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The recombinant globular head domain of the measles virus hemagglutinin protein as a subunit vaccine against measles

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ABSTRACT

Despite the availability of live attenuated measles virus (MV) vaccines, a large number of measles-associated deaths occur among infants in developing countries. The development of a measles subunit vaccine may circumvent the limitations associated with the current live attenuated vaccines and eventually contribute to global measles eradication. Therefore, the goal of this study was to test the feasibility of producing the recombinant globular head domain of the MV hemagglutinin (H) protein by stably transfected human cells and to examine the ability of this recombinant protein to elicit MV-specific immune responses. The recombinant protein was purified from the culture supernatant of stably transfected HEK293T cells secreting a tagged version of the protein. Two subcutaneous immunizations with the purified recombinant protein alone resulted in the production of MV-specific serum IgG and neutralizing antibodies in mice. Formulation of the protein with adjuvants (polyphosphazene or alum) further enhanced the humoral immune response and in addition resulted in the induction of cell-mediated immunity as measured by the production of MV H-specific interferon gamma (IFN-γ) and interleukin 5 (IL-5) by *in vitro* re-stimulated splenocytes. Furthermore, the inclusion of polyphosphazene into the vaccine formulation induced a mixed Th1/Th2-type immune response. In addition, the purified recombinant protein retained its immunogenicity even after storage at 37 °C for 2 weeks.

1. Introduction

Vaccination against measles with the current licensed live attenuated virus (LAV) vaccines has resulted in a significant decline in the measles cases in many countries and measles elimination in large geographical regions. However, in some countries of Africa and South East Asia measles remains a leading cause of high mortality among young infants [1]. This is partially due to the limitations of the currently used LAVs. For instance, effective immunization of infants below the age of 9 months is unsuccessful due to the presence of interfering maternal antibodies [2]. Furthermore, a cold chain must be maintained to support anti-measles vaccinations because LAVs are thermally unstable [3].

Subunit vaccines are among those currently being considered as alternative measles vaccine candidates [4]. The ISCOM and Protollin formulations incorporating measles virus (MV) glycoproteins have been shown to induce both humoral and cell-mediated immune responses, as well as antiviral protection in rodents and macaques [5–10]. In addition, ISCOM formulations have been shown to be protective in the presence of passively acquired MV-specific antibodies [8]. However, to our knowledge, the measles virus antigens used in these studies have been expressed in mammalian cells based on viral vectors or were derived from a split virus antigen preparation. The major drawback of these expression systems is the safety concern associated with the use of live viruses for the production of the MV glycoproteins.

The hemagglutinin (H) protein of MV plays a critical role in viral tropism, receptor binding, hemagglutinating activity and the induction of protective immunity against viral infection [11]. It is a 617-residue type II integral membrane glycoprotein that consists of a C-terminal globular head domain, a long stalk, an N-terminal transmembrane domain and a short cytoplasmic tail [11]. Although the H protein and the F (fusion) protein are both thought to be important for the induction of effective MV-specific immunity, the majority of neutralizing antibodies are generated against the MV H

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protein; in fact, these antibodies against the H protein neutralize MV *in vitro* and provide protection against MV *in vivo* [9,12–17].

The X-ray crystal structure of the MV H protein globular head domain demonstrated that a significant area of the protein is covered with N-linked sugars [18,19]. As such, a subunit vaccine would ideally be produced in a mammalian cell expression system as its antigenicity and immunogenicity may depend on its glycosylation state [20]. Since a cell line-based technology has added advantages in terms of safety, scalability and productivity, we generated a stable human cell line (HEK293T) producing a secreted form of the globular head domain of the MV H protein. The protein and its formulations with different adjuvants were found to induce both humoral and cellular MV-specific immune responses in mice.

2. Materials and methods

2.1. Construction of the recombinant MV H protein expression plasmid

The pUC-MV-H plasmid containing a codon optimized MV H gene was constructed by GeneScript Corporation (Piscataway, USA). The DNA of this plasmid served as a template to amplify a part of the MV H gene encoding the globular head domain of the protein (amino acids 156–617 of Edmonston strain) by PCR using the Phusion High-Fidelity PCR Kit (New England Biolabs). Briefly, PCR was carried out in 50 μl containing 25 pmol of each primer (MV-H-head-FOR) 5′-TTGGCCGGCCAGACGTTGCCGCCGAAGAGTT-3′ and (MV-H-head-REV) 5′-ATTTGCGGCCGCTCGGCGATTAGTGCCATCTT-3′. The reaction conditions were as follows: enzyme activation for 1 min at 95 °C followed by 30 cycles of denaturation for 1 min at 95 °C, annealing for 1 min at 56 °C, and extension for 1 min at 72 °C. The cycling protocol included a final extension for 1 min at 72 °C. The product was then digested with *Fse*I and *Not*I (New England Biolabs) and the resulting fragment was ligated into expression vector, pPA-TEV [21], in frame with the transin [22] leader, the protein A purification tag and the tobacco etch virus (TEV) protease cleavage site resulting in pProtA-MV-H156/617. The sequence of the recombinant gene was confirmed by nucleotide sequencing.

2.2. Generation of a stable cell line expressing the globular head domain of MV H protein

Human embryonic kidney (HEK) 293T cells (ATCC CRL-1573) were plated in a 6-well plate (10^6 cells/well) and maintained in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) supplemented with 10% heat-inactivated FBS (Lonza), 0.1 mM non-essential amino acids (Invitrogen), 10 mM HEPES buffer (Invitrogen) and 50 μg/ml gentamicin (Invitrogen). When the monolayers were 70–80% confluent, the cells were transfected with 6 μg of pProtA-MV-H156/617 using the Calcium Phosphate Transfection Kit (Promega) according to the manufacturer's instructions. To produce stably transfected cell lines, puromycin (Invitrogen) was added one day after transfection to a final concentration of 5 μg/ml. Resistant clones were clearly visible 13 days following transfection. The culture media were collected from individual clones and analyzed by Western blotting with a protein A-specific monoclonal antibody (Sigma–Aldrich, Cat # 2921).

2.3. Western blotting

To detect the expression of the recombinant ProtA-MV-H156/617 protein, medium from the cloned cell lines was incubated at 96 °C for 5 min in 2× loading buffer containing 4% β-mercaptoethanol (Sigma–Aldrich) and separated by 10% SDS-PAGE. The separated proteins were transferred onto a nitrocellulose membrane (Bio-Rad). The membrane was blocked overnight with 5% skim milk in TBS containing 0.05% Tween-20 at 4 °C, followed by incubation for 4 h at room temperature with the protein A-specific mAb diluted 1:5000. Bound antibodies were detected by incubating the blot with goat anti-mouse HRP-conjugated secondary antibody (1:3000) (GE Healthcare) at room temperature for 1 h, and the protein bands were detected using the ECL-plus reagent (GE Healthcare). The purified recombinant protein MV-H156/617 (without the protein A tag) was analyzed by 10% SDS-PAGE under reducing conditions with Coomassie blue G-250 staining.

2.4. Purification of the recombinant protein

The recombinant ProtA-MV-H156/617 protein was produced in HEK 293T cells (clone 14) grown in DMEM/F12 (50:50, Invitrogen) containing 3% FBS (Invitrogen), 100 U/ml penicillin, 0.1 mg/ml streptomycin, 1 mg/l puromycin, and 1 mg/l aprotinin. The media were filtered and concentrated 10-fold on a TIFF prepscale concentrator (Millipore). The concentrated medium was incubated with IgG sepharose beads overnight at 4 °C. The beads were then washed with buffer (50 mM Tris pH 7.5, 150 mM NaCl) followed by an overnight on-column cleavage with TEV protease to release the H protein fragment (MV-H156/617). The H protein fragment was exchanged into 50 mM Tris pH 7.5, 50 mM NaCl and purified on a HiTrap Q (GE Healthcare) column. The resulting protein was concentrated (to 1 mg/ml) and exchanged into sterile PBS using a 10 K concentrator (Millipore) for the immunization studies. The purified protein yield was ~1.8 mg per 2 L of harvested cell culture medium.

2.5. Mouse immunizations

Three independently performed immunization studies were conducted in 6- to 8-week-old female C57BL/6 mice. In the first experiment, mice were randomly allocated to seven groups of five animals each and vaccinated two times subcutaneously (SC) at a 4-week interval. Three groups of mice (1–3) were immunized with 100 μl solutions containing 0.3 μg, 3 μg or 15 μg of purified MV-H156/617 per mouse. The other three groups (4–6) were immunized with 100 μl solutions containing 0.3 μg, 3 μg, or 15 μg of the protein formulated with alum (Alhydrogel "85", Brenntag Biosector; 2.5 μl/μg of the protein). Group 7 (negative control) was vaccinated SC with 100 μl of PBS. Sera were collected prior to the first immunization and again at week 4 (prior to the boost) and week 7. In the second experiment, mice were randomly allocated to four groups of six animals each and vaccinated twice SC at a 4-week interval, with one of the following: (group 1) 9 μg MV-H156/617 protein with alum, using protein stored at 4 °C prior to immunizations; (group 2) 9 μg MV-H156/617 protein with alum, using protein incubated at 37 °C for 1 week prior to immunizations; (group 3) 9 μg MV-H156/617 protein with alum, using protein incubated at 37 °C for 2 weeks prior to immunizations. The fourth group of mice was immunized with saline. Sera were collected prior to the first immunization and again at week 7. In the third experiment, mice were randomly allocated to four groups of five animals each and vaccinated twice SC at a 4-week interval. The individual groups of mice were either immunized with PBS, antigen alone (3 μg of the MV-H156/617 protein per dose), the same dose of protein formulated with alum, or the same dose of protein formulated with 50 μg poly[di(sodiumcarboxylatoethylphenoxy)phosphazene] (PCEP). Mice were bled prior to the first immunization, and again at week 4 (prior to the boost) and week 7. Following euthanasia, spleens were collected and examined for the number of antigen-specific cytokine-secreting cells.

All procedures involving animals were approved by the University of Saskatchewan's Animal Research Ethics board, and adhered