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Vaccination with a human parainfluenza virus type 3 chimeric FHN glycoprotein formulated with a combination adjuvant induces protective immunity

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ABSTRACT

Human parainfluenza virus type 3 (PIV3) is a major cause of lower respiratory disease i.e. bronchitis, bronchiolitis or pneumonia, in infants and young children. Presently there is no licensed vaccine against PIV3. To produce an effective subunit vaccine, a chimeric FHN glycoprotein consisting of the N-terminal ectodomain of the fusion (F) protein linked to the haemagglutinin-neuraminidase (HN) protein without transmembrane domain, and secreted forms of the individual F and HN glycoproteins, were expressed in mammalian cells and purified. Mice and cotton rats were immunized intramuscularly (IM) with FHN or both F and HN proteins (F + HN), formulated with poly(I:C) and an innate defense regulator peptide in polyphosphazene (TriAdj). Significantly higher levels of systemic virus-neutralizing antibodies were observed in mice and cotton rats immunized with FHN/TriAdj when compared to animals immunized with the combination of F and HN proteins (F + HN/TriAdj). As PIV3 is a pneumotropic virus, another goal is to produce an effective mucosal subunit vaccine. Intranasal (IN) administration with FHN/TriAdj resulted in mucosal IgA production in the lung and virus neutralizing antibodies in the sera. After PIV3 challenge no virus was detected in cotton rats immunized with FHN/TriAdj regardless of the route of delivery. Protective immunity against PIV3 was also induced by FHN/TriAdj in hamsters. In conclusion, the FHN protein formulated with TriAdj has potential for development of a safe and effective vaccine against PIV3.

1. Introduction

Human parainfluenza viruses (PIVs) are common respiratory pathogens and a major cause of respiratory disease such as bronchiolitis and pneumonia in infants, young children and immunocompromised adults globally. Parainfluenzaviruses are second only to respiratory syncytial virus (RSV) as causative agents of acute respiratory tract illness in children under 5 years of age. They belong to the family *Paramyxoviridae* and are genetically and antigenically divided into four major serotypes, PIV1 to PIV4. Among all PIVs, PIV3 is the most virulent and is associated with significant morbidity and mortality [1,2].

Parainfluenzavirus-3 is an enveloped virus with a single-stranded, non-segmented, negative-sense RNA genome. This virus encodes six proteins: nucleo (N), phopho (P), matrix (M), fusion (F), haemagglutinin-neuraminidase (HN) and large (L) proteins, among which two glycoproteins, F and HN, represent major targets for neutralizing antibodies [1,3]. The HN glycoprotein initiates the viral infection in host cells by binding to sialic acid on the plasma membrane, and is responsible for the haemagglutination and neuraminidase activities. The F glycoprotein mediates penetration and fusion of the viral envelope to host cells, syncytium formation and haemolysis [2]. During viral entry, the HN protein binds to its receptor and triggers the F protein to mediate fusion with the target cell membrane [3]. Since both the HN and F glycoproteins play a crucial role in the pathogenesis of PIV3 infection, they have been identified as major subunit vaccine candidates. Serological studies have also revealed that inhibition of PIV3 replication correlates to the development of antibodies to both the HN and F glycoproteins in animals and humans [4–6].

A number of vaccine candidates, including live attenuated and recombinant viruses, vector-based and subunit protein vaccines

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have been developed and evaluated in rodents and humans, but there are still no licensed vaccines available for prevention of human PIV3 infection [4,7–13]. In the present study we expressed truncated, secreted forms of the F and HN proteins, individually and as chimeric FHN protein, and formulated them with poly(I:C), innate defense regulator peptide (IDR) and poly[di(sodium carboxylatoethylphenoxy)]-phosphazene (PCEP) (TriAdj). The chimeric FN protein was more immunogenic than the combination of F and HN proteins. Furthermore, intramuscular (IM) immunization with FHN/TriAdj elicited complete protection from PIV3 challenge in cotton rats and hamsters. Intranasal (IN) immunization with FHN/TriAdj was also evaluated in cotton rats, which developed both systemic and mucosal immune responses, resulting in protection from PIV3 challenge.

2. Materials and methods

2.1. Cells and virus

LLC-MK2 monolayer cell cultures (ATCC, Manassas, VA, USA) were maintained in minimal essential medium (MEM, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 10 mM HEPES buffer (Thermo Fisher Scientific), 0.1 mM nonessential amino acids (Thermo Fisher Scientific) and 50 μg/ml gentamicin (Thermo Fisher Scientific). The PIV3 C243 strain (ATCC) was propagated in LLC-MK2 cells.

2.2. Preparation of secreted forms of F, HN and FHN proteins

The ORFs of the truncated F and HN (human PIV3 strain J; accession number Z11575) were codon optimized and synthesized by GenScript (Piscataway, NJ, USA). The F protein ORF was designed to express a truncated F (amino acids 23-466) with the carboxyl terminal transmembrane domain replaced by a gsgsg(h)12 tag to facilitate purification; the native signal peptide was replaced with that for human tissue plasminogen activation factor (TPA) to enhance secretion. The HN ORF was designed to express a truncated version (amino acids 87-572) with the amino terminal transmembrane domain replaced by a TPA signal peptide and ending with a gsgsg(h)12 tag. The chimeric truncated FHN ORF expresses F (amino acids 23-466) linked to HN (amino acids 87-572) via a glycine-serine linker (BamHI site); the protein is preceded by TPA and ends with gsgsg(h)12. All ORFs are proceeded by a Kozak sequence and cloned downstream of a human CMV promoter and intron contained within an in-house episomal vector. The vector contains the EBNA-1 antigen ORF and P origin; elements downstream of cloned ORFs include a woodchuck hepatitis post-transcriptional regulatory element and bovine growth hormone poly-adenylation site (accession no MG182339). HEK293T cells were transfected with the constructs and episomal maintenance was selected for by puromycin resistance. HEK293T stably expressing protein were grown in serum-free medium (SFM4HEK293, Thermo Fisher Scientific). Supernatant was concentrated and dialyzed by tangential flow and recombinant protein was purified using His60 Ni superflow resin (Clontech, Mountain view, CA, USA) according to the manufacturer's instructions. The proteins were analyzed by SDS-PAGE. Proteins were transferred to nitrocellulose and incubated with PIV3 F- and HN-specific rabbit sera (in-house), followed by incubation with alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG (KPL, Gaithersburg, MD, USA) and development with SIGMAFAST™ BCIP/NBT tablets (Sigma-Aldrich).

2.3. Vaccination and challenge

Female BALB/c mice (8–10 weeks of age) (Charles River Laboratories, Saint-Constant, QC, Canada), cotton rats (*Sigmodon hispidus*) (Envigo, Indianapolis, IN, USA) and golden Syrian hamsters (Charles River Laboratories, Saint-Constant, QC, Canada) were vaccinated IM with a combination of 1 μg of F and HN protein each or with 2 μg of FHN protein formulated with TriAdj. In all experiments there were five animals per group. The TriAdj contains 10 μg poly(I:C) (Invivogen, San Diego, CA, USA), 20 μg IDR peptide 1002 (Genscript), and 10 μg PCEP (Idaho National Laboratory, Idaho Falls, ID, USA) in phosphate-buffered saline (PBS, Gibco). One additional group of animals received PBS only. For IN immunization, cotton rats were vaccinated with a combination of 3 μg of F and HN protein each or 6 μg of FHN protein formulated with TriAdj. Three weeks after the second immunization mice were euthanized and serum samples were collected. Cotton rats and hamsters were challenged IN with PIV3 strain C243 (1×10^6 PFU/100 μl) three weeks after the second vaccination and euthanized, respectively four and three days after challenge for detection of virus in the lungs. Blood samples were obtained under anesthesia prior to each vaccination, and before and after challenge. The University Animal Ethics Committee approved all procedures in accordance with the standards stipulated by the Canadian Council on Animal Care.

2.4. Lung fragment cultures and enzyme-linked immunosorbant assay (ELISA)

Lung fragment cultures were prepared as previously described [14]. Briefly, lungs of challenged cotton rats were cut into pieces and cultured in complete medium for 5 days at 37 °C. Cell-free supernatants were collected and stored at −80 °C. PIV3 F- and HN-specific IgG, IgG1, IgG2a and IgA were evaluated in serum and lung fragment culture supernatants by ELISA. Briefly, 96-well microtiter plates (Thermo Fisher Scientific) were coated with F or HN protein (50 ng/ml) overnight at 4 °C. After washing, 4-fold serially diluted samples were applied to the protein-coated plates and incubated overnight at 4 °C. Bound F- and HN-specific antibodies were detected with biotinylated anti-mouse IgG1, IgG2a (Southern Biotech, Birmingham, AL, USA) or IgA (Thermo Fisher Scientific), anti-cotton rat IgG (Immunology consultants lab, Portland, OR, USA), or anti-hamster IgG (Southern Biotech, Birmingham, AL, USA). Lastly, the substrate *p*-nitrophenyl phosphate (Sigma-Aldrich) or TMB (Thermo Fisher Scientific) was added, and after incubation plates were read in a SPECTRAmax 340 PC Microplate Reader (Molecular Devices, CA, USA).

2.5. Virus neutralization assay

PIV3-specific neutralization titers were determined by plaque reduction assays. Sera were serially diluted in 96-well plates (Corning Incorporated, Corning, NY, USA), and then mixed with PIV3 for 75 min at 37 °C. The sample-virus mixtures were transferred to duplicate LLC-MK2 cell monolayers and incubated for 3 h at 37 °C. After incubation, sample-virus mixtures were removed from the plates and cells were overlaid with MEM supplemented with 2% heat-inactivated FBS (Thermo Fisher Scientific) and 2% methylcellulose (Sigma-Aldrich). Four days after incubation, the overlay medium was removed and the cells were fixed with 75% ethanol/25% acetic acid. Plaques were visualized with rabbit anti-PIV3 antibody (in-house) followed by AP-conjugated goat anti-rabbit IgG (KPL), and developed with an AP conjugate substrate kit (Bio-rad).

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