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Immunogenicity, protective efficacy and mechanism of novel CCS adjuvanted influenza vaccine

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ABSTRACT

We optimized the immunogenicity of adjuvanted seasonal influenza vaccine based on commercial split influenza virus as an antigen (hemagglutinin = HA) and on a novel polycationic liposome as a potent adjuvant and efficient antigen carrier (CCS/C-HA vaccine). The vaccine was characterized physicochemically, and the mechanism of action of CCS/C as antigen carrier and adjuvant was studied. The optimized CCS/C-HA split virus vaccine, when administered intramuscularly (i.m.), is significantly more immunogenic in mice, rats and ferrets than split virus HA vaccine alone, and it provides for protective immunity in ferrets and mice against live virus challenge that exceeds the degree of efficacy of the split virus vaccine. Similar adjuvant effects of optimized CCS/C are also observed in mice for H1N1 swine influenza antigen. The CCS/C-HA vaccine enhances immune responses via the Th1 and Th2 pathways, and it increases both the humoral responses and the production of IL-2 and IFN-γ but not of the pro-inflammatory factor TNFα. In mice, levels of $CD4^+$ and $CD8^+$ T-cells and of MHC II and CD40 co-stimulatory molecules are also elevated. Structure–function relationship studies of the CCS molecule as an adjuvant/carrier show that replacing the saturated palmitoyl acyl chain with the mono-unsaturated oleoyl (C18:1) chain affects neither size distribution and zeta potential nor immune responses in mice. However, replacing the polyalkylamine head group spermine (having two secondary amines) with spermidine (having only one secondary amine) reduces the enhancement of the immune response by ~50%, while polyalkylamines by themselves are ineffective in improving the immunogenicity over the commercial HA vaccine. This highlights the importance of the particulate nature of the carrier and the polyalkylamine secondary amines in the enhancement of the immune responses against seasonal influenza. Altogether, our results suggest that the CCS/C polycationic liposomes combine the activities of a potent adjuvant and efficient carrier of seasonal and swine flu vaccines and support further development of the CCS/C-HA vaccine.

1. Introduction

Influenza remains a pervasive public health problem in spite of the widespread use of the i.m. trivalent inactivated vaccine [1,2], which is based on split viral envelopes having the protein hemagglutinin (HA) as the main vaccine antigen. Examples of such vaccines are Fluvirin and Aggripal (Novartis Vaccines, USA) as well as Vaxigrip (Sanofi Pasteur, USA). While such vaccines prevent infection in ~80–90% of adult vaccinees, they are less efficacious in the most vulnerable population, the elderly (≥age of 65), in which the average efficacy rate is ~50–70% [3–5]. In general, in the elderly population, the levels of antibodies against HA elicited by one vaccine dose are rather low and immunity is short term. Therefore, a vaccine that overcomes these deficiencies is needed.

Abbreviations: APC, antigen presenting cells; C, cholesterol; CCS, ceramide carbamoyl-spermine; CCSD, ceramide carbamoyl spermidine; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; EM, electron microscopy; F-HA, free HA (non-liposomal associated); FSC, forward scatter; HA, hemagglutinin; HI, hemagglutination inhibition; HPLC, high performance liquid chromatography; i.m., intramuscular; INFγ, interferon γ; i.n., intranasal; IL-2/4/5, interleukin 2/4/5; MOA, mechanism of action; NMR, nuclear magnetic resonance; OCCS, N-oloeyl-D-erythro-sphingosyl-carbamoyl-spermine; SPF, specific pathogen free; SSC, side scatter; TEM, transmission electron microscopy; TLC, thin-layer chromatography; TNFα, tumor necrosis factor α; UHV, unsized heterogeneous vesicles.

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The common way for increasing vaccine potency is the use of a suitable carrier and/or adjuvant. In order to optimize the use of such a carrier/adjuvant, it is important to understand its mechanism of action (MOA). Such understanding should include issues such as which targets are most important to enhance, which cytokines are involved, and what is the balance between humoral and cellular responses. Recently, one intranasal (i.n.) and two i.m. new influenza vaccines, having different antigen carriers, were approved for human use. The first is a live attenuated influenza vaccine (Flumist: formulated for i.n. delivery) [6,7]. The second is FluAd, in which the HA antigen is formulated in MF59 (an oil-in-water emulsion) as a carrier/adjuvant (Novartis Vaccines). This i.m. vaccine (licensed in Europe) modestly increases (~1.1–1.4-fold) the geometric mean serum anti-HA (hemagglutination inhibiting, HI) antibody titer in young adults and the elderly. However, increased reactogenicity, including local pain, erythema, induration, fever and myalgia, has been reported [8]. The third is an i.m. "virosomal" flu vaccine (Inflexal, Crucell, The Netherlands), which is based on purified HA incorporated into the membrane of unilamellar liposomes composed of viral lipids mixed with external phosphatidylcholine (PC) and phosphatidylethanolamine (PE). This vaccine has similar or slightly higher immunogenicity based on HI antibody titers than the standard vaccine [9,10].

Since 1990, numerous reviews on liposomes as carriers and potential adjuvants have shown that liposome-based vaccines promote both humoral and cell-mediated immunity to a wide variety of pathogens [11]. The activity of the liposomes is highly dependent on their physicochemical properties that control the *in vivo* fate of vesicles, including the mode of antigen interaction with antigen presenting cells (APCs). The surface electrical charge (particle electrostatics) has been shown to have a major influence on adjuvanticity. Recent *in vivo* studies demonstrate the superiority of the cationic liposomes over anionic and neutral liposomes [12–14]. This is thought to be related to the electrostatic interaction between the cationic liposome carriers and the negatively charged cell membranes, which significantly increases liposomal uptake by APC and thus enhances antigen-specific immune responses [15]. Furthermore, the ability of cationic liposomes to destabilize endosomes allows delivery of antigens into the cytosol of the cells and the eliciting of specific cellular immune responses [16].

Based on these findings, we have designed several lipid-based formulations with the hope of combining antigen delivery and adjuvanticity, which together will result in superior potency to current commercial split or subunit HA vaccines [13]. Various lipid-based influenza vaccine formulations (using multi-lamellar large lipid vesicles (MLV) > 0.2 μm in diameter) were compared in mice with the commercial split HA vaccine administered i.n. or i.m. with/without additional adjuvants. It was found that only formulations based on cationic liposomes composed of the lipids DOTAP and DMTAP or on our novel CCS (*N*-palmityol-D-*erythro*-sphingosyl-Carbamoyl- Spermine) lipid meet our criteria of significantly increased immune responses without the need of additional adjuvant. The most effective formulation consisted of CCS mixed with the neutral "helper" lipid cholesterol (C) in the form of cationic liposomes.

In the current study, we show that the commercial influenza split virus vaccine formulated with cationic CCS/C liposomes (also referred to as "VaxiSome") administered i.m. is highly effective in inducing strong humoral and cellular immune responses, including higher levels of serum HI antibodies. It increases the percentage of responder animals and seroconversion rates in mice, rats and ferrets, and it provides enhanced protection against live virus challenge in ferrets (an established model of influenza infection [17]). The i.m. administration of CCS/C-HA vaccine (VaxiSome-HA) also enables a significant dose-sparing effect of the influenza antigen. *In vivo* studies, performed to clarify the MOA, suggest that the CCS/C formulation upregulates levels of MHC II and CD40 co-stimulatory molecules and induces production of IL-2 and IFN-γ but not of the pro-inflammatory factor TNFα.

2. Materials and methods

2.1. Animals

Mouse and rat experiments were performed in the animal facility of the Hebrew University, Jerusalem, Israel. Specific pathogen free (SPF) female and male BALB/c mice, female C3H/HeNHsd mice, female C57BL/6JOlaHsd mice (8–9 weeks old), and female Sprague–Dawley rats (~9 weeks old) were obtained from Harlan (Jerusalem, Israel). Studies using ferrets were performed in two places: Retroscreen Virology, London UK and Statens Veterinärmedicinska Anstalt (SVA), Uppsala Sweden. Healthy female ferrets (*Mustela furo*, outbred, fitch and/or albinos), ~4–6 months old, 3–8 per group, were obtained from Highgate Farm, Highgate UK for the first site and from Thomas Sävsjö, a private Swedish ferret breeder, for the second site. Animals were maintained under a controlled ferret diet in a clean animal unit.

All animal studies were approved by Ethics Committees and the Institutional Animal Care and Use Committees at the sites of the specific experiments.

2.2. Influenza antigens (HA)

Commercial trivalent split vaccine (Vaxigrip, Aventis Pasteur or Sanofi Pasteur) from several vaccination seasons was used throughout the study. The three strains of the vaccine were those recommended by the World Health Organization for each respective influenza season: (i) A/New Caledonia/20/99 (H1N1)-like strain derived from IVR-116, A/Fujian/411/2002 (H3N2)-like strain derived from X-147 derived from A/Wyoming/3/2003, B/Shanghai/361/2002-like strain used B/Jiangsu/10/2003 (season 2004/2005, used for the physicochemical characterization of the CCS/C vaccine); (ii) A/New Caledonia/20/99 (H1N1), A/California/7/2004-like strain (H3N2) derived from NYMC-X-157, B/Jiangsu/10/2003—a B/Shanghai/361/2002-like strain (season 2005/2006, used for the ferrets study in Retroscreen, UK, the rats and the CCS:helper lipid ratios experiments); (iii) A/New Caledonia/20/99 (H1N1), A/Wisconsin/67/2005 (H3N2), B/Malaysia/2506/2004 (season 2006/2007, used for the comparison of CCS isomers, lipid:HA ratios and mechanism of action experiments in mice, and for the ferret study in Sweden). (iv) A/Solomon Islands/3/2006 (H1N1)-like virus; A/Wisconsin/67/2005 (H3N2)-like virus; B/Malaysia/2506/2004-like virus (season 2007/2008, used for the mice strains and gender comparison studies and clearance rate of ^{3}H-CCS/C vaccine experiments). A/Brisbane/59/2007 (H1N1)-like virus; (v) A/Brisbane/10/2007 (H3N2)-like virus; B/Florida/4/2006-like virus (season 2008/2009, used for the biodistribution and the N-palmitoyl-sphingosyl-spermidine experiments). The level of HA referred to throughout this study is based on HA quantification as described in the commercial vaccine insert. In all seasonal vaccines, the HA content was ~50% of total split vaccine protein (quantified by the Lowry method [18–21]). The swine influenza antigen H1N1 was A/California/7/2009 (H1N1)v (NYMC-X179A), egg-derived, formalin-inactivated, whole virus partially purified (NIBSC, UK) code 09/146, supplied at 50 μg/ml HA. The H1N1 influenza virus New Caledonia/20/99 Recombinant, Prospec, Israel, was labeled using Alexa 647, at a fluorophore:protein molecular ratio of 5.5–7:1 and used for the time-dependent biodistribution experiment.