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Methylglycol chitosan and a synthetic TLR4 agonist enhance immune responses to influenza vaccine administered sublingually

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ABSTRACT

Influenza is a vaccine-preventable contagious respiratory illness caused by influenza (flu) viruses which can lead to hospitalization and sometimes even death. Current flu vaccines delivered intramuscularly (IM) or intradermally (ID) are less effective at eliciting protective mucosal immune responses and vaccines delivered intranasally (IN) possess potential safety concerns. Sublingual (SL) vaccination is a promising alternative route for vaccine delivery which has been indicated as safe and effective at inducing protective immune responses in both systemic and mucosal compartments. We evaluated the efficacy of methylglycol chitosan (MGC) and a synthetic toll-like receptor 4 agonist (CRX-601), alone or in combination, for improving systemic and mucosal immune responses to a monovalent detergent-split flu virus vaccine delivered SL. SL vaccination of mice with split-flu vaccine formulated with either MGC or CRX-601 resulted in specific serum IgG and mucosal IgA titers that were significantly greater than titers from non-adjuvanted vaccination and equivalent to or greater than titers in mice vaccinated IM. Our results demonstrate that SL vaccination utilizing MGC or CRX-601 as adjuvants is a viable alternative route of vaccination for flu which can elicit systemic immune responses equivalent to or greater than IM vaccination with the added benefit of stimulating a robust specific mucosal immune response.

1. Introduction

Influenza is a common contagious respiratory tract infection responsible for notable morbidity and mortality during recurrent epidemics and occasional pandemic outbreaks. Annual vaccination is currently the most effective strategy for preventing or containing flu infections [1,2]. Current licensed vaccines against flu viruses are principally live attenuated, whole inactivated, split virion or subunit vaccines. Available vaccines are administered either intramuscularly (IM), intradermally (ID) or intranasally (IN) and promote a humoral immune response against the viral surface glycoprotein hemagglutinin (HA) and neuraminidase (NA) [2,3]. Vaccines administered parenterally are generally effective at stimulating systemic antibody mediated immune responses but are less effective at inducing mucosal immunity [4–6]. Since flu viruses enter the host via the respiratory tract mucosa, prospective improved vaccination strategies should not only elicit an effective systemic immune response but also neutralizing mucosal antibodies, particularly IgA, at the initial site of infection.

Mucosal vaccination has been explored as an alternative strategy to parenteral administration to more efficiently elicit mucosal and systemic immune responses [7–10]. Mucosal vaccination is generally associated with oral or IN routes. Vaccines delivered orally are potentially degraded by gut microflora or stomach pH during the passage through the gut and require specialized

Abbreviations: Flu, influenza; MGC, methylglycol chitosan; IM, intramuscular; ID, intradermal; IN, intranasal; SL, sublingual; Ig, immunoglobulin; HA, hemagglutinin; NA, neuraminidase; AGP, aminoalkyl glucosaminide 4-phosphate; TLR4, toll-like receptor 4; MGC-CRX-601, MGC co-formulated with CRX-601; HPLC, high-performance liquid chromatography; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; ZP, zeta-potential; PDI, polydispersity indices; DLS, dynamic light scattering; 14dp2°, 14 days post-secondary; 14dp3°, 14 days post-tertiary; ELISA, enzyme-linked immunosorbent assay; HAI, hemagglutination inhibition assay; DPBS, Dulbecco's phosphate buffered saline; RBC, red blood cell; RDE, receptor destroying enzyme; RT, room temperature; LLOQ, lower limit of quantification; VW, vaginal wash; TW, tracheal wash; Th, T helper; NT, not tested; Uni, unimodal; Multi, multimodal; Bi, bimodal; S.C., seroconversion; THF, tetrahydrofuran.

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formulations to prevent degradation while antigens or adjuvants delivered IN can potentially be redirected to the central nervous system through the olfactory nerve epithelium, thereby causing adverse side effects and reducing vaccine safety [11–16]. Sublingual (SL) administration is a promising alternative mucosal vaccination method which bypasses the potential degradation in the gut or olfactory bulb redirection and has been demonstrated as safe and effective for both bacteria and virus vaccines, including influenza [17–27].

Although delivery through the oral mucosa avoids alteration by gastric fluids and enzymes present in the gastrointestinal tract, various factors exist which act as barriers and hinder absorption of certain vaccine components, especially larger molecules. These barriers include the permeability of the mucosa to the vaccine components, saliva, mucus, membrane coating granules, basement membrane, etc., all of which can limit the absorption through the mucosa, depending on the physiochemical characteristics of the vaccine components [8]. In order to address the challenge of delivering vaccine components via the SL route and eliciting an adequate immune response, a number of enhancement strategies have been explored, including the use of adjuvants and/or improved vaccine formulations [8,9]. SL vaccination with flu vaccine in particular has been shown to require high doses of antigen and/or use of an adjuvant to elicit a robust immune response [6].

CRX-601 is an aminoalkyl glucosaminide 4-phosphate (AGP), a new class of synthetic lipid A mimetics engineered to effectively trigger toll-like receptor 4 (TLR4), which has recently been shown to enhance the immune response against flu vaccination following IN administration [28]. In addition, chitosan and chitosan derivatives have shown promise as safe and effective adjuvants and delivery systems for enhancing immunogenicity of mucosally administered vaccines [29–41]. Chitosan is the generic term for a family of linear polysaccharides which exist as copolymers of β-(1–4)-linked glucosamine and N-acetylglucosamine and is produced from the exoskeletons of crustacean or the cell walls of fungi. Chitosan possess the favorable biological properties for formulation with mucosal vaccines such as biocompatibility, biodegradability, mucoadhesive properties and permeation-enhancing ability; however, chitosan has limited solubility at physiological pH. Therefore, various chitosan derivatives with improved solubility profiles more suited for inclusion in vaccine formulations have been explored [30,42–44].

In this study, we evaluated chitosan derivatives formulated with a monovalent detergent-split flu vaccine to be delivered mucosally via the SL route. We also evaluated the compatibility of lead chitosan derivatives for co-formulation with the synthetic mucosal adjuvant CRX-601. The immunogenicity of SL flu vaccines containing either methylglycol chitosan (MGC) or CRX-601 was evaluated in a mouse model and compared to flu vaccine administered IM. In addition, to determine if combinations of MGC and CRX-601 confer any added immunological benefits, vaccines containing MGC co-formulated with CRX-601 (MGC-CRX-601) were tested for immunogenicity following SL administration in mice.

2. Materials and methods

2.1. Chitosan derivatives and CRX-601

Synthesis, purification and analysis of the AGP CRX-601 have been described previously [28,45]. Aqueous buffered dispersions of CRX-601 were prepared by suspending CRX-601 (0.05–2 mg/mL) in 10 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid buffered saline (HEPES-saline, pH 7.0) in a borosilicate glass vial (2–5 mL batch) and sonication on a water bath sonicator (Elma-Lab line, Singen, Germany). The bath temperature was maintained below 45 °C, and the dispersion was sonicated (1–2 h) until a clear to slightly hazy dispersion with an average particle size (as measured by dynamic light scattering, DLS) of 60–100 nm was obtained. The aqueous dispersion was further sterile filtered using a 0.22 μm Millex-GV filter (Millipore, Bedford, MA). The concentration of CRX-601 in formulations was determined by ion-pair reverse phase high-performance liquid chromatography (RP-HPLC, Waters Alliance 2690/2695, Milford, MA) on a C8 column (Ace 3, 3 μm, 50 mm × 3.0 mm; Mac-Mod Analytical, Chadds Ford, PA) and UV detection at 210 nm (Waters model 2487 or 996 PDA detector). Elution consisted of a linear gradient at 0.8 mL/min from 50% to 100% B over 10 min and 100% B for 5 min. Solvent A consisted of 8% ACN, 2% buffer and 90% water. Solvent B consisted of 2% buffer in ACN. Buffer was prepared from 62.5 mL of 0.4 M tetrabutylammonium hydroxide in water with pH adjustment to 6.0 with 15 M phosphoric acid and a final volume of 100 mL. Samples were diluted in tetrahydrofuran (THF) (1:1, v/v) and analyzed against a set of CRX-601 standards (0.25, 0.5, 1, 1.5, and 2 mg/mL in THF) with system suitability injections at the start and the end of the run. The chitosan derivatives methylglycol chitosan (MGC), chitosan oligosaccharide lactate (CO) and glycol chitosan (GC, Sigma-Aldrich, St Louis, MO), were prepared in either 10 mM HEPES (pH 7.0) or 10 mM HEPES-saline (0.9% saline, pH 7.0) and sterile filtered using a 0.22 μm filter. For preparation of chitosan-CRX-601 complexes, the solution of a chitosan derivative was admixed with aqueous CRX-601 dispersion and vortexed for 30 s. Zeta-potential (ZP), particle size, and polydispersity indices (PDI) of formulations were determined by DLS using a Malvern Zetasizer Nano ZS (Malvern Instruments, Westborough, MA). Samples (8 μL) were diluted with 800 μL ultrapure water before measurement.

2.2. Vaccination of mice

Stock solutions of MGC were prepared at either 1.0 or 5.0 mg/mL in 10 mM HEPES-saline (0.9% saline, pH 7.0). CRX-601 was prepared in HEPES-saline at 1 mg/mL, with or without MGC at either 1.0 mg/mL or 5.0 mg/mL. For the mouse study involving MGC formulated with a suboptimal dose of CRX-601, complexes were prepared at a higher concentration of MGC (12.5 mg/mL) and lower concentrations of CRX-601 (0.005 and 0.05 mg/mL). These preparations did not exhibit any precipitation and were used as such without further characterization. For mouse dosing, MGC and/or CRX-601 were diluted in HEPES-saline and admixed with H3N2 monovalent detergent-split flu (A/Victoria/210/2009) and mixed by vortexing immediately prior to each study to obtain final vaccine formulations which were visually inspected for flocculation or precipitation (none observed for any formulations used). Final formulations were administered to mice within 2 h of antigen addition. Female BALB/c mice (6–8 weeks of age) were obtained from Charles River Laboratories, Wilmington, MA. For vaccinations, mice anesthetized by intraperitoneal administration of ketamine (80–150 mg/kg) and xylazine (8–15 mg/kg) were given vaccine by either SL administration (6 μL/mouse deposited under the tongue toward the floor of the mouth) or IM administration (50 μL/mouse in the quadricep muscle) at 21 day intervals. Mice receiving vaccine by SL administration were vaccinated a total of either two or three times on days 0, 21 and 42, respectively. Mice receiving vaccine by IM administration were vaccinated two times on either days 21 and 42 or days 0 and 21. For each vaccination, mice (except for the naïve group) received the indicated dose of flu antigen (0.3 or 3.0 μg) alone or in combination with the indicated dose of either MGC, CRX-601, or CRX-601 co-formulated with MGC. All animals were treated in accordance with guidelines established by the U.S. Department of Health and Human Services Office of Laboratory