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A new intranasal influenza vaccine based on a novel polycationic lipid-ceramide carbamoyl-spermine (CCS). II. Studies in mice and ferrets and mechanism of adjuvanticity

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ABSTRACT

We recently showed that lipid assemblies comprised of a novel polycationic sphingolipid (ceramide carbamoyl-spermine, CCS) are an effective adjuvant/carrier when complexed with cholesterol (CCS/C) for influenza and other vaccines administered parenterally and intranasally (i.n.) in mice. Here we expand these studies to ferrets, an established model of influenza infection. We also address the question of why the CCS/C-based liposomal vaccine (also known as VaxiSome™) in mice is superior to vaccines based on liposomes of other lipid compositions (neutral, anionic or cationic). Ferrets immunized i.n. with CCS/C-influenza vaccine produced significantly higher hemagglutination inhibition (HI) antibody titers compared to ferrets immunized intramuscularly with the unadjuvanted influenza vaccine, indicating that the CCS/C-based vaccine is very immunogenic. Furthermore, the i.n. adjuvanted vaccine was shown to significantly reduce the severity of influenza virus infection in ferrets following homologous viral challenge as determined by weight loss, temperature rise and viral titer. No adverse reactions were observed. Pharmacokinetic and biodistribution studies following i.n. administration in mice of CCS/C-based vaccine showed that both the lipids and antigens are retained in the nose and lung for at least 24 h, and it appears that this retention correlates with the superior immunogenicity elicited by the adjuvanted vaccine formulation. The CCS lipid also increases production of cytokines (mainly IFN gamma, IL-2 and IL-12) and co-stimulatory molecules' expression, which might further explain the robust adjuvantation of this liposome-based vaccine.

1. Introduction

Influenza, a highly contagious viral disease of the upper respiratory tract, is a serious human affliction that can cause localized epidemics and global pandemics resulting in very high socio-economic burdens [1]. Each year the influenza virus is responsible for 3300–49,000 deaths and up to 300,000 hospitalizations in the US alone [2–4]. Current split-virion or subunit influenza vaccines administered intramuscularly (i.m.) are efficacious in preventing hospitalization and death. Since the elderly respond poorly to these vaccines [3], improved vaccines are required. Sporadic human outbreaks of avian H5N1 [5] and of the recent "swine flu" pandemic [6] further highlight the need for more immunogenic vaccines and dose-sparing vaccines.

Abbreviations: APC, antigen-presenting cells; BD, biodistribution; CCS, ceramide carbamoyl spermine (N-palmitoyl D-erythro-sphingosyl carbamoyl-spermine); C, cholesterol; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; EM, electron microscopy; F-HA, free HA (non-adjuvanted); HA, hemagglutinin; HI, hemagglutination inhibition; INFγ, interferon γ; IL-2/5/12, interleukin 2/5/12; i.m., intramuscular; i.n., intranasal; i.p., intraperitoneal; NALT, nasal-associated lymphoid tissue; NO, nitric oxide; PK, pharmacokinetics; SPF, specific pathogen-free; TEM, transmission electron microscopy; TNFα, tumor necrosis factor α.

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Intranasal vaccines, and mucosal vaccines in general, may offer several potential advantages over traditional parenteral vaccines [7–13]. These include: (a) the possibility of generating a "first line of defense" at the sites of entry of the majority of human pathogens, as well as systemic immunity, thereby aborting the primary infection and not just morbidity; (b) vaccine delivery is relatively simple and painless and avoids the reuse of needles and syringes, which are potential sources of infection; (c) the mucosal immune system may be less affected by aging as compared with systemic immunity; (d) the possibility of eliciting cross-protective immunity against strain variants; and (e) the vaccine may be self-administered.

The use of lipid assemblies such as liposomes, micelles or emulsions as adjuvant/carrier for vaccines has several distinct advantages [reviewed in 14–21]. These include: (a) biocompatibility, biodegradability and low or no toxicity; (b) the effective passive targeting of encapsulated antigens to antigen-presenting cells (APC); (c) the slow release of the antigen(s), which may provide longer-term immune-stimulation protection using a single-dose vaccine; (d) the possibility of co-formulating another adjuvant with antigen in the same vesicles; (e) induction of serum and secretory antibodies as well as cellular responses; (f) the feasibility of extended shelf life; and (g) excellent stability of freeze-dried formulations.

We have previously reported [22] that efficacious i.n. immunization can be demonstrated with the appropriate lipid. The use of the novel polycationic sphingolipid N-palmitoyl D-*erythro*-sphingosyl-carbamoyl-spermine triacetate salt (CCS), in the form of micelles or liposomes with cholesterol (CCS/C, also known as VaxiSome™) that include influenza HA antigen, stimulates increased humoral and cellular immune responses in mice and protects against influenza virus infection [22]. By comparing various neutral, anionic and cationic liposomal formulations with influenza vaccine administered i.n., we found that the CCS/C-based formulations are the most immunogenic, and are able to elicit protective responses both systemically and mucosally. These responses protect not only against the viral strains included in the vaccine, but also against several drift variants of these strains. Furthermore, the CCS/C liposomal vaccine is effective in both young (~2 months old) and older (18 months old) mice, and the humoral response is prolonged (>9 months). We systematically analyzed [22] many factors that can affect immunogenicity, i.e., electrical charge, lipid/antigen ratio, cholesterol, instillation volume, frequency and number of administrations, route of administration, and dose of antigen and lipid. Based on those results, we were able to optimize the vaccine formulation. The CCS/C liposomal formulation administered i.n. was also effective for hepatitis A vaccine in mice [23].

The aims of the current study were two-fold: Firstly, to confirm the superiority of CCS/C-influenza vaccine administered i.n. in the ferret model. This is a more relevant and established animal model in which influenza infection is induced by natural human influenza viruses and the severity of illness can be assessed using combined measurements of nasal and systemic signs, temperature and nasal cellular infiltration. Furthermore, influenza infection in the ferret model closely mimics human influenza with regard both to the sensitivity to infection and the clinical response [24]. Secondly, to elucidate the mechanism of action of this vaccine formulation administered i.n. to mice by trying to relate the physical attributes of the vaccine to its superior immunogenicity. By studying biodistribution, pharmacokinetics and immunomodulatory effects following i.n. administration to mice, we attempt to explain how this novel vaccine formulation is able to evoke a robust and protective response.

2. Materials and methods

2.1. Materials

2.1.1. Antigens

Monovalent subunit antigen preparations derived from influenza A/New Caledonia/20/99-like (H1N1) and A/Panama/2007/99-like (H3N2) strains were generously provided by Drs. Glück and Zurbriggen, Berna Biotech, Bern, Switzerland. These preparations consisted of 80–90 wt% hemagglutinin (HA), 5–10 wt% neuraminidase and trace amounts of NP and M1 proteins (Berna Biotech certificate of analysis). In addition, two commercial split-virion trivalent vaccines from several vaccination seasons were used for which HA content is ~50% of total protein – Fluvirin (Evans Vaccines Ltd., Liverpool, UK) and Vaxigrip (Sanofi Pasteur, Lyon, France). In mouse experiments, for formulation with VaxiSome™, the two trivalent vaccines were concentrated eight-fold using an Eppendorf Concentrator 5301 (Eppendorf AG, Hamburg, Germany). The concentration process resulted in ~80% recovery of the HA as determined by the HI assay. For simplicity, all vaccines are referred to as HA or HA vaccines.

2.1.2. Lipids

The phospholipids dimyristoyl phosphatidylcholine (DMPC) and dimyristoyl phosphatidylglycerol (DMPG) were purchased from Lipoid GmbH, Ludwigshafen, Germany. The monocationic lipids dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP) and dimyristoyl-3-trimethylammonium-propane (chloride salt) (DMTAP) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Cholesterol (C) was purchased from Sigma, and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) was obtained from Avanti Polar lipids or Lipoid. The novel polycationic sphingolipid N-palmitoyl D-erythro sphingosyl-carbamoyl-spermine (ceramide carbamoyl-spermine, CCS) [22] was synthesized as an acetate salt by Biolab Ltd., Jerusalem, Israel. N-^{3}H-palimitoyl D-*erythro* sphingosyl carbamoyl spermine was prepared at a specific activity of 4.44 μCi/mg. Where indicated, the helper lipid C was used at a lipid/helper lipid mole ratio of 1/1 for DMTAP and DOTAP or at mole ratios of 3/2, 2/1 and 3/1 for CCS-based vaccines. The helper lipid DOPE was used at a CCS/helper lipid mole ratio of 2/1. Recent H^+ NMR analysis showed that CCS is actually a mixture of two position isomers: N-palmitoyl-D-*erythro*-sphingosyl-1-0-carbamoyl-spermine (1-0-CCS) and N-palmitoyl-D-*erythro*-sphingosyl-3-0-carbamoyl-spermine (3-0-CCS), in a 4:1 mole ratio. For more details on the analysis and the similar performance of the separate isomers and their mixture see [25].

2.1.3. Mice

Specific pathogen-free (SPF) female 8–9-week-old BALB/c mice were used. Animals were maintained under SPF conditions. All animal studies were approved by the Institutional Animal Care and Use Committee and were consistent with the guide for the care and use of laboratory animals.

2.1.4. Ferrets

Healthy female ferrets (outbred, fitch and albinos), approximately 6 months old and with body weights 450–1000 g, were obtained from Retroscreen Virology repositories. Animals were maintained under a controlled ferret diet in a clean animal unit. All animal work was conducted according to H.M. Home Office guidelines.