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Intranasal immunization with a proteoliposome-derived cochleate containing recombinant gD protein confers protective immunity against genital herpes in mice

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ABSTRACT

The purpose of this study was to investigate the potential of intranasal (*IN*) immunization with *Neisseria meningitides* B proteoliposome (AFPL1) and AFPL1-derived cochleate (AFCo1), containing glycoprotein D (gD) of herpes simplex virus type 2 (HSV-2) for induction of protective immunity against genital herpes infection in mice. We could show that *IN* immunization with both AFPL1 and AFCo1 containing gD induced gD-specific IgG antibody and lymphoproliferative responses. However, IFN-γ response could only be detected in $CD4^+$ splenic cells and genital lymph node cells of the AFCo1gD immunized mice upon recall antigen stimulation *in vitro*. Importantly, *IN* immunization with AFCo1gD could elicit a complete protection against an otherwise lethal vaginal challenge with HSV-2, while the AFPL1gD immunized mice were only partially protected. Further, we could show that the IFN-γ response and protective immunity observed after *IN* immunization with AFCo1gD are mediated via the adaptor molecule myeloid differentiation factor 88. These data may have implications for the development of a mucosal vaccine against genital herpes.

1. Introduction

Herpes simplex virus type 2 (HSV-2) is the major cause of genital ulcer diseases world-wide. The incidence and prevalence of genital herpes infection continue to increase globally [1]. While many individuals experience life-long infection characterized by sporadic, sometimes frequent, ulcerative outbreaks, others have less severe, more infrequent outbreaks, with eventual long-term control of symptoms associated with development of HSV-specific adaptive immunity, in particular a Th1-type cell-mediated immunity [2–4]. Importantly, genital herpes infection is associated with an increased risk of acquisition of a human immunodeficiency virus (HIV) infection [1,5]

Although antiviral drugs are effective for the treatment of HSV-related symptoms, they require daily use and drug resistant HSV strains have emerged. The development of vaccines to prevent genital herpes is predominantly directed towards the viral surface glycoproteins, especially glycoproteins (g) B and gD with promising results [6]. However, clinical trials based on intramuscular immunization with gB and/or gD adjuvanted in alum or ASO4 adjuvant showed no or only limited promise [6,7]. Thus, there remains a need to develop new strategies to prevent genital herpes infection. Given the fact that HSV-2 is mainly transmitted via exposure at the genital tract mucosa, development of mucosal vaccine for genital herpes is an attractive strategy for which development of effective and safe mucosal adjuvants is required. It has been previously shown that some of the TLR agonists are capable of eliciting innate immunity to genital herpes infection [8–11]. Further, we and others have previously documented the potential of the TLR9 agonist CpG ODN as mucosal adjuvant for generation of protective immunity to genital herpes in mice [12,13].

Delivery systems such as nano- and microparticles as well as liposomes enhance the amount of antigen reaching antigen-presenting cells; however, they lack the ability of efficiently stimulating innate immune responses required for the development of humoral and cell-mediated adaptive immune responses. It is therefore desirable to develop vaccine adjuvants comprising delivery system and immunopotentiators to ensure that both antigen and immunostimulator are delivered into the

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same antigen-presenting cells [14]. Proteoliposome (PL) derived from outer membrane of *Neisseria meningitidis* B is a nanoparticle (~70 nm) containing major outer membrane proteins, lipopolysaccharide (LPS)/lipoolygosaccharide, and phospholipids. PL constitutes the main antigen of anti-meningococcal vaccine VA-MENGOC-BC™ developed by Finlay Institute [15] with more than 60 million doses given to humans. Previous studies have demonstrated the ability of PL as potent inducer of Th1-type immune response in mice and humans [16]. The PL adjuvant (AFPL1, Adjuvant Finlay PL 1) has shown a strong ability to activate dendritic cells (DC) and macrophages and to induce cross-presentation of a heterologous antigen incorporated into its vesicular structure [17,18]. AFPL1 contains several natural TLR ligands, including porins (TLR2 ligands) and native LPS (a TLR4 ligand) naturally inserted in the membrane as well as traces of bacterial DNA (a TLR9 ligand) [19].

AFPL1-derived cochleate (AFCo1) is a cochlear structure derived from the interaction of anionic lipid of PL with divalent cations, such as Ca^{2+} [20]. Cations establish an ionic bridge between the two negative charges of lipids from adjacent lipid membranes to stabilize the AFCo1 structure forming microparticles that contain main components of AFPL1 including its TLR ligands. In addition to the immunostimulatory property of AFCo1 shared with AFPL1, AFCo1 has an antigen delivery property inherent of cochlear structures [21,22]. Previous studies have shown that AFPL1 and AFCo1 could function as adjuvants to elicit a Th1-type immune response to co-administrated or incorporated antigens [16,19,22]. Particularly, AFCo1 was shown to be more potent as mucosal adjuvant than AFPL1, owing to its increased stability at mucosal surfaces [17].

The aim of this study was to investigate the potential of AFPL1 and AFCo1 as nasal-mucosal adjuvants for induction of protective immunity against genital herpes infection in mice.

2. Materials and methods

2.1. Mice

Six- to 8-week-old female C57Bl/6 mice (Taconic, Denmark) and MyD88$^{-/-}$ mice on C57Bl/6 background were used for experiments. Animals were housed in microisolators under specific-pathogen-free conditions at the Experimental Biomedicine animal facility, Sahlgrenska Academy at University of Gothenburg, Sweden. All experiments were performed with the approval from the Ethical Committee for Laboratory Animals in Gothenburg, Sweden.

2.2. Preparation of proteoliposome

PLs were prepared and supplied as ethanol precipitate by the vaccine production unit of Finlay Institute, Havana, Cuba, as for the *N. meningitidis* B vaccine. Briefly, PLs were obtained from the outer membranes of *N. meningitidis* serogroup B Cu 385-83 strain (B:4:P1.19.15;L3,7,9) by gentle extraction with 10% deoxycholate (DOC, Merck, Darmstadt, Germany). Bacterial debris was removed by centrifugation and nucleic acids were eliminated by enzymatic treatment with deoxyribonuclease and ribonuclease (5 μg/mL) (Merck). PLs were purified by gel filtration chromatography on Sephacryl S-300 (Pharmacia Fine Chemicals, Uppsala, Sweden) followed by precipitation with 96% ethanol. PLs are membrane vesicles that contain major outer membrane proteins (PorA and PorB), a complex of proteins from 65 to 95 kDa, LPS, phospholipids and traces of bacterial DNA. These structures were manufactured under GMP conditions at Finlay Institute, Havana, Cuba

2.3. Construction and expression of recombinant HSV-2 gD protein

The gene encoding the extracellular part of HSV-2 gD (aa 1–342) was PCR amplified using DNA from HSV-2 strain 333. The PCR product with an added C-terminal His_6-tag was then cloned in pcDNA6 expression vector (Invitrogen, Carlsbad, CA) using T4 DNA ligase (Fermentas) and *Hin*dIII and *Age*I restriction enzymes according to the standard protocol, and the DNA sequence of the resulting gene was confirmed (MWG Biotech, Ebersburg, Germany). The expression vector was transfected into CHO K1 cells cultured in Iscove's Modified Dulbecco's medium with 10% fetal bovine serum (Lonza Biologicals), using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Stable clones were generated in the presence of 10 μg/ml blasticidin (Invitrogen, Carlsbad, CA) and evaluated for secretion of gD protein by ELISA and Western blot using monoclonal anti HSV gD antibody (Abcam, UK). One clone was then re-cloned to obtain a stable clone and this was then adapted to suspension growth in ProCHO 4 with ProHT supplement, 4 mM L-glutamine (Lonza Biologicals) and 10 μg/ml blasticidin, by repeated passages in spinner flasks. The fully adapted clone was then cultured in a perfusion bioreactor with bubble-free aeration (Applikon) using the following set-points: 37 °C, pH 6.9 and pO_2 40%. Cells were retained in the reactor by a 10 μm spin filter and the cell supernatants were harvested and concentrated using tangential flow filtration in a Pellicon™-2 system with a 5 kDa filter (Millipore). The protein was purified using HiTrap Chelating HP column (GE Healthcare).

2.4. Incorporation of recombinant gD protein into AFPL1

Incorporation of gD protein within AFPL1 was performed by detergent disruption of PL followed by re-assembly in the presence of gD protein. Briefly, AFPL1 was dissolved in 30 mM Tris buffer (Fluka, Switzerland) containing EDTA (2 mM) (BDH, Poole, UK) and 0.5% DOC to give a final protein concentration of 1 mg/ml. The concentration of DOC in the AFPL1 suspension was increased to 1.5% in order to disrupt the vesicle structure. Recombinant gD protein was added to give a final concentration of 0.5 mg/mL. The mixture was dialyzed against five changes of Tris buffer for 24 h. AFPL1 cotaining gD protein was purified by ultracentrifugation (40 000 g 4 h). The presence of gD protein in the vesicles was verified by western blot using an anti-gD monoclonal antibody (Abcam, UK). The amount of incorporated gD was quantified by Lowry method.

2.5. Preparation of AFCo1 with recombinant gD protein incorporated

PLs were dissolved in a buffer containing 30 mmol/L Tris, 10 mmol/L EDTA and 1.5% DOC to a final protein concentration of 1 mg/mL. Recombinant gD was added to the PL solution at a concentration of 0.5 mg/mL and the mix was subjected to AFCo1 formation. AFCo1 formation was performed by detergent elimination and Ca^{2+} incorporation by a rotational dialysis methodology by using a washing buffer containing 30 mmol/L Tris, 100 mmol/L NaCl and 5 mmol/L $CaCl_2$, pH 7.4. The formation of cochleate was visualized by the appearance of a white suspension in the preparation and light microscopy analysis as described elsewhere [23]. The resulting AFCo1 was centrifuged at 3000 × *g* for 10 min and the pellets were washed with $CaCl_2$ buffer to eliminate non-incorporated gD protein. The presence of gD protein in the AFCo1 was determined by western blot using an anti-gD monoclonal antibody (Abcam, UK), and protein concentration in the AFCo1gD preparation was determined by Lowry method. The efficiency of incorporation was estimated by comparing the protein quantities in precipitate and supernatant, and was shown to be more than 85% in all cases.