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CTA1-M2e-DD: A novel mucosal adjuvant targeted influenza vaccine

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Summary At present few vaccine candidates exists against potentially pandemic influenza virus infections. We provide compelling evidence that a targeted fusion protein based on the CTA1-DD adjuvant and containing tandem repeats of the matrix protein 2 (M2e) ectodomain epitope, CTA1-3M2e-DD, confers strong protective immunity against a potentially lethal challenge infection with influenza virus in mice. The formulation was highly effective for mucosal immunizations and promoted high M2e-specific serum IgG and mucosal IgA antibody titers and an hitherto unknown anti-M2e CD4 T cell immunity. This novel CTA1-3M2e-DD fusion protein combines adjuvant and a conserved influenza A antigen in a promising candidate for a universal anti-influenza vaccine.

Introduction

Novel approaches for the design of effective vaccines for mucosal delivery are much warranted. Although there are few examples of licensed mucosal vaccines, several candidates are being evaluated at present [1,2]. Recently a live attenuated nasal-spray influenza vaccine (FluMist™) was licensed in the United States and results using this seasonal vaccine are promising [3–5]. However, live influenza vaccines are dependent on the susceptibility to infection of the vaccine, appear to suffer from interference between A and B strains, and may have a limited ability to elicit strong humoral immunity [6]. On the other hand, injectable inactivated influenza virus vaccines generally give weak cell-mediated immunity and little or no mucosal immunity [6]. Development of improved vaccine formulations and adjuvants, as well as procedures to enhance the immunogenicity of influenza virus proteins and peptides could result in

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improved stimulation of both humoral and cell-mediated immunity [7—13]. The benefits of such non-living mucosal influenza vaccines would be several including, ease of administration, lower reactogenicity and probably better take in adult and elderly populations as well as improved mucosal immunity, the portal site of viral entry [6,14—19]. In addition, they would also mean lower production costs, especially important for developing countries, less regulatory complications, less risks of cross-contamination, and less dependence on the cold-chain for distribution [20]. Current mucosal vaccine candidates, though, suffer from a costly need for high antigen load, lack a safe and sufficiently strong adjuvant and depend on formulations to which the population may already be pre-immune [2,21].

A highly attractive goal for a vaccine against influenza virus would be the stimulation of long lasting heterosubtypic protection [22]. At present, no vaccine is available, which guarantees protection against new pandemic influenza virus infections [3,23]. However, we have previously reported on a universal human influenza A vaccine candidate based on the external domain of the third integral membrane protein, matrix protein 2 (M2e) [24—26]. M2e is highly conserved in all human influenza A virus strains [27—29]. It is expressed in low copy numbers on the viron, but infected target cells abundantly express M2 on their surface and readily bind specific antibodies [27—29]. Because of these properties, M2e based vaccines are considered promising vaccine candidates for the induction of intra- and heterosubtypic immunity against influenza A infection. Experiments in mice have shown that antibodies directed against the 23 amino acids long M2e peptide, is sufficient to stimulate protective immunity [25]. In our previous studies we expressed multiple copies of the M2e-peptide in genetic fusion with the Hepatitis B virus core subunit (HBc) and immunizations with the resulting virus like particles (VLPs) were highly protective against homo- and heterosubtypic virus challenge. Furthermore, when a potent mucosal adjuvant was incorporated in the vaccine formulation, efficient protection was also obtained following intranasal administration [13,25]. Complete protection and reduction of morbidity were observed when the CTA1-DD adjuvant was added together with the M2e-HBc preparation [13]. Whereas only a single human CTL epitope has been reported for M2e-peptides it is known to host at least one B cell epitope (aa 4-16) [28,30,31]. Protection correlates closely with the presence of anti-M2e antibodies, in particular the IgG2a subclass antibodies [25,32].

The CTA1-DD adjuvant is a genetic fusion protein consisting of the cholera toxin (CT) subunit A1 (CTA1), a strong ADP-ribosylating agent, and a dimer of the D-fragment of *Staphylococcus aureus* protein A [33]. It was constructed to retain the potent adjuvant function of CT but without its toxic side effects, by excluding the CTB moiety, binding to the GM1 ganglioside receptor present on all nucleated cells. The latter characteristic of CT and *E. coli* heat-labile toxin (LT) is responsible for the recently reported accumulation of toxin-based adjuvants in the brain following intranasal administration [34]. In extensive studies we have documented the non-toxic nature of CTA1-DD and its targeted action, causing no reactogenicity at the site of administration and no binding to, nor accumulation in the nervous tissues [35]. CTA1-DD binds to the Ig-receptor on B cells and exerts potent immunoenhancing functions on specific antibody production as well as class I and class II MHC restricted T cell immunity after intranasal or systemic administrations [35,36].

Because M2e is a promising candidate for a universal vaccine against human influenza A infections and CTA1-DD is strong adjuvant for mucosal vaccine delivery we combined the two into one fusion protein with the purpose of developing a cost-effective, safe and reliable non-toxic protective influenza vaccine. By combining M2e and CTA1-DD into one fusion protein, CTA1-M2e-DD we hoped to achieve a formulation that carried the immunomodulator, the vaccine Ag and the targeting unit in one molecule, thereby reducing both Ag-requirement and limiting possible side-effects. However, because there is ample evidence that B cell epitope recognition is conformation-dependent and even sequential epitopes are sensitive to the molecular environment including flanking residues, further experimental evidence supporting the concept was desirable [37,38]. Therefore, the major questions addressed in this study were whether B cell recognition of M2e was achieved when it was expressed as an integral part of the CTA1-M2e-DD fusion protein and whether the adjuvant properties were retained after the M2e-insertion.

Material and methods

Mice and immunizations

BALB/c mice were obtained from Charles River (Maastricht, Netherland) (Ghent University) or Taconic (M&B, Denmark) and B cell deficient (JHD) mice, back-crossed onto Balb/c mice, were bred in ventilated cages at the Laboratory for Experimental Biomedicine, University of Göteborg (Göteborg, Sweden). All mice were maintained under specific pathogen-free conditions. Age- and sex matched mice were immunized intranasally (i.n.) twice, 3 weeks apart, with 20—50 μl containing 10 μg of the M2e-HBc particle 1818 admixed with 5 μg CTA1-DD-adjuvant, 10 μg of HBc admixed with 5 μg CTA1-DD-adjuvant, a range of doses as indicated were tested; CTA1-M2e-DD, CTA1-3M2e-DD (carrying three copies of M2e), CTA1-DD adjuvant alone or PBS. Groups with 3—12 individuals in each experiment were used as indicated. Challenge experiments were performed in Ghent. Before the first, and 2 weeks after each immunization, blood samples were collected from the ventral tail vein. The final bleeding of surviving mice was performed 2 weeks after challenge. Blood clotting was allowed to continue for 60 min at 37 °C, samples were placed on ice and serum was separated by collecting the supernatant of two consecutive centrifugations. Immunizations and challenge experiments were authorized by the Institutional Ethical Committee on Experimental Animals (Ghent University).

Preparation of fusion proteins

CTA1-DD, CTA1-M2e-DD or CTA1-3M2e-DD (carrying three copies of M2e; amino acid sequence: SLLTEVETPIRNEWGSRSNDSSD) were produced by Biovitrum AB.

The fusion proteins were expressed in *E. coli* DH5 cells, transformed with the expression vectors for the CTA1-DD,