administration of influenza vaccine and cholera toxin adjuvant by EPI induced both serum and mucosal antibody responses, which are considered important for protection against influenza [10–12].

The anatomical structure and immunocompetence of the skin, which vary from animal species to animal species, may influence the quality and the magnitude of the immune responses to antigens administered by EPI. Mice are known to have a very thin skin and respond extremely well to vaccines administered by the skin route [13,14]. Non-human primates are more relevant animal models for evaluating human vaccines and adjuvants because of the resemblance of their immune system and skin structure to humans [13,15]. In the present study, we first selected a best-performing adjuvant-containing influenza vaccine formulation based on its ability to augment the serum and mucosal antibody responses to influenza vaccine in mice and its acceptable tolerability at the delivery site in pigs; and then, evaluated the immunogenicity of the formulation in rhesus macaques.

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2. Materials and methods

2.1. Vaccines and adjuvants

All studies used commercial trivalent human influenza vaccine from 2000 to 2001 season (Evans Vaccines, Speke, UK). This vaccine is composed of A/New Caledonia/20/99 IVR-166 (H1N1) (A/New Caledonia), A/Panama/2007/99 RESVIR-17 (H3N2) (A/Panama), and B/Yamanashi/166/98 (B/Yamanashi) viruses.

The QS-21 adjuvant was obtained from Antigenics (Woburn, MA); LTR72 was obtained from Chiron Vaccines (Emeryville, CA); and cholera toxin (CT) was purchased from Sigma Scientific Chemicals (St. Louis, MO). LTR72 is a genetically detoxified heat-labile toxin from *E. coli* (alanine-to-arginine substitution at position 72 in the A subunit) with only residual enzymatic activity of the wild-type toxin [16].

2.2. Powder formulation and characterization

The bulk vaccine was concentrated to 87.5 mg protein/ml by ultrafiltration using a combination of a Tangential Flow Filtration System (Labscale™, Millipore, Bedford, MA) and a Centriprep® with a 10 kDa cellulose membrane (Millipore). The concentrated bulk vaccine was combined with an excipient solution containing trehalose, mannitol and dextran (10,000 kDa) at 3:3:4 ratio (w/w/w). The adjuvant was added to the aqueous vaccine formulations before preparing dry powders.

The formulation solution was sprayed into liquid nitrogen (−196 °C) using an ultrasonic atomizer (Sono-Tek Corporation, Milton, NY) at a 60 kHz frequency. The resultant frozen powder of the trivalent bulk vaccine was lyophilized in a freeze-drier (Dura-Stop, FTS System, Stone Ridge, NY) [17].

Vaccine powders were characterized using previously reported methods [18]. The external morphology of coated particles was examined using an Amray 1810T scanning electron microscope (Amray, Bedford, MA). The mean geometric/aerodynamic diameter of the particles in the volume distribution was determined using a time-of-flight particle size analyzer (Aerosizer, API, Minneapolis, MN). The tap density of the powder sample was determined as previous described [18]. The potency of the influenza vaccine was determined by measuring the hemagglutinin (HA) content using a standardized single radio-immunodiffusion (SRID) assay [19]. The QS-21 content in the powder formulation was determined by HPLC analysis.

2.3. Immunization and challenge

Mice: Female 6-week-old Balb/c mice (four animals per group) from Harlen–Sprague–Dawley (Indianapolis, IN) were fully anesthetized by intraperitoneal injection of ketamine and xylazine and their abdominal skin clipped prior to EPI. Since only a small dose of vaccine is required to immunize mice, blended powder formulations were prepared by mixing placebo (prepared with excipients without vaccine or adjuvant) and active powder formulation at a 9:1 ratio so that each mg of powder formulation contained 1.5 μg of HA per virus (×3 viruses) alone, or antigen plus 5 μg of QS-21, 2.5 μg of cholera toxin, or 2.5 μg of LTR72.

The EPI procedure and the PowderJect® ND device have been previously described [8]. In brief, the device was placed against the shaved abdominal skin of animals and actuated by releasing compressed helium from the gas cylinder. Each administration delivered 1 mg of blended powder. Immunizations were performed on days 0 and 28. Blood, saliva, nasal, and bronchio-alveolar lavage (BAL) fluids were collected on day 42 using previously described methods [11].

Control mice (four animals per group) received IM injection of the influenza vaccine without an adjuvant, mimicking the current practice of influenza immunization. Each animal was injected with 50 μl liquid influenza vaccine (1.5 μg of HA per virus × 3 viruses) into the quadricepes muscle using a 26-gauge needle.

Monkeys: Rhesus macaques (six animals per group, mixed ages) were immunized by EPI with 1 mg of powders containing trivalent influenza vaccine alone (15 μg HA per virus) or trivalent influenza vaccine and 50 μg of QS-21 on the inner upper arm skin on days 0 and 30. The same PowderJect® ND used for the mouse study was used to immunize monkeys. Six animals were injected IM into the quadriceps muscle with the same dose of liquid vaccine without an adjuvant. Blood samples were collected on days 0, 14, 30, 44, 60, 74, and 88 to assess antibody responses by HI assays.

All immunized monkeys and 8 naive controls were challenged on day 60 by intranasal instillation of 1.0 ml (0.5 ml per nostril) solution containing 10^6 $TCID_{50}$ of A/Sydney/5/97-like virus (H3N2). The challenge virus had been passed several times in monkeys by intranasal inoculation. Nasal swabs were made on days 61, 62, 63, 64, 65, 66, and 67 for measuring $TCID_{50}$ in MDCK cells.

Mice and monkeys were housed in specific-pathogen-free facilities, provided free access to water and food, and cared for by adherence to the USDA Animal Welfare Act, the Guide and PHS policy.

2.4. Hemagglutination inhibition (HI) assay

The HI assay was performed using a previously described method [20]. Animal sera were treated with receptor destroying enzymes from *Vibrio cholera* (Takeda Chemical Ind., Osaka, Japan) to remove non-specific inhibition. Chicken red blood cells were purchased from Charles River Spafas (New Franklin, CT). Assays were performed in 96-well microtiter plates (Dynex Technologies, VA). The HI titer was defined as the reciprocal of the highest serum dilution that completely inhibited hemagglutination of the chicken red blood

cells by four hemagglutination units of influenza virus. HI titer was determined using individual serum and geometric mean titers of the group were calculated.

2.5. ELISA

The IgG and IgA antibody titers to influenza vaccine in the mucosal secretions were measured using ELISA as previously described [11]. Briefly, a 96-well Costar medium binding plate (Fisher Scientific Products, Pittsburgh, PA) was coated with 1 μg of influenza virus antigen (the same trivalent vaccine used for immunization) per well in 30 mM phosphate buffered saline (PBS, pH 7.4) overnight at 4 °C. Plates were washed and then incubated with test samples diluted in PBS containing 5% dry milk for 1.5–2 h at room temperature, followed by three washes and a 1 h incubation with biotin-labeled goat anti-mouse IgG. Following three additional washes, plates were incubated with a streptavidin–horseradish peroxidase conjugate (Southern Biotechnology) for 1 h at room temperature. Finally, plates were washed and developed for 15 min with the substrate 3,3′5,5′-tetramethylbenzidine (Bio-Rad Laboratories, Melville, NY). The reaction was stopped by the addition of 1 N sulfuric acid. The plates were read at 450 nm wavelength using an Emax plate reader (Molecular Devices, Sunnyvale, CA).

2.6. Statistical analysis

The HI titers of individual monkey samples were logarithmically (log 2) transformed before statistical analysis using the one-tailed Student t test (JMP, SAS Institute, Inc., NC). *P*-values of less than 0.05 were considered statistically significant.

3. Results

3.1. Powder formulation and characterization

The dry powder contains spherical particles under light and scanning electron microscopy with a mean particle size of 46 μm (D_{10} = 31 μm, D_{90} = 63 μm) with a density of approximately 1 mg/ml, and a moisture content of 2.0% (w/w). Each mg of powder contained 15 ± 2 μg of HA for each vaccine virus (a total of 45 μg of HA) as determined by the SRID assay. Each mg of the adjuvanted powder formulation contained 50 μg QS-21 determined by HPLC analysis, 25 μg of CT, or 25 μg of LTR72 as determined by microbicinchoninic acid assay (Pierce, Rockford, IL). These physical and chemical characteristics of powder formulations, when stored as bulk powder in a sealed glass vial at 40 °C, remained unchanged for at least 16 weeks, the longest time point examined. The potency of the vaccine as determined by SRID assay was also unchanged during the 16-week storage (Fig. 1).

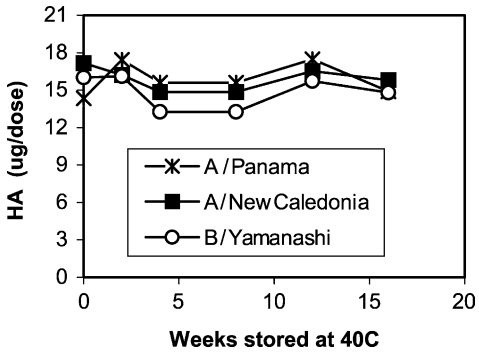

Fig. 1. Stability of spray–freeze–dried influenza vaccine. Trivalent influenza vaccine was formulated with trehalose, mannitol, and dextran, and spray–freeze–dried. Powder samples were stored in sealed glass vials at 40 °C with 75% humidity. Data is HA content in 1 mg of spray–freeze–dried powder determined at different storage times using the SRID assay.

3.2. Serum and mucosal antibody responses in mice

Three adjuvants, QS-21, CT, and LTR72, were compared in the mouse study for their ability to enhance the serum and mucosal antibody responses to the trivalent influenza vaccine administered by EPI. Control animals received influenza vaccine alone by either IM or EPI. Compared to IM injection, EPI with influenza vaccine alone elicited approximately three-fold higher serum HI titers against the B/Yamanashi and A/Panama viruses (Fig. 2A, $P < 0.05$, *t*-test), and comparable HI titers to the A/New Caledonia virus. EPI using QS-21, LTR72, or CT elicited significantly higher HI titers against all three-vaccine viruses than influenza vaccine alone administered by EPI ($P < 0.05$) or IM injection ($P < 0.05$). There were no differences in HI titers against corresponding virus among the animals receiving different adjuvant formulations. Only the mice immunized via EPI with an adjuvant formulation had HI titers in the BAL fluid, in particular against the B/Yamanashi and A/Panama viruses (Fig. 2B).

IgG (Fig. 2C) and IgA (Fig. 2D) antibodies to influenza vaccine in saliva, nasal, and BAL fluid were detected by ELISA using trivalent influenza vaccine as detection antigens. Trivalent vaccine without an adjuvant administered IM or by EPI elicited low levels of antigen-specific IgG but no IgA antibodies. However, significantly higher IgG and detectable IgA antibodies were detected in the mucosal lavage fluids of the mice immunized with adjuvanted vaccine formulations via EPI.

All three adjuvants, QS-21, LTR72, and CT, appear to be well-tolerated and are equivalently potent in enhancing the serum and mucosal antibody responses to influenza vaccine following EPI in mice. However, when these vaccine formulations were administered to pigs by EPI, CT and, to a less extent, LTR72 caused erythma and induration at the target sites that lasted for up to 2 weeks (unpublished observations). In contrast, QS-21 formulation following EPI was well tolerated. This observation, along with the potential

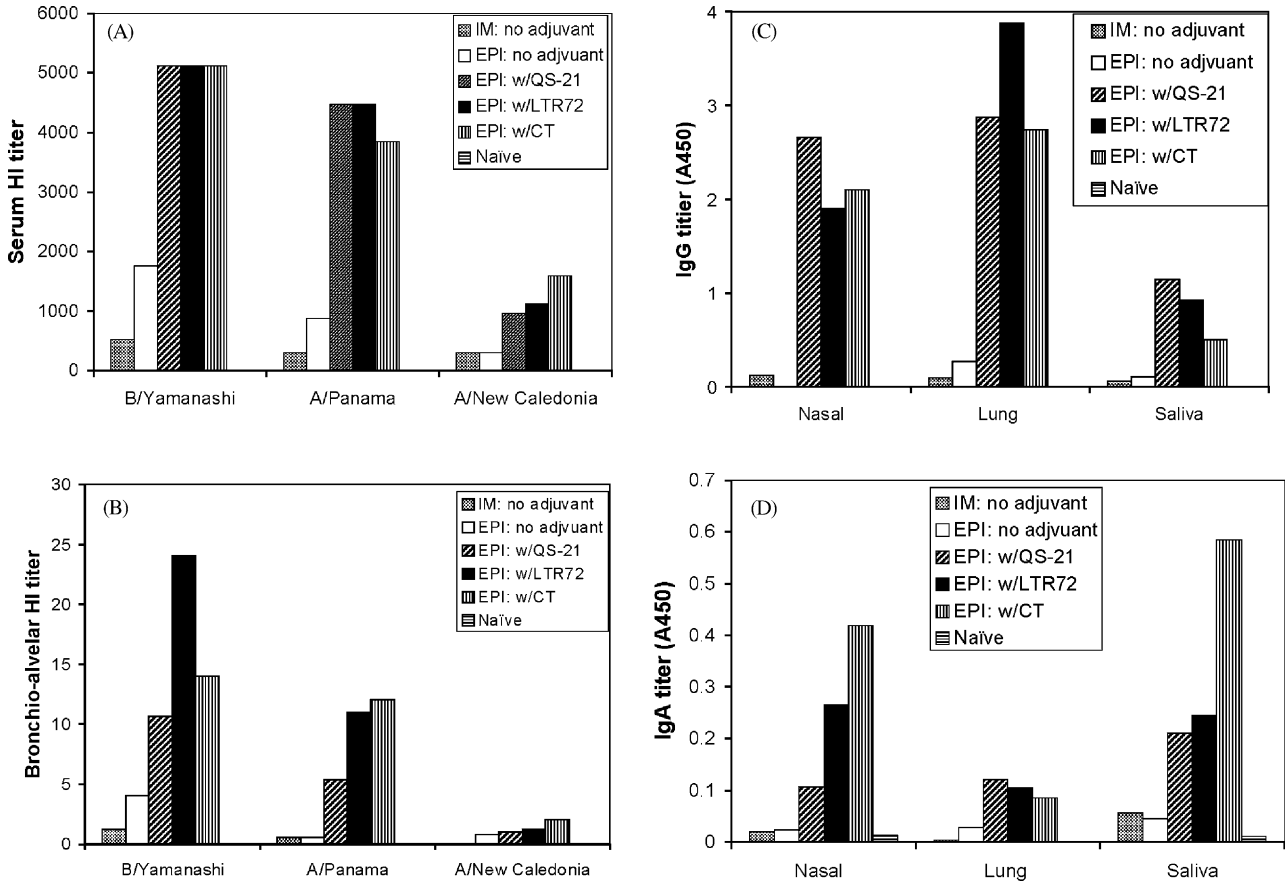

Fig. 2. Serum and mucosal antibody responses in mice following EPI. Immunizations were given on days 0 and 28. Each EPI with an ND1 device under a 40 bar pressure delivered 1 mg of powders containing trivalent influenza vaccine (1.5 μg HA per virus) alone or the same dose of vaccine plus 5.0 μg of QS-21, 2.5 μg of LTR72, or 2.5 μg of CT. Control animals were injected IM with 50 μl of liquid vaccine. Data are mean antibody titers from four animals in the serum and mucosal lavage fluids collected on day 42. Data are mean serum HI titers (A), HI titers in the bronchio-alveolar fluid (B), IgG antibodies in the mucosal lavage fluid measured by ELISA (C), and IgA antibodies in the mucosal lavage fluid measured by ELISA (D).

toxicity of CT after incidental mucosal exposure, has prompted us to select the QS-21 formulation for the primate study.

3.3. Serum antibody responses and challenge result in monkeys

Prior to immunization, all but one monkey had no detectable HI titers to the vaccine viruses (Fig. 3). At days 14 and 30 following the first dose of vaccine, a small portion of animals that received EPI with antigen alone (without an adjuvant) had detectable HI titers. In contrast, 100% of the animals that received EPI with the influenza vaccine and QS-21 had a HI titer to A/Panama (Fig. 3A) and B/Yamanashi (Fig. 3C) viruses, and 67% (four out of six) to the A/New Caledonia virus (Fig. 3B). The mean HI titers to each vaccine antigen at 30 days post-immunization were significantly different between the two groups of animals ($P < 0.05$, *t*-test), indicating the strong adjuvant activity of the QS-21 adjuvant. EPI using the influenza vaccine and QS-21 elicited approximately two-fold higher HI titers against each virus than the IM injection control; however, the difference was not statistically significant ($P > 0.05$). The post-prime HI titers between the IM control and EPI with antigen alone were also not statistically different.

A second dose of vaccine on day 30 boosted the HI titers in all groups of animals (Fig. 3). Peak titers were seen on day 44 (14 days after the booster immunization). EPI with influenza vaccine and QS-21 elicited significantly higher serum HI titers against each vaccine virus than that evoked by IM injection or EPI without an adjuvant ($P < 0.05$). Immunization with antigen alone without an adjuvant by EPI or IM injection elicited comparable HI titers to each virus ($P > 0.05$). By day 60, HI titers in all groups were stable or slightly lower when compared to the titers on day 44.

None of the animals developed fever or any other clinical symptoms during the 7-day monitoring period after the challenge. Measurable virus titers were seen in every animal at least at one time point, indicating that all animals had been infected. The inherent variability of virus recovery from nasal samples prevented the determination of whether or not there was an accelerated clearance in the immunized animals.

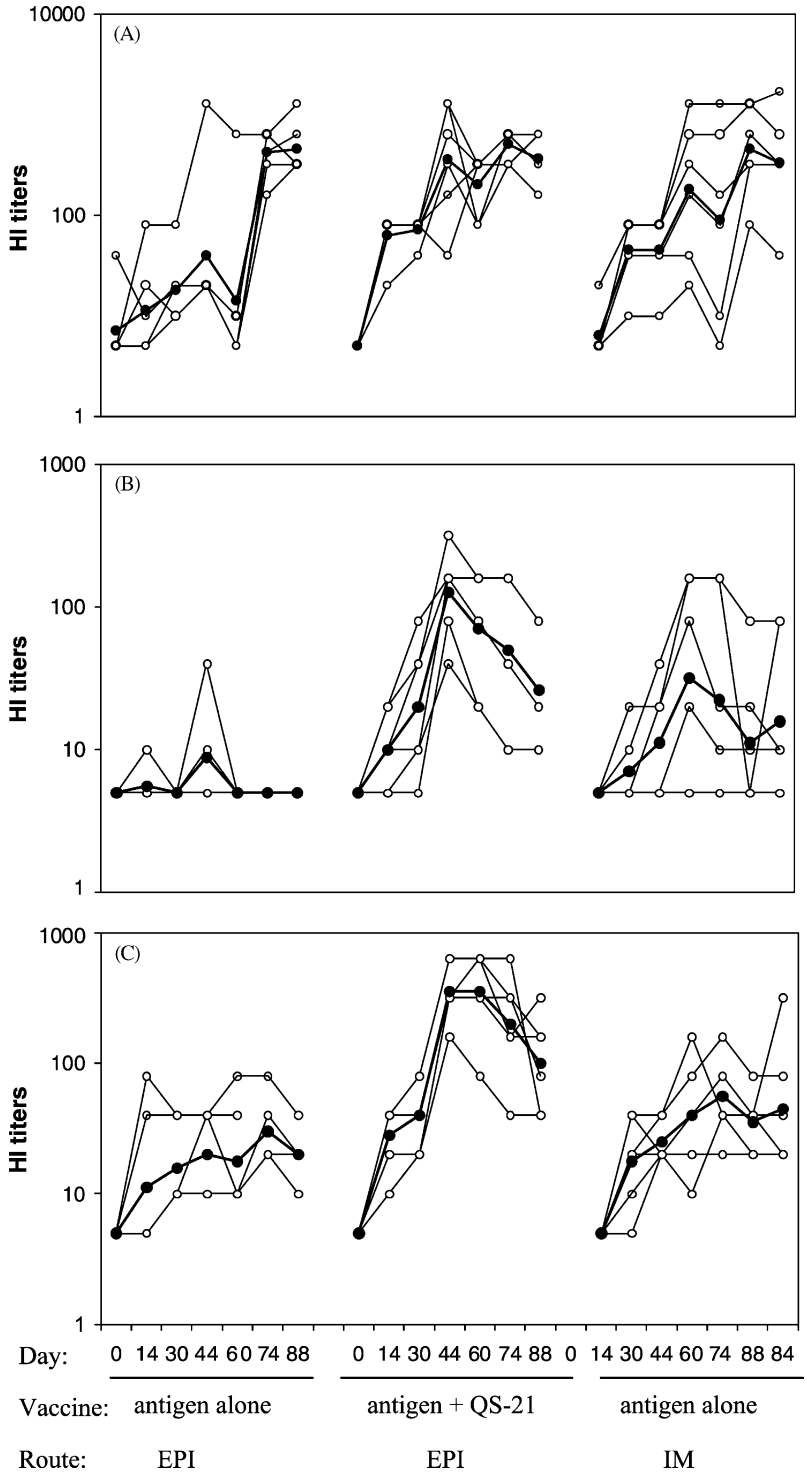

Fig. 3. Serum HI antibody responses in monkeys following EPI. Animals (six per group) were immunized on days 0 and 30. Each EPI with an ND1 device under a 45 bar pressure delivered 1 mg of powders containing a trivalent influenza vaccine (15 μg HA per virus) alone or plus 50 μg of QS-21 to the skin in the inner upper arm. Control animals received IM injection of the same dose of trivalent vaccine without QS-21. Challenge was performed on day 60 by intranasal instillation of 2.0 ml solution (1 ml per nostril) containing 10^6 $TCID_{50}$ of A/Sydney (H3N2). Data are HI titers of individual animals (open circle/light line) or geometric mean titers of the group (filled circle/heavy line) at different time points to A/Panama virus (A), A/New Caledonia virus (B), and B/Yamanashi virus (C).

Following the challenge with A/Sydney virus, HI titers to the antigenically related A/Panama virus became elevated on days 70 and 84 (Fig. 3A), indicating that an immune response was mounted to the challenge virus. The increases in HI titers were more evident for the groups receiving antigen alone by EPI or IM injection than the group receiving adjuvanted vaccine by EPI. HI titers to the antigenically unrelated A/New Caledonia and B/Yamanashi viruses were unchanged or slightly lower.

In the primate study, the sites of EPI were monitored by visual examination. Erythema was observed for all sites immediately after treatment. Complete resolution was seen within 2–3 days. The degree and duration of erythema between the antigen alone group and the adjuvanted group were similar. There were no other discernable local or systemic reactions.

4. Discussion

Non-human primate is considered a useful animal model for evaluating the immunogenicity of a vaccine and the safety and potency of adjuvants prior to a clinical study. In this study, we demonstrated that EPI of monkeys with influenza vaccine plus QS-21 induced high levels of serum antibody responses. The serum HI antibody titers against each vaccine virus elicited by EPI were significantly higher than those induced by IM injection of the standard liquid vaccine. In general, the serum HI antibodies are considered protective and correlate with the efficacy of the influenza vaccine [10,12]. Mucosal antibodies to influenza vaccine may be important for protection of upper respiratory tract against infection by influenza virus in humans and animals [10]. We have previously shown that CT helps to induce a mucosal antibody response to co-administered influenza vaccine by EPI [11]. The present study shows that induction of mucosal immunity in mice by EPI is not unique to the use of CT, it can be achieved with other adjuvants such as genetically detoxified LTR72 and QS-21. To our knowledge, this is the first study to demonstrate an antigen-specific mucosal responses following immunizations using these adjuvants via a non-mucosal route, although these adjuvants have been shown to help induce a mucosal immune response upon delivery via a traditional mucosal route [21,22].

Many adjuvants have been evaluated in the past and were found to be unsuitable for use in human vaccines because of the safety issues or insufficient adjuvant activity following a traditional route of delivery. Only a few are considered potential candidates for human vaccines. Delivering adjuvant to the skin may have a safety over deeper tissue injection. We have demonstrated in a previous study that 50 μg of CT was well-tolerated by mice following EPI whereas 10 μg of CT was lethal for nasal delivery or IM injection [20]. Others have shown that high doses of LT delivered to skin was well-tolerated in humans [23]. Nevertheless, CT and CT-related molecules may be toxic to humans upon incidental mucosal exposure and adjuvants without toxicity are preferred for human vaccination [24]. QS-21 has been evaluated with numerous vaccine antigens in several thousand human subjects by needle injection. Occasionally, QS-21 causes pain at the injection site [25]. This side effect may not be seen with EPI since the target tissue (epidermis) contains little if any sensory nerve endings. In the present study, using both mice and monkeys, we observed no grossly detectable local or systemic side effects with EPI other than a transient erythema at the site of immunization. Administering QS-21 to epidermis may have a potency advantage over deeper tissue injection. As shown in Fig. 2, EPI of mice using 5 μg QS-21 significantly enhanced antigen-specific serum and mucosal antibody responses. The same dose of QS-21 failed to boost serum antibody titer to influenza vaccine when administered by IM injection (our unpublished observation).

EPI with influenza vaccine and QS-21 elicited high levels of serum antibody in the monkeys, however, EPI did not generate sterilizing immunity against an intranasal challenge using A/Sydney, an antigenic variant virus. A/Sydney virus was chosen for the challenge because it had previously induced a fever in young macaques when the virus was inoculated intranasally (unpublished observations). However, in the present study, the virus only resulted in asymptomatic infections in the unimmunized animals. The immunized animals, regardless of the vaccine formulation and immunization route, all shed virus. The A/Panama virus (H3N2) in the vaccine was presumably to confer protection against the challenge virus due to similarity of the HA proteins. Antibodies raised against A/Panama virus had cross-reactivity to A/Sydney virus in the HI assay, but the titers were approximately four-fold lower than that against the homologous virus (data not shown). Therefore, induction of sterilizing immunity against challenge with an antigenically variant influenza A strain may not be achievable. Unfortunately, the inherent variability of the animal model and assay techniques prevent us from assessing accelerated virus clearance in the immunized animals, which may be a more likely outcome.

In summary, this study demonstrated that EPI is an efficient method to immunize non-human primates and that QS-21 is a potent adjuvant for the influenza vaccine. The mouse study demonstrated that EPI with QS-21 adjuvanted influenza vaccine, in addition to augmenting the serum antibody titer, induces mucosal antibody responses. The stability of powdered vaccine has important implications for extending vaccinations to areas of the world where cold-chain for transportation and storage of vaccines does not exist. Thus, EPI may be a viable alternative to syringe and needle injection for human vaccination.

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