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Research paper

Preformulation studies with the *Escherichia coli* double mutant heat-labile toxin adjuvant for use in an oral vaccine

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ABSTRACT

Double mutant heat-labile toxin (dmLT) is a promising adjuvant for oral vaccine administration. The aims of our study were to develop sensitive methods to detect low concentrations of dmLT and to use the assays in preformulation studies to determine whether dmLT remains stable under conditions encountered by an oral vaccine. We developed a sandwich ELISA specific for intact dmLT and a sensitive SDS-PAGE densitometry method, and tested stability of dmLT in glass and plastic containers, in saliva, at the pH of stomach fluid, and in high-osmolarity buffers. The developed ELISA has a quantification range of 62.5 to 0.9 ng/mL and lower limit of detection of 0.3 ng/mL; the limit of quantification of the SDS-PAGE is 10 μg/mL. This work demonstrates the application of dmLT assays in preformulation studies to development of an oral vaccine containing dmLT. Assays reported here will facilitate the understanding and use of dmLT as an adjuvant.

1. Introduction

Oral vaccines have a number of advantages over traditional parenteral vaccines, including the ability to elicit a protective mucosal immune response, ease of administration, and simplicity of manufacturing compared to vaccines intended for parenteral injection (Lal and Jarrahian, 2016). Oral vaccines also can help facilitate vaccine coverage improvements, as they can sometimes be provided by community health workers outside of formal clinical settings. This is a distinct advantage over vaccines administered by injection that require higher levels of training and can result in needlestick injuries (Aziz et al., 2007; Silin et al., 2007). Despite these advantages, only a few oral vaccines are currently licensed for use: cholera, typhoid, polio, and rotavirus. Challenges to producing effective oral vaccines include a higher likelihood of immune tolerance for antigens delivered via the mucosal route and degradation of vaccine antigen in the harsh environment of the human stomach before it reaches target sites in the intestine (Lamm, 1997; Rhee et al., 2012).

Adjuvants frequently are added to vaccines to improve immunogenicity and produce a long-term protective effect (Mestecky and McGhee, 1989; Pavot et al., 2012). For oral vaccines, adjuvants not only increase immunogenicity but also help overcome the natural tolerance to antigens introduced at mucosal portals of entry. To prevent degradation of vaccine antigens, antacid buffers can be added to raise stomach pH, either by incorporating antacid into the vaccine formulation or by providing it in an accompanying container. However, the added volume, higher pH, and increased osmolarity of the adjusted formulation may interfere not only with the stability of vaccine antigens, but also with that of any adjuvants. In addition, the large volume of antacid buffer required to maintain pH stability for the time it takes a vaccine to transit the stomach can prevent its use in infant populations. The ideal infant oral vaccine candidate would consist of a vaccine antigen and adjuvant formulation packaged with a single small dose of antacid buffer.

Adjuvants that increase the immunogenicity of co-administered vaccine antigens include bacterial enterotoxins such as cholera toxin produced by *Vibrio cholera*, and the closely related heat-labile toxin (LT) produced by *Escherichia coli* (*E. coli*; (Clements et al., 1988; Elson, 1989)). Initial studies found that even low doses of these toxins were effective adjuvants, but when delivered orally, they produced side effects such as diarrhea and vomiting (Banerjee et al., 2002; Elson, 1989). In order to reduce toxicity while maintaining adjuvant activity, amino acid substitutions were introduced into LT, resulting in the double mutant adjuvant (R192G/L211A; (De Magistris et al., 1998; Dickinson

Abbreviations: BSA, bovine serum albumin; CV, coefficient of variation; dmLT, double mutant heat-labile toxin; DPBS, Dulbecco's phosphate buffered saline; *E. coli*, *Escherichia coli*; ELISA, enzyme-linked immunosorbent assay; HCl, hydrochloric acid; LD, limit of detection; LT, heat-labile toxin; mOsm, milliosmole; NT, not tested; OD, optical density; RT, room temperature; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

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and Clements, 1995; Norton et al., 2011)). Double mutant heat-labile toxin (dmLT) has been shown to have little to no toxicity while maintaining adjuvanticity similar to that of LT, and it is under evaluation in combination with a number of vaccine candidates at preclinical and clinical stages (El-Kamary et al., 2013; Holmgren et al., 2003; Leach et al., 2012; Newsted et al., 2015; Norton et al., 2015a; White et al., 2014). The dmLT molecule consists of a single A-subunit attached to a pentameric B-subunit, a structure thought to be essential for adjuvant activity (Norton et al., 2015b). There are currently no marketed oral vaccines that contain dmLT as an adjuvant.

In order to determine whether dmLT would remain stable under conditions encountered by an oral vaccine, we developed an enzyme-linked immunosorbent assay (ELISA) that can detect intact dmLT but not the dissociated A- or B-subunits (Norton et al., 2011; Norton et al., 2012; Ristaino et al., 1983; Yolken et al., 1977; Toprani et al., 2017). This ELISA had to be sensitive enough to measure the small amounts of dmLT present in samples of putative vaccine formulations, which are anticipated to contain as low as 2.5 μg per dose (Kaminski et al., 2014; Many et al., 2016; Walker, 2015). We also developed a gel densitometry method with high sensitivity to detect dmLT. We then applied these assays in preformulation studies, to investigate the effects of container type, saliva, pH, stomach acid, and salt concentration on the stability of the dmLT molecule. This paper reports the development of the two assays and their use in preformulation studies to help determine the parameters for oral administration of dmLT in a vaccine.

2. Materials and methods

2.1. dmLT stock and development of assays

dmLT in the form of 700 μg lyophilized cakes in 3 mL glass vials was produced and provided by the Walter Reed Army Institute of Research (Silver Spring, MD, lot #1735, technical batch manufactured on November 21, 2011). Endotoxin testing was performed and found to be < 2.4 × 104 EU/mL and host cell protein was also performed and found to be 224 ng host cell protein per milligram of dmLT. For reconstitution, each vial of dmLT was diluted with 0.7 mL water-for-injection to achieve a final dmLT concentration of 1 mg/mL.

Purified A and B-subunits of dmLT and dmLT specific rabbit sera used in assay development were provided by Tulane University (Provided by Dr. John Clements, Tulane University, New Orleans, LA). Each of the dmLT subunits were produced as recombinant proteins as detailed in Norton, E, et al. 2012. (Norton et al., 2012).

We developed two sensitive tests for dmLT content of samples, a sandwich ELISA specific for intact dmLT molecules down to approximately 1 ng/mL and a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) densitometry method able to detect dmLT in sample concentrations of 10 μg/mL.

2.2. ELISA

The dmLT ELISA was designed to capture the B-subunit of dmLT and then to detect the A-subunit of dmLT, making it a method that detects the intact molecule. For this test, GM1 ganglioside (Sigma-Aldrich, St Louis, MO, cat #G7641), the putative cell receptor for the B-subunit of dmLT, was used to coat 96-well plates (Costar®, Corning®, Corning, NY, cat #9018) by diluting to 1 μg/mL in Dulbecco's phosphate buffered saline (DPBS, pH 7; HyClone™, Fisher Scientific, Waltham, MA, cat #SH30378.02) and adding 0.1 mL/well. Plates were sealed and incubated overnight at 2 °C–8 °C. Plates were then washed with DPBS with 0.05% (*v*/*v*) Tween® 20 (Fisher Scientific, cat #337-500) using a SkanWasher 300-plate washer three times (Molecular Devices, Sunnyvale, CA) and blocked with 0.2 mL/well of DPBS with 1% (*w*/*v*) bovine serum albumin (BSA; Roche, Basel, Switzerland, cat #100350) for 1 h at room temperature (RT; 20 °C–25 °C). After repeating plate washing, the diluted test samples, internal control, and dmLT standards (62.5–0.5 ng/mL) were added to ELISA plates at 0.1 mL/well, and the plates were sealed and incubated for 2 h at RT. dmLT standards and internal control samples were diluted to a starting concentration of 62.5 ng/mL in assay buffer (DPBS with 1% BSA and 0.05% Tween® 20, Sigma-Aldrich, cat #P2287) followed by six two-fold dilutions to generate a titration curve. Test samples were diluted to a starting concentration within the linear range of the assay.

Detection antibody was prepared by diluting rabbit anti-dmLT A-subunit sera (Provided by Dr. John Clements, Tulane University, New Orleans, LA) 1:2000 in assay buffer. Plate washing was repeated for a total of five wash cycles; 0.1 mL/well of diluted detection antibody was added; and plates were incubated for 1 h at RT. Secondary antibody was prepared by diluting biotin-labeled donkey anti-rabbit antibody (SouthernBiotech, Birmingham, AL, cat #6440-08) 1:10,000 in DPBS. Plate washing was repeated for a total of five wash cycles; diluted secondary antibody was added to ELISA plates at 0.1 mL/well; and plates were incubated for 1 h at RT. Plate washing was repeated for a total of five wash cycles; ExtrAvidin® peroxidase (Sigma-Aldrich, cat #E2886) diluted 1:10,000 in DPBS was added to ELISA plates at 0.1 mL/well; and plates were incubated for 1 h at RT. Plates were washed for a total of five wash cycles and 0.1 mL/well of pre-warmed tetramethylbenzidine substrate (Sigma-Aldrich, cat #T0440) was added to plates. Plates were kept in the dark at RT for 15 min. Reactions were stopped with 0.1 mL/well of 1 M sulfuric acid and plate absorbance was read at 450 nm using a SpectraMax® M2 plate reader (Molecular Devices). A standard curve was generated for interpolation of test samples using a four-point logistic fit (4-parametric) in SoftMax® Pro (Molecular Devices). ELISA analysis was performed on background subtracted data. For an assay to be considered acceptable, the average optical density (OD) of the blank wells must be < 0.15 absorbance units and the coefficient of variation between replicate wells must be < 15%. A minimum of four points are required to generate a standard curve using a 4-parametric equation. The regression coefficient must be > 95% and the back calculation of the reference OD data must be within 10% of the expected values. The adjuvant concentration of a test sample is determined from interpolation of at least three points on the standard curve. In order for a sample measurement to be valid, it must give an OD of two times the background and the sample curve must be parallel to the standard curve. Parallelism is tested by looking at the ratio of highest to lowest calculated potency value in a dilution series (Rezapkin et al., 2005). The final interpolated dmLT concentration for each sample is the average of all the acceptable dilutions tested.

2.3. SDS-PAGE

A sensitive SDS-PAGE densitometry method was developed for detection of dmLT down to 10 μg/mL. Each SDS-PAGE gel included a standard curve (with five points), an internal control, and the test sample tested at three dilutions within the range of the standard. Five concentrations of a dmLT standard were prepared by dilution in normal saline (Teknova, Hollister, CA, cat #S5815) with 0.05% (*v*/*v*) Tween® 80 (ACROS™ Organics, Fisher Scientific, cat #278632500) to 0.104, 0.052, 0.026, 0.013, and 0.007 mg/mL. Three dilutions of test samples (10, 5, and 2.5 μg/mL) were also prepared by dilution in saline with Tween® 80. Diluted standards, internal control, and test samples were mixed with 4 × Laemmli sample buffer (Bio-Rad, Hercules, CA, cat #1610747) with 400 mM dithiothreitol at a 3:1 ratio, and heated at 95 °C for 5 min. A total of 16 μL of each diluted standard was loaded per well, for a total of 1.25, 0.624, 0.312, 0.156, and 0.078 μg/lane, onto a 4–20% tris-glycine gel (Bio-Rad, cat #456-1094). A total of 48 μL per well of diluted test samples was loaded per well, for a final concentration of 0.36, 0.18, and 0.09 μg/lane. Gels were run at 100 V for 5 min followed by 150 V for 45 min. Gels were stained with colloidal blue stain (Fisher Scientific, cat #LC6025) for 3 h at RT with shaking, followed by shaking with water for 20 h. Gels were imaged using AlphaImager HP® (ProteinSimple, San Jose, CA) and analyzed using US

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