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Respiratory syncytial virus subunit vaccine based on a recombinant fusion protein expressed transiently in mammalian cells

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ABSTRACT

Although respiratory syncytial virus (RSV) causes severe lower respiratory tract infection in infants and adults at risk, no RSV vaccine is currently available. In this report, efforts toward the generation of an RSV subunit vaccine using recombinant RSV fusion protein (rRSV-F) are described. The recombinant protein was produced by transient gene expression (TGE) in suspension-adapted human embryonic kidney cells (HEK-293E) in 4 L orbitally shaken bioreactors. It was then purified and formulated in immunostimulating reconstituted influenza virosomes (IRIVs). The candidate vaccine induced anti-RSV-F neutralizing antibodies in mice, and challenge studies in cotton rats are ongoing. If successful in preclinical and clinical trials, this will be the first recombinant subunit vaccine produced by large-scale TGE in mammalian cells.

1. Introduction

Respiratory syncytial virus (RSV) causes severe lower respiratory tract infections in infants and adults at risk [1,2]. Although a vaccine against RSV is expected to reduce the disease burden considerably, no licensed RSV vaccine is yet available because of two main reasons. First and tragically, enhanced disease was observed in infants after immunization with a formalin inactivated vaccine in the 60 s, which impeded research because the mechanisms for enhanced disease needed to be clarified [3,4]. Second, RSV is naturally weakly immunogenic, and it is a challenge to develop a vaccine that induces long-term immunization [5,6]. An attractive candidate subunit vaccine is the viral fusion protein (RSV-F) because of its ability to induce a protective immune response in animals when administered with an adjuvant that supports the induction of a safe, effective, and balanced immune response [7,8]. Immunostimulating reconstituted influenza virosomes (IRIVs) have been proven to be safe and are approved as a vaccine adjuvant. IRIVs are spherical, unilaminar liposomal vesicles bearing the influenza virus hemagglutinin (HA) and neuraminidase (NA) proteins but devoid of any viral genetic material [9]. IRIVs with intercalated viral RSV-F (vRSV-F) induced a balanced immune response in mice and did not cause the pathology observed with other RSV candidate vaccines [10].

The production of vRSV-F, however, is hampered by low virus titers in cell culture and by biosafety concerns. An alternative approach proposed here is based upon the production of recombinant RSV-F (rRSV-F) in mammalian cells by large-scale transient gene expression (TGE). Mammalian cells are the preferred host system for the production of therapeutic proteins of high complexity since they are able to perform correct post-translational modifications such as glycosylation, disulphide bond formation, and oligomerization. The standard method of recombinant protein production in mammalian cells involves the generation of a recombinant cell line that stably expresses the protein [11]. The establishment and characterization of a stable cell line is time consuming, and this approach may not be feasible for rRSV-F since it induces the formation of syncytia, which may impair cell growth and decrease cell viability [12]. In contrast to stable gene expression, TGE allows rapid recombinant protein production in mammalian cells within a short time period (2–10 days) from the time of DNA delivery [13]. Until now, however, TGE has only been used for the production of recombinant proteins for analytical and preclinical studies. There are no approved and marketed recombinant therapeutic proteins produced by TGE so far. To our knowledge, this is the first study reporting the application of large-scale TGE for the manufacture of a recombinant subunit vaccine envisioned for human use.

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2. Materials and methods

2.1. Plasmid DNA and PEI

pcDFsyn was obtained from Prof. Grunwald, Department of Molecular and Medical Virology at the Ruhr-Universität Bochum. It was constructed by cloning the codon-optimized RSV-F cDNA from strain A2 into pcDNA3.1 (Invitrogen AG, Basel, Switzerland) as described [14]. Purified plasmid DNA for transfection was produced by Fasteris SA (Geneva, Switzerland). Linear 25 kDa polyethyleneimine (PEI; Polysciences, Eppelheim, Germany) was dissolved in water at a concentration of 1 mg/mL at pH 7 and filter sterilized.

2.2. Cell culture

Suspension-adapted HEK-293E cells were routinely grown in orbitally shaken, square-shaped glass bottles (Schott Glass, Mainz, Germany) in Ex-Cell293 medium (SAFC Biosciences, St. Louis, USA) supplemented with 4 mM glutamine. Cells were incubated at 37 °C in 5% CO_2 with orbital shaking at 110 rpm [15]. The VoluPAC system (Sartorius AG, Göttingen, Germany) was used to determine packed cell volume which was correlated to cell density as previously described [16].

2.3. Transfections

One day prior to transfection, cells were seeded at 1×10^6 cells/mL in Ex-Cell293 medium with 4 mM glutamine as described above. Small-scale transfections were performed in "CultiFlask 50" tubes (Sartorius AG, Göttingen, Germany) with a final working volume of 10 mL [17]. On the day of transfection, cells were pelleted by centrifugation and resuspended in 0.5–1.5 mL of RPMI 1640 medium (Lonza, Vervier, Belgium) containing 25 mM Hepes, 4 mM glutamine, and 0.1% Pluronic F68 (AppliChem, Darmstadt, Germany) at a cell density of 20×10^6 cells/mL. DNA and PEI were sequentially added to the cultures at the concentrations indicated in the text. The tubes were incubated at 37 °C in an atmosphere with 5% CO_2 and 85% humidity with orbital shaking at 180 rpm (ISF-4-W incubator, Kühner AG, Birsfelden, Switzerland). After 3 h cells were diluted to a cell density of $1–3 \times 10^6$ cells/mL to a final volume of 10 mL with Ex-Cell293 medium containing 4 mM glutamine as indicated in the text. Incubation was continued as before.

The 4-L transfection was performed in a 10-L square-shaped polycarbonate disposable container (Biotainer Carboy, Cellon, Luxembourg) that was fitted with a two-port cap [18]. One of the ports was connected to a 0.22 μm sterile filter (Sartorius AG, Göttingen, Germany) for gas exhaust. The second port was connected to a membrane pump, and a constant air flow with 5% CO_2 was pumped into the headspace of the container through a sterile filter. On the day of transfection, cells were pelleted by centrifugation and resuspended in 600 mL of RPMI 1640 medium containing 25 mM Hepes, 4 mM glutamine, and 0.1% Pluronic F68 in a 5 L glass bottle (Schott Glass, Mainz, Germany) at a cell density of 20×10^6 cells/mL. 10 mg of plasmid DNA and 32 mg of PEI were sequentially added to the cell culture. Cells were incubated at 37 °C with orbital shaking at 110 rpm (ISF-4-W incubator, Kühner AG, Birsfelden, Switzerland) for 3 h and then transferred to the 10-L polycarbonate container where they were diluted to a cell density of 3×10^6 cells/mL with Ex-Cell293 medium containing 4 mM glutamine. Incubation was continued at 37 °C with orbital shaking set at 64 rpm.

2.4. rRSV-F quantification by ELISA

0.5 mL samples of cell cultures were collected in eppendorf tubes, cells were pelleted by centrifugation and the cell culture medium discarded. The cell pellet was resuspended in PBS containing 1% Triton X-100 and a cocktail of protease inhibitors (Roche Applied Sciences, Indianapolis, USA) and incubated at 4 °C with agitation for 30 min. Solubilised protein was recovered in the supernatant following centrifugation. Microtest 96-well ELISA plates (BD Biosciences, Bedford, USA) were coated with an anti-RSV-F monoclonal antibody in coating buffer. A rabbit anti-RSV-F polyclonal antibody was used as a secondary antibody, and the detection antibody was a horseradish peroxidase-conjugated goat anti-rabbit IgG monoclonal antibody (Bio-Rad Laboratories, Hercules, USA). o-Phenylenediamine in stable peroxide substrate buffer (Pierce, Rockford, USA) was added as a detection substrate and incubated at room temperature. The reaction was stopped by addition of sulphuric acid to 0.5 M (Sigma, Saint-Louis, USA), and absorption was measured at 490 nm using a microplate reader (SpectraMax plus, Bucher Biotech, Basel, Switzerland). vRSV-F purified from Vero cells infected with wild type RSV strain A2 [19] was used in concentrations from 0.02 to 1000 ng/mL for the standard curve.

2.5. Protein purification and formulation in IRIVs

rRSV-F purification was achieved by a combination of chromatography steps to be described in detail in a separate publication. vRSV-F was purified from Vero cells infected with wild type RSV strain A2 as described [19]. IRIVs were prepared as described [20,21]. Briefly, 32 mg of egg phosphatidylcholine (Lipoid, Cham, Switzerland) and 8 mg of 1-oleoyl-3-palmitoyl-rac-glycero-2-phosphoethanolamine (Bachem, Bubendorf, Switzerland) were dissolved in 3 mL of PBS containing 100 mM octaethyleneglycol-mono-(n-dodecyl)ether (OEG-PBS, Fluka, Buchs Switzerland). Inactivated influenza A/H1N1 virus HA and NA proteins (2 mg) were centrifuged at $100{,}000 \times g$ for 1 h at 4 °C, and the pellet was dissolved in 1 mL of OEG-PBS. The detergent-solubilised phospholipids and virus proteins were mixed, sonicated for 1 min, and centrifuged at $100{,}000 \times g$ for 1 h at 18 °C. For virosome formation by detergent removal, the supernatant was removed and mixed twice with 1.5 g of wet SM2 Bio-Beads (BioRad, Reinach, Switzerland) each for 1 h at room temperature with agitation. The virosomes were then sterile filtered (0.22 μm, Supor membrane, Pall, Basel, Switzerland). RSV-F-containing IRIVs were prepared in a similar way. Purified viral or recombinant RSV-F (0.4–1.6 mg) was added to the mixture of lipids and HA/NA before detergent removal. The amounts of HA and RSV-F in each IRIV preparation were determined by single radial immunodiffusion assay and ELISA, respectively.

2.6. Immunization of mice

BALB/c mice were pre-immunized intramuscularly with 0.1 mL of the commercial whole virus influenza vaccine Inflexal BernaTM (Berna Biotech, Bern, Switzerland). Three weeks later, mice were immunized twice with an interval of 2 weeks with IRIVs containing 7 μg viral or recombinant RSV-F. Blood samples were collected 2 weeks after the final injection.

2.7. Quantification of anti-RSV-F antibodies

Anti-RSV-F antibody concentration in the serum of immunized mice was measured by ELISA. MaxisorpTM plates (Nunc, Fisher Scientific, Wohlen, Switzerland) were coated overnight at 4 °C with 100 μl per well of a 1 μg/mL solution of vRSV-F in carbonate buffer. After washing with PBS containing 0.05% Tween-20