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A novel non-toxic combined CTA1-DD and ISCOMS adjuvant vector for effective mucosal immunization against influenza virus☆

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ABSTRACT

Here we demonstrate that by using non-toxic fractions of saponin combined with CTA1-DD we can achieve a safe and above all highly efficacious mucosal adjuvant vector. We optimized the construction, tested the requirements for function and evaluated proof-of-concept in an influenza A virus challenge model. We demonstrated that the CTA1-3M2e-DD/ISCOMS vector provided 100% protection against mortality and greatly reduced morbidity in the mouse model. The immunogenicity of the vector was superior to other vaccine formulations using the ISCOM or CTA1-DD adjuvants alone. The versatility of the vector was best exemplified by the many options to insert, incorporate or admix vaccine antigens with the vector. Furthermore, the CTA1-3M2e-DD/ISCOMS could be kept 1 year at 4 °C or as a freeze-dried powder without affecting immunogenicity or adjuvanticity of the vector. Strong serum IgG and mucosal IgA responses were elicited and CD4 T cell responses were greatly enhanced after intranasal administration of the combined vector. Together these findings hold promise for the combined vector as a mucosal vaccine against influenza virus infections including pandemic influenza. The CTA1-DD/ISCOMS technology represents a breakthrough in mucosal vaccine vector design which successfully combines immunomodulation and targeting in a safe and stable particulate formation.

1. Introduction

Although pre-clinical work has developed a number of highly promising non-injectable vaccine candidates for mucosal delivery, few of these vaccines have made it to the market [1–3]. This is mainly because of a lack of safe and clinically effective mucosal adjuvants [4]. The search for better, more efficacious substances that function as mucosal adjuvants has gone on for over two decades, but clinical testing has been slow and few non-living mucosal vaccines are at present commercially available [1,3]. In fact, there is no general consensus as to how mucosal vaccines should be formulated and delivered, let alone which adjuvant components would make them effective in humans [5,6]. An exception to this statement are vaccine formulations based on the enterotoxins, cholera toxin (CT) and the closely related *Escherichia (E). coli* heat-labile toxin (LT), which have proven highly effective when given orally or intranasally (i.n.) [7,8]. However, because of severe toxicity problems such vaccines are no longer on the market [9,10]. Indisputably, though, these molecules host strong mucosal adjuvant properties, and hence many research groups have embarked on strategies to circumvent the toxicity problem [5,6,11]. It is now well established that mutations that affect the enzymatically active A-subunit of these AB_5-complexed molecules have substantially reduced toxicity, while significant adjuvanticity was retained [11]. Unfortunately, the B-subunits of these mutant molecules still bind the ganglioside receptors present on all nucleated cells, including nerve cells, which may reduce their clinical attractiveness as was concluded when severe cases of facial nerve paralysis (Bell's palsy) stopped an i.n. trial using LTK63 [9].

To circumvent this receptor binding problem we made a fusion protein composed of the A1-subunit from CT and a dimer of

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the D-fragment from *Stapylococcus aureus* protein A [12]. The resulting CTA1-DD adjuvant has now been tested extensively in many pre-clinical immunization protocols and has proven to be as effective as CT in augmenting a wide range of cell- and humoral immune responses to many infectious disease relevant antigens [13–15]. Contrary to the holotoxins, the CTA1-DD adjuvant was completely non-toxic and safe in mice, guinea pigs and monkeys and it did not accumulate in the central nervous tissues when given intranasally [12,16,17]. In an effort to allow also for effective oral immunizations using the CTA1-DD adjuvant we developed the combined CTA1-DD/ISCOMS vector, which was stable and exceptionally effective at stimulating immune responses after oral delivery with nanogram doses of antigen [17]. Also responses following intranasal or parenteral immunizations were greatly enhanced using the combined CTA1-DD/ISCOMS vector [17]. Immune stimulating complexes (ISCOMs) are stable, protein-containing cage-like structures composed of cholesterol, phospholipids and saponins from the *Quillaja saponaria Molina* tree, which exert adjuvant effects [18]. The corresponding structure without incorporated protein is called ISCOM-Matrix [19]. ISCOMS and ISCOM-Matrix have been found to have good tolerability and safety profiles and are currently used in commercial veterinary vaccines as well as in a few human vaccine candidates that have reached different stages of clinical testing [20–27]. In the course of development Quil A was introduced as a more defined and better characterized mixture of saponin fractions and today manufacturers use even more refined fractions termed QHA, QHB and QHC for making ISCOMS [28,29]. Unfortunately, some of these fractions are highly toxic and testing of ISCOMS may not be possible as the experimental animals may die and adverse reactions in clinical trials using the QHC fractions are a major concern [30,31].

Because CTA1-DD and ISCOMS represent two mechanistically different and complementary immunomodulating systems that when combined provide a stable, highly effective mucosal adjuvant hosting novel and unique adjuvant properties, we were interested in developing the vector further, ideally reducing toxicity while improving adjuvanticity [32]. Therefore, in the present study we wanted to explore these functions further in a relevant mucosal vaccine model [32]. We selected the influenza vaccine model, which we had used earlier when establishing the combined vector technology [14,17]. The aim of this work was to reduce toxicity of the combined vector while augmenting the stability and function through optimizing its composition. Furthermore, we have analyzed functional requirements for an enhanced adjuvant effect of the vector following intranasal immunizations. Protective immunity against conserved epitopes of the influenza virus has been shown to have potential as a "universal" vaccine [33–35]. Previously, we developed such a candidate vaccine against influenza by incorporating the M2e-peptide, from the influenza A matrix 2 protein (M2), into our adjuvant molecule. We found that the CTA1-M2e-DD fusion protein administered i.n. to mice stimulated strong protective immunity against live viral challenge following intranasal immunizations [14]. Other than protective M2e-specific serum IgG antibodies, a few studies have, in fact, suggested that mucosal IgA antibodies may play a critical cross-protective role against seasonal and pandemic influenza, arguing for mucosal immunizations rather than traditional parenteral vaccination [36,37]. Therefore, in the present study we have developed a mucosal "universal" influenza vaccine candidate based on the combination vector CTA1-3M2e-DD/ISCOMS and evaluated its immunogenicity and impact on vaccine-induced protection against infection. The novel vaccine vector was found to be highly potent, exceptionally stable and allowed freeze-drying without loss of function.

2. Materials and methods

2.1. Mice and immunizations

BALB/c mice were obtained from Taconic (Denmark) and kept under pathogen-free conditions at the Laboratory for Experimental Biomedicine (EBM). OVA T cell receptor (TCR) transgenic mice on C57Bl/6 background (OT II) with $CD4^+$ T cells expressing a TCR specific for the $peptide_{323–339}$ fragment of OVA were bred in ventilated cages at EBM at University of Gothenburg and used as a source of immune cells for *in vitro* experiments. Age- and sex-matched groups of BALB/c mice were immunized intranasally (i.n.) 2–3 times, 1–3 weeks apart with 20 μl containing indicated doses of CTA1-DD/ISCOM, CTA1-3M2e-DD/ISCOM, CTA1-3M2e/ISCOM, CTA1(R7K)-3M2e-DD/ISCOM and CTA1-DD adjuvant alone, ISCOM alone or PBS. All immunizations were given together with 2 μg Puerto Rico (PR8) influenza whole cell antigens or 2 μg tetanus toxoid (TT) (Statens Serum Institute, Copenhagen, Denmark) at a final volume of 20 μl in PBS. Where indicated, mice were given 10 μg of CTA1-DD protein or 10 μg CTA1-DD protein admixed with 2 μg CTA1-DD/ISCOM. Groups with 3–12 individuals in each experiment were used as indicated in figure legends. One week after the final immunization, mice were killed and cells, serum and bronchoalveolar lavage (BAL) were collected. Specimens were freshly used (cells) or stored at −20 °C (serum and lavage) until analyzed. To study the *in vivo* toxicity three naive mice were immunized subcutaneously (s.c.) at the base of the tail with 5 μg ISCOM-Matrix made with either of the different *Quillaja* saponin compositions fraction QHA, fraction QHC or a mixture of fractions QHA and QHC. Mortality and survival of all mice were monitored daily after the s.c. immunization. Live influenza challenge experiments were performed in the Department of Molecular Biology (Ghent, Belgium). Before the first and 2 weeks after each immunization, blood samples were collected from the lateral tail vein. The final bleeding of surviving mice was performed 2 weeks after challenge and collected serum samples were kept at −20 °C before analysis. Immunizations and challenge experiments were authorised by the Institutional Ethical Committee on Experimental Animals (Ghent University).

2.2. Preparation of fusion proteins

CTA1-DD fusion protein alone or CTA1-DD constructions containing a single copy of $OVA_{323–339}$ peptide, CTA1-OVA-DD, or three copies of the influenza virus antigen M2e (amino acid sequence: SLLTEVETPIRNEWGSRSNDSSD), CTA1-3M2e-DD (with enzymatic activity), the enzymatically inactive mutant M2e-fusion protein, CTA1(R7K)-3M2e-DD or CTA1-3M2e lacking the DD-domain were produced in *Escherichia coli* as previously described [14,32,38]. ADP-ribosyltransferase enzymatic activity was tested using the NAD:agmatine assay as described previously [39,40]. PR8 antigens were produced from the influenza virus envelope glycoproteins, haemmagglutinin and neuraminidase of mouse adapted human influenza A virus strain PR/8/34 (H1N1), as described [41]. Protein analysis was performed with SDS-PAGE, and concentrations were determined using the Bio-Rad DC protein assay, according to the manufacturer's instructions.

2.3. Preparation of ISCOMS

ISCOMS and ISCOM-Matrix with different *Quillaja* saponin compositions (QHA and QHC) were prepared as described by Lövgren-Bengtsson and Morein [19]. Briefly, ISCOMs were prepared by mixing 1.0 mg of antigen (OVA, CTA1-DD or CTA1-3M2e-DD, CTA1-3M2e or CTA1(R7K)-3M2e-DD) with 1.0 mg of cholesterol (C-8503, Sigma, St. Louis, USA), 1.0 mg of phosphatidylcholine of egg-origin (Lipoid Gmb, GERMANY) and 5.0 mg of *Quillaja*