



The corn smut-made cholera oral vaccine is thermostable and induces long-lasting immunity in mouse



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ABSTRACT

The use of corn smut for the production of recombinant vaccines has been recently implemented by our group. In this study, the stability and immunogenic properties of the corn smut-based cholera vaccine, based on the cholera toxin B subunit (CTB), were determined in mouse. The immunogenic potential of distinct corn smut CTB doses ranging from 1 to 30 μg were assessed, with maximum humoral responses at both the systemic (IgG) and intestinal (IgA) levels at a dose of 15 μg . The humoral response last for up to 70 days after the third boost. Mice were fully protected against a challenge with cholera toxin after receiving three 15 μg -doses. Remarkably, the corn smut-made vaccine retained its immunogenic activity after storage at room temperature for a period of 1 year and no reduction on CTB was observed following exposure at 50 °C for 2 h. These data support the use of the corn smut-made CTB vaccine as a highly stable and effective immunogen and justify its evaluation in target animal models, such as piglet and sheep, as well as clinical evaluations in humans.

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1. Introduction

Cholera is an acute intestinal infection derived from the ingestion of food or water contaminated with *Vibrio cholerae*, a gram negative bacteria; which produces the cholera toxin (CT) responsible for causing a copious, painless, watery diarrhea. Subsequently, patients can present severe dehydration and even death (WHO). CT consists of two subunits: the A subunit (CTA) and the B subunit (CTB). The latter forms a 55 kDa homopentameric non-toxic protein that binds the GM1 ganglioside on mammalian cells and induces a potent humoral immunity that leads to CT neutralization in the gut (Kopic and Geibel 2010). Cholera remains as a global threat, being poor countries the most affected since cholera transmission is associated to the lack of potable water sources and adequate sanitation of public services. In 2012, a WHO report estimated that 2.8

million cases of cholera (uncertainty range: 1.2–4.3 million) and about 91,000 deaths (uncertainty range: 28 000–142,000) occur in endemic countries every year. In contrast, 87,000 cases and 2500 deaths occur in non-endemic countries. The burden of cholera is greatest in Africa and southern Asia, where poor economies do not achieve adequate access and/or sanitization of fresh water (Mohammad et al., 2012).

To prevent cholera, safe and effective oral cholera vaccines have been licensed and used by affluent tourists for more than a decade. Thus far, oral vaccines consist on rCTB-alone or rCTB combined with two dominant domestic killed *V. cholerae* strains (O1 Ogawa El Tor and O1 Inaba El Tor) plus one standard *V. cholerae* strain (O1 Ogawa classic ATCC 14035). Both formulations have shown an acceptable protection against toxigenic *V. cholerae* (Boustanshenas and Bakhshi, 2014). Currently, two oral cholera commercial vaccines are available. Dukoral is internationally licensed and prequalified by the WHO for purchase by United Nations agencies. Dukoral is formulated with inactivated *Vibrio cholerae* O1 whole cells plus recombinant cholera toxin B subunit (BS-WC). In the 1980's, this vaccine showed to be safe and highly protective (~85%) in a large-scale field trial in Bangladesh (Clemens et al., 1986, 1990).

Abbreviations: CTB, cholera toxin B subunit; CT, cholera toxin; FA, fluid accumulation.

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Moreover, Shanchol is a much less expensive killed oral cholera vaccine licensed in Vietnam and India and is undergoing the WHO prequalification. The Shanchol vaccine, consists of WC without recombinant cholera toxin B subunit and proved to be safe and efficacious in a double-blind, placebo-controlled trial (Sridhar 2009; Sur et al., 2009).

The emergence of large and prolonged outbreaks, particularly in sub-Saharan Africa, make WHO to reconsider the recommendation of not oral cholera vaccination once an outbreak had started (Bhattacharya et al., 2009). Therefore, the limited economic resources in the affected countries limit the current supply of cholera vaccines to implement national vaccination campaigns and are not sufficient to meet endemic and epidemic worldwide needs (Deen et al., 2015). Thus, the development of the research and development of new affordable vaccines is crucial to prevent cholera outbreaks. Given the relevance of CTB as protective immunogen, it has been expressed in several recombinant systems, including plant species, such as rice (Yuki et al., 2013); yeast (Arzanlou et al., 2005; Jung-Gu and Hyo-Sang 2008); and silkworm (Li et al., 2014). We have recently reported on the expression of CTB using corn smut or 'huitlacoche' as a new platform observing competitive yields (Juárez-Montiel et al., 2015) The present study aimed at characterizing the immunogenic properties of the corn-smut CTB vaccine, which induced long-lasting immunoprotective responses at low doses.

2. Materials and methods

2.1. Corn smut production

Corn smut expressing CTB was produced as previously described (Juárez-Montiel et al., 2015). Briefly, FB1 WT and FB2-CTB3 *Ustilago maydis* strains were grown in either liquid YPD (2% yeast extract, 1% peptone, and 1% glucose) or YEPSL (0.4% yeast extract, 0.4% peptone, and 2% sucrose) medium at 28 °C and shaking at 250 g. Crosses of paired strains were performed as previously reported (Holliday 1974). 4–6 days after silking, a 10 mL volume of each strain mixture was injected into the silk channel of primary ears in order to induce maize ear galls. Inoculated maize plants were maintained under greenhouse conditions. 'Huitlacoche' was harvested 18 days post inoculation and subjected to lyophilization. Samples were processed in a LABCONCO freeze-dry system (FreeZone 6 L) during 48 h at a –50 °C collector temperature. Dry material was subsequently milled and stored at room temperature until further use.

2.2. Thermostability assessment

Freeze-dried corn smut samples, which were maintained at approximately 25 °C during one year, were subjected to 37 °C, 50 °C, 60 °C, and 80 °C treatments for 2 h. CTB levels and integrity were assessed using Western blot assays and ELISA. Protein extracts of corn smut galls were obtained by resuspending 30 mg of freeze-dried tissue in 300 µL of the extraction buffer consisting of 750 mM Tris-HCl pH 8.0, 15% (w/v) sucrose, 100 mM β-Mercaptoethanol and 1 mM PMSF (Franklin et al., 2002). Then, protein extracts were centrifuged at 16,000g for 15 min at 4 °C. Supernatants were separated and 30 µL aliquots were mixed with reducing loading buffer. Samples were denatured at 95 °C for 5 min and SDS-PAGE was performed in 4–12% polyacrylamide gels. The gel was blotted onto PVDF membranes (Pall Corporation, <http://www.pall.com>), which were blocked with a 5% fat-free milk (Carnation, www.nestle.com) solution prepared in phosphate saline buffer (PBS) plus 0.01% Tween 20 (PBST). Primary labelling was performed overnight using a mouse anti-CTB antiserum (1:200 dilution) followed by labelling with a horseradish peroxidase-conjugated secondary anti-mouse

antibody 1:2000 dilution (Sigma, <http://www.sigmaaldrich.com>) during 2 h at room temperature. Immunodetection was completed by using the SuperSignal West Dura solution following the manufacturer's instructions (Thermo Scientific, <http://www.thermoscientific.com>). Pure CTB was included as positive control (Sigma).

For ELISA analysis, 50 mg of lyophilized corn smut subjected to thermal treatments were resuspended in 500 µL of protein extraction buffer (50 mM Tris pH 8, 40 mM NaCl, 0.1% Tween 20, 1 mM PMSF). Samples were centrifuged at 16,000g for 15 min at 4 °C and supernatants were diluted 1:2 in carbonate buffer (0.2 M, pH 9.6) and used for coating GM1-ELISA plates by an overnight incubation at 4 °C. After washing, plates were blocked for 2 h at room temperature with a 5% fat-free dry milk solution. Plates were washed and primary labelling was conducted by adding an anti-CTB mouse serum diluted 1:800 in PBS and incubating overnight at 4 °C. The secondary labelling was conducted by incubating the plates at 25 °C during 2 h with a goat anti-mouse horseradish peroxidase-conjugated antibody diluted 1:2000 (Sigma). After washing, a substrate solution of 0.3 mg/mL 2-20-azino-bis-3-ethylbenzotiazoline-6-sulphuric acid (ABTS; Sigma) and 0.1 mM H₂O₂, was added for 30 min at 25 °C. Optical density (OD) was read in an iMark™ microplate reader (BIO-RAD, Hercules, CA, USA) at 405 nm.

2.3. Immunogenicity assay

Experimental procedures in test mice were approved by the Institutional Animal Care and Use Committee (Protocol number: CEID-2013-004). Five groups (n=4) of 12 week-old female BALB/c mice were established, and received by the oral route one of the following treatments: 1, 8.5, 10 or 25 mg of freeze-dried FB2-CTB3 galls containing approximately 1, 10, 15, and 30 µg of CTB, respectively; or 25 mg of freeze-dried WT galls. The corn smut used in this experiment was previously maintained at 25 °C during one year period before conducting this experiment. The vaccine consisted of the corresponding amount of corn smut resuspended in 200 µL PBS, and administered to mice on days 0, 7, 14, and 21. Mice were bled on days 21, 61 and 91 to conduct ELISA analysis for determining anti-CTB IgG levels.

In order to determine IgG, IgG1, IgG2a, IgA and IgM antibody levels by ELISA analysis, two groups of immunized mice as aforementioned, one with 10 mg CTB corn smut (15 µg of CTB) and another with 10 mg WT corn smut, were bled at days 21, 61 and 91 after the first immunization. For IgA determination feces were collected at the same time points (Rosales-Mendoza et al., 2008).

ELISA assay was conducted using ninety six-well polystyrene plates coated overnight with CTB (0.25 µg/well) at 4 °C. After blocking with 5% fat-free milk for 2 h, plates were incubated overnight at 4 °C with serial dilutions of mice sera (1:20–1:160). Anti-IgG, –IgG1, –IgG2a, –IgA and –IgM horseradish peroxidase-conjugated secondary anti-mouse antibodies (1:2000 dilution, Sigma) were applied for 2 h at room temperature, and after washing, signals were detected following incubation with an ABTS substrate and 0.1 mM H₂O₂ for 15 min (Sigma). Optical density values were measured at 405 nm using a Microplate reader (Thermo). Antibody titers were determined as the reciprocal of the higher serum dilution with an OD value above the mean OD value of the WT group plus 2 times its standard deviation.

2.4. CT challenge

Cholera toxin (CT) challenge experiment was performed according to a previously described method (Richardson et al., 1984; Rosales-Mendoza et al., 2008). The following test mice groups (n=5) were set up: mice treated with WT 'huitlacoche' (challenged); mice treated with transgenic 'huitlacoche' (challenged);

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