PCPP-Formulated H5N1 Influenza Vaccine Displays Improved Stability and Dose-Sparing Effect in Lethal Challenge Studies

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ABSTRACT: The potential impact of an influenza pandemic can be mitigated through the realization of a successful vaccination program. The implementation of antigen stabilization and dose-sparing technologies is an important step in improving availability of vaccines at the time of a pandemic outbreak. We investigated poly[di(carboxylatophenoxy)phosphazene] (PCPP) as a potential stabilizing and immunostimulating agent for H5N1 influenza vaccine. Physicochemical characterization of PCPP-formulated H5N1 influenza vaccine revealed macromolecular complexation in the system, whereas single radial immunodiffusion assay verified antigenicity of the formulation *in vitro*. PCPP-enhanced formulation displayed a fourfold increase in the half-life at 40°C compared with a nonadjuvanted vaccine. Lethal challenge studies in ferrets demonstrated 100% protection for low-antigen dose PCPP-adjuvanted formulations (1 μg of hemagglutinin) and at least a 10-fold antigen-sparing effect. Therefore, PCPP demonstrated an ability to improve thermal stability of H5N1 influenza vaccine in solutions and provide for a substantial dose-sparing effect *in vivo*. © 2010 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 100:1436–1443, 2011

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INTRODUCTION

The emergence of highly pathogenic avian influenza A/H5N1 virus and the increasing number of cases of its direct transmission to humans poses a major pandemic threat.[1–3] It is expected that safe and effective vaccines will be the single most important public health instrument for reducing the morbidity, mortality, and economic impact of pandemic influenza.[1,4] Despite extensive experience with vaccines against human influenza viruses, researchers face serious challenges in developing successful vaccines against avian influenza with pandemic potential.[3] One of the challenges stems from the fact that, for unknown reasons, hemagglutinin (HA) proteins of avian subtypes of influenza A viruses are not as immunogenic as those of human subtypes.[5] As more research is ongoing to understand biological basis for the poor immunogenicity of avian HA glycoproteins, a strategy for enhancing immunogenicity of avian influenza vaccine and implementing dose-sparing technologies through the use of adjuvants appears to be of high importance.[1,3,6]

Potential challenges in pandemic vaccine manufacturing also lie in the uncertainties associated with predicting the time of the potential outbreak and even the pandemic virus itself. To maximize preparedness, the concept of vaccine stockpiling has become a part of a global pandemic plan, and there is a strong focus on improving shelf life of vaccine and standardization of assays for their monitoring.[7–9] Improvement of vaccine stability is also of prime importance for seasonal strains as reduced dependence on cold-chain facilities, as well as the diminished risk of vaccine losses caused by "off-label" storage can lead to enormous annual savings.[10,11]

Water-soluble polyphosphazenes, such as poly[di(carboxylatophenoxy)phosphazene] (PCPP), have been extensively investigated *in vivo* as vaccine adjuvants for seasonal influenza vaccines.[12–14] In

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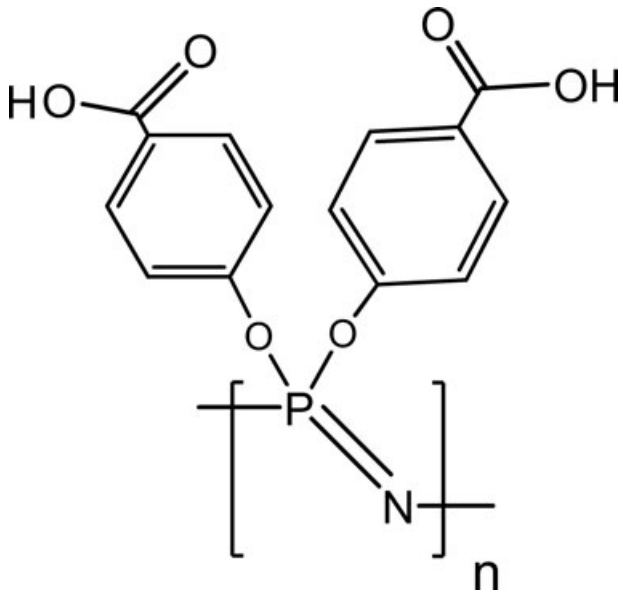

Figure 1. Molecular formula of poly[di(carboxylatophenoxy)phosphazene].

these studies, PCPP has demonstrated the ability to significantly increase hemagglutination inhibition and serum antigen-specific immunoglobulin G titers, as well as displayed a substantial dose-sparing effect. PCPP-formulated vaccines have been also tested in clinical trials and reported to be immunogenic and well tolerated with no vaccine-related, serious adverse events.[14,15] From the physicochemical and formulation standpoint, PCPP (Fig. 1) is a well-defined compound produced by a controlled synthetic process and capable of forming water-soluble complexes with antigens.[16,17] However, to this date, there have been no reports on the effect of this adjuvant on H5N1 influenza vaccines.

In the present paper, we investigated PCPP-formulated H5N1 influenza vaccines in regard to their physicochemical behavior in solution. We assessed *in vitro* potency using a single radial immunodiffusion (SRID) assay. Furthermore, we studied the effect of PCPP on thermal stability of H5N1 antigen in solution and the resistance of such formulation to drying. Finally, we evaluated the *in vivo* activity of PCPP-formulated H5N1 vaccine in lethal challenge studies in ferrets.

EXPERIMENTAL

Materials

PCPP (Sigma–Aldrich, St. Louis, Missouri) was purified by multiple precipitations using sodium chloride to produce polymer with weight-average molecular weight of 855,000 g/mol and polydispersity parameter of 2.5, as determined by gel permeation chromatography using poly(acrylic acid) standards, which is typically in a good correlation with light scattering data.[18] The following reagents were obtained through the NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH: monovalent influenza subvirion vaccine, rgA/Vietnam/1203/2004 (H5N1), NR-4143; polyclonal anti-influenza virus H5 HA, A/Vietnam/1203/04 (H5N1) (antiserum, goat), NR-2705. A37 agarose (molecular biology grade, IBI Scientific, Peosta, Iowa); *N*-lauroylsarcosine sodium salt, polyoxyethylene (20) sorbitan monolaurate (Tween) (TCI America, Portland, Oregon); Coomassie Brillant Blue R-250 (J.T. Baker, Phillipsburg, New Jersey); Dulbecco's phosphate buffered saline (DPBS) without magnesium and calcium (Thermo Scientific, Logan, Utah); glacial acetic acid, methanol, 10× PBS concentrate (EMD Chemicals, Gibbstown, New Jersey); acetonitrile (Fisher Scientific, Pittsburgh, Pennsylvania); and sodium azide (VWR International, West Chester, Pennsylvania) were used as received.

Methods

Physicochemical Characterization

Size-exclusion chromatography (SEC) of the formulation was performed using a Hitachi high performance liquid chromatography (HPLC) system with an L-2450 diode array detector (Hitachi LaChrom Elite System, Hitachi, San Jose, California) equipped with an ultrahydrogel linear size exclusion column (Waters Corporation, Milford, Massachusetts) at 25°C using 0.1× PBS with 10% acetonitrile as a mobile phase with a flow rate of 0.75 mL/min. Samples were prepared by mixing vaccine formulation with solution of PCPP in PBS to obtain a final HA concentration of 0.015 mg/mL (0.076 μM) and PCPP concentration of 0.006 mg/mL (0.007 μM). The solution was gently vortexed immediately after mixing and incubated at room temperature for at least 1 h prior to analysis. Peak area and retention time of the peak attributed to PCPP ($\lambda_{max} = 235$ nm) was monitored.

Ultraviolet-visible light spectroscopy was conducted using a Hitachi U-2810 Spectrophotomer (Hitachi, San Jose, California) at 25°C in a 1 cm quartz cuvette (NSG Precision Cells Inc., Farmingdale, New York). Samples were prepared at a concentration of 0.005 mg HA/mL (0.025 μM) and 0.006 mg/mL (0.007 μM) of PCPP. The solutions were gently vortexed prior to the analysis. Optical density was recorded during wavelength scans from 400 to 200 nm in 1 nm steps. All spectra are the result of average of two repeated scans for each sample.

Single-Radial Immunodiffusion Assay

The SRID technique utilized to determine influenza HA concentrations was used as described