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Immunologic characterization of a novel inactivated nasal mumps virus vaccine adjuvanted with Protollin

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ABSTRACT

An inactivated, mucosal mumps virus (MuV) vaccine would address many of the problems associated with current live-attenuated formulations. Protollin (Prl)-based adjuvants (containing TLR2 and TLR4 ligands) are well-suited for nasal administration. We sought to develop an inactivated whole-virus nasal vaccine for MuV using the Prl adjuvant/delivery vehicle and to test tolerability and immunogenicity in a mouse model. BALB/c mice exhibited signs of transient reactogenicity (hunched posture, erect fur, weight loss ≤10% of total body weight) following administration of intranasal MuV-Prl vaccines, though most of these manifestations resolved within 24 h. Compared to high-dose unadjuvanted vaccine (8 μgMuV), administration of high-dose adjuvanted formulation (8 μgMuV-Prl) induced greater MuV-specific serum IgG (3.26E6 ng/mL *vs.* 2.2E5 ng/mL, 8 μgMuV-Prl *vs.* 8 μgMuV, $p < 0.001$) and mucosal IgA (128 ng/mL *vs.* 45 ng/mL, 8 μgMuV-Prl *vs.* 8 μgMuV, $p < 0.05$). Serum IgG isotypes and splenocyte cytokine secretion induced by MuV-Prl suggested a predominant T helper cell (Th)1-type immune response. This response was characterized by: (1) ≥four-fold increase of IgG2a levels compared to IgG1; and (2) high IL-2 (644 pg/mL)/IFN-γ (228 pg/mL) and low IL-5 (31 pg/mL) secretion in MuV-restimulated splenocytes from animals receiving MuV-Prl formulations. MuV-Prl vaccination induced higher levels of serum antibodies capable of neutralizing MuV *in vitro* than MuV alone, particularly for high-dose 8 μg formulations (357 neutralizing units (NU)/mL *vs.* 32 NU/mL, 8 μgMuV-Prl *vs.* 8 μgMuV, $p < 0.001$). Thus, nasal MuV-Prl vaccines are fairly well-tolerated and highly immunogenic in mice.

1. Introduction

Introduction of mumps virus (MuV)-containing vaccines into national vaccine programs has dramatically reduced mumps incidence in many areas of the world [1]. However, continued mumps outbreaks in even highly-vaccinated populations have highlighted limitations of current vaccines and vaccination programs [2]. Also, currently licensed live-attenuated MuV vaccines can cause rare but serious adverse events [3,4], providing further support for continued development efforts to improve MuV vaccines.

An inactivated-nasal MuV vaccine would be an appealing alternative to current live-attenuated formulations [3]. Changing either the nature of the vaccine or the administration route may plausibly circumvent problems associated with current vaccines. The intranasal (IN) route would not only avoid needles (painful/biohazardous) [5], it would also simplify vaccine administration, mimic more closely the route of natural infection, and potentially avoid maternal antibody interference [6]. Finally, an inactivated formulation might avoid some of the serious side-effects associated with the live-attenuated vaccines.

The Protollin adjuvant (Prl) enhances immune responses to a range of inactivated respiratory virus vaccines [7–9]. The Prl nanoparticle is composed of outer-membrane proteins (OMP) purified from *Neisseria meningitidis* and hydrophobically complexed to lipopolysaccharide (LPS) derived from *Shigella flexneri* [9]. Prl-based vaccines are produced by simple mixing of Prl with antigen immediately prior to administration. Numerous features of Prl likely contribute to its immunostimulatory capabilities including the presence of at least two TLR ligands (TLR2:porB/TLR4:LPS) [10,11]. Both the nanoparticulate nature of Prl vaccines and their presentation of antigens in immunologically-relevant arrays are also major advantages.

Given the promising results with several other enveloped, respiratory viruses, we set out to develop a Prl-based MuV vaccine for IN delivery and to test its tolerability and immunogenicity in a BALB/c mouse model.

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2. Materials and methods

2.1. Cell growth and virus propagation

MuV Jones strain (ATCC#VR-365, m.o.i. = 0.0001) was grown in Vero cells (ATCC# CCL-81) at 33 °C, 5% CO_2, seeded in Corning 10-level CellSTACK culture chambers (infection media: EMEM supplemented with 3% fetal bovine serum (FBS), 1% HEPES, and 50 μg/mL gentamicin; Wisent Corporations, St. Bruno, QC). Culture supernatants were stored at −80 °C until purification of virions.

2.2. Preparation of detergent-inactivated MuV antigen

Thawed supernatants were clarified (2100 × *g*, 10 min, 4 °C) and centrifuged (25,000 × *g*, 4 °C, 7 h). For crude antigen preparations (to be used for ELISA and *ex vivo* re-stimulation of cryopreserved splenocytes), virus pellets were resuspended in sterile PBS (Wisent) and stored at −80 °C until further use. For vaccine-grade antigen (to be used in both MuV-Prl and MuV vaccines), virus pellets were resuspended in cold sterile TNE buffer (25 mM TrisCl, 150 mM NaCl, 5 mM EDTA), placed on a 60%:20% biphasic sucrose gradient, and ultracentrifuged (200,000 × *g*, 4 °C, 90 min). Virions were collected at the interface and pelleted by ultracentrifugation (200,000 × *g*, 4 °C, 90 min). Pellets were resuspended in 1% detergent (Empigen EMD BioScience Inc., La Jolla, CA) and placed on ice for 1 h. Dialysis of detergent-inactivated virus and protein quantitation of both crude and vaccine-grade antigen preparations were performed as described previously [7]. Preparations contained no infectious virus (assessed by $TCID_{50}$).

2.3. Characterization of MuV vaccine-grade antigen

Preparations were separated by electrophoresis on a 7.5% polyacrylamide gel under denaturing conditions and visualized with Coomassie Brilliant Blue staining (American Chemicals Ltd.). Individual protein amounts were estimated by quantitative densitometry of stained gels using Image J software (National Institutes of Health, Bethesda, MD). Proteins run on a parallel gel were transferred to PVDF membranes (ImmunoBlot PVDF membrane, Bio Rad Laboratories, Hercules, CA) for immunoblotting. Membranes were blocked (1 h, room temperature, in 5% milk in PBS containing 0.05% Tween (PBS-T) or 3% bovine serum albumin (BSA – Sigma–Aldrich Inc., St. Louis, MO)), washed with PBS-T, and probed with MuV-specific antibodies (polyclonal rabbit anti-F and anti-HN, donated by Dr. Kathryn Carbone, Food and Drug Administration, Bethesda, MD) overnight at 4 °C. After washing with PBS-T, membranes were incubated with goat anti-rabbit IgG-horseradish peroxidase (HRP) conjugate (Santa Cruz Laboratories, Santa Cruz, CA) (1 h, room temperature). Proteins were detected by chemiluminescence and visualized by autoradiography (both methods described previously [7]).

MuV-Prl formulations were characterized by electron microscopy as previously described [7], using the following antibodies: polyclonal rabbit anti-F/anti-HN polyclonal antibodies (same as for immunoblotting) and goat anti-rabbit IgG conjugated with 10 nm gold (Sigma–Aldrich, Saint Louis, MO).

2.4. Formulation of detergent-inactivated MuV vaccines

Vaccine-grade MuV antigen was used in both adjuvanted and unadjuvanted formulations. The Prl adjuvant was formulated as previously described [12] and obtained from ID Biomedical of Canada. For MuV-Prl vaccines, Prl (4 μg) was added to 4 or 8 μgMuV vaccine-grade antigen and mixed gently by pipetting. All vaccine preparations were performed immediately prior to vaccine administration. The final Prl amount was based on LPS content measured by the KDO (2-keto-3-deoxyoctonate) assay [13] while the final MuV amount was based on estimation of surface glycoprotein content (F/HN).

2.5. Mouse immunization

All animal procedures were approved by the McGill University Animal Care and Use Committee. Vaccine schedules/regimens and study groups are described in Fig. 2. Animals were vaccinated IN/IT under isofluorane anesthesia by pipetting 12.5 μL of formulation into each nare during quiet breathing. For intramuscular (IM) vaccination, 40 μL of formulation was administered into the hind thigh muscle of anaesthetized mice.

2.6. Animal procedures and sample collection

Mice were monitored throughout the experiment for weight loss and signs of reactogenicity. On the last day of each study, mice were euthanized by CO_2 asphyxiation and exsanguinated by cardiac puncture. Sera were obtained from blood samples by centrifugation and lung mucosal secretions were collected by bronchoalveolar lavage (BAL) as previously described [7]. Splenocytes were collected as previously described [7], pooled by group, and cryopreserved in liquid nitrogen.

2.7. Antibody responses in serum and BAL measured by ELISA

Serum total IgG, IgG1 and IgG2a and mucosal IgA levels were determined by enzyme-linked immunosorbant assays (ELISA), as described previously [7]. Crude MuV antigen was used for coating. Secondary antibodies used were: goat anti-mouse IgG-HRP (Jackson Immunoresearch Laboratories, West Grove, PA), goat anti-mouse IgG1-HRP (Southern Biotechnologies Associates, Birmingham, AL) and goat anti-mouse IgA-HRP (Sigma–Aldrich, Saint Louis, MO). All incubations were performed at room temperature. Antibody titers were calculated based on standard curves run on each plate. Selected wells were coated with either goat anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA) or goat anti-mouse IgA (Sigma–Aldrich Canada, Oakville, ON). Purified murine IgG (Sigma–Aldrich Canada, Oakville, ON), IgG1 (Pharmingen BD, San Diego CA), IgG2a (Pharmingen BD, San Diego, CA) or IgA (Bethyl Laboratories, Montgomery, TX) was then serially diluted (1:2) and plated onto wells containing anti-IgG or anti-IgA antibodies, in duplicate for each concentration.

2.8. Serum neutralizing antibody responses

Sera from individual mice were diluted ten-fold in flat-bottomed 96-well MICROTEST tissue culture plates, and serially diluted two-fold. All dilutions were performed in quadruplicate in EMEM supplemented with 5% FBS, 1% HEPES and 50 μg/mL gentamicin (complete EMEM). MuV Jones strain was added (100 $TCID_{50}$ units/well) and plates were incubated at 37 °C, 5% CO_2 for 30 min. Vero cells were then added (1E5 cells/well) and plates were incubated at 37 °C, 5% CO_2 for four days. Wells demonstrating no cytopathic effect were recorded manually and titers were calculated using the Kärber method [14] to determine 50% end-point neutralization.

2.9. Cytokine secretion of re-stimulated splenocytes

Cryopreserved splenocytes were thawed, washed, and seeded in quadruplicate to flat-bottomed 96-well MICROTEST tissue culture plates (6E5 cells/well). Cells were stimulated with 1 μg/mL crude MuV antigen and incubated (37 °C, 5% CO_2,