Recombinant Shiga toxin B subunit-keyhole limpet hemocyanin conjugate vaccine protects mice from Shigatoxemia

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Abstract
Enterohemorrhagic Escherichia coli (EHEC) cause hemorrhagic colitis in humans and, in a subgroup of affected subjects, more severe conditions called hemolytic-uremic syndrome. These conditions arise because EHEC produces two immunologically distinct forms of Shiga toxin (Stx1 and Stx2), that form dimers. Administration of a purified recombinant Stx2 B subunit preparation to protect Shigatoxemia in adult rabbits. This study revealed that effective immunization could only be achieved if endotoxin was included with the vaccine antigen. Since the presence of endotoxin would be unacceptable in a human vaccine, the object of the studies described herein was to investigate ways to safely augment, in mice, the immunogenicity of the recombinant Stx2 B subunit conjugate without exceeding 2 endotoxin units per mg. The study revealed that sera from mice immunized with such a preparation, conjugated to keyhole limpet hemocyanin, administered with the 15-adjuvant system, displayed the highest Shiga toxin 2 B subunit-specific IgG1 and IgG2a ELISA titers and cytokine neutralizing activities in Balb/c mice with 100% of the animals vaccinated with this preparation were substantially protected from a lethal dose of Stx2 holotoxin. These results support further evaluation of a Stx2 B subunit-based human EHEC vaccine.

Materials and Methods
Toxin purification and cell lines. Stx2 B subunit and Stx2 holotoxin were expressed and affinity-purified as described previously (1, 2). Endotoxin was removed from all the preparations using a Detox gel LPS affinity column. Ramos Burkitt’s lymphoma B cells were generously provided by Dr. Andrew Shaw (Cross Cancer Institute, University of Alberta).

Conjugation of Stx2 B subunit to Keyhole limpet hemocyanin (KLH). The recombinant KLH was conjugated to the Stx2 B subunit using 1-Ethyl-3-[3-Dimethylaminopropyl] carbodiimide Hydrochloride.

Figure 2. Analysis of the recombinant Stx2 B subunit-KLH conjugate by SDS-PAGE. Lane A, low MW pre-stained standard proteins used to calibrate the gel; lane B, KLH; lane C, Stx2 B subunit; lane D, Stx2 B-subunit-KLH conjugate vaccine. The gel was stained with Coomassie.

Figure 3. ELISA analysis for antigen-specific IgG1 (panels A and B) and IgG2a (panels C and D) titer of sera from individual mice after two injections of KLH only (or, for the 3rd B subunit-KLH conjugate (panels A and C) or RAS-TDM+KLH conjugate (panels B and D). The data represent the average of triplicate determinations for each point. Analysis of variance (ANOVA) analysis by the Ramos B cell cytotoxicity neutralization assay. Five uL of mouse serum were pre-incubated with 100 ng of Stx2 holotoxin in five uL of saline and then inoculated in 10 uL of 2.5 x 104 plated Ramos B cells. After 2 hours, the cells were washed and incubated a further 16 hours. The percentage of apoptotic Ramos cells was then determined by flow cytometry.

The murine Shigatoxemia model. Fourteen days after their final vaccination, the mice received a subcutaneous LD100 (0.2 mg/kg body weight) injection of Stx2. The mice were immediately euthanized by CO2 asphyxia when signs of Shigatoxemia became apparent.

Conclusions
- Cloned Stx2 B subunit admixed with different adjuvant combinations failed to induce a significant protective anti-Stx2 holotoxin immune response in mice (Fig. 5).
- Stx2 B subunit conjugated to KLH induced a protective anti-Stx2 immune response (Figs. 3, 4, and 5).
- 100% of mice immunized with Stx2 B-KLH conjugate admixed with RAS-TDM survived a subsequent challenge with a lethal dose of Stx2 holotoxin (Fig. 3).
- 90% of mice immunized with Stx2 B-KLH conjugate admixed with Alhydrogel survived a subsequent challenge with a lethal dose of Stx2 holotoxin (Fig. 5).
- Survival correlated with higher anti-Stx2 IgG1 titers (Figs. 3 and 5).
- Most of the sera samples from mice immunized with Stx2 B-KLH conjugate admixed with RAS-TDM neutralized the cytokine activity of Stx2 holotoxin in vitro (Fig. 4).
- These results contribute to the development of a Stx2 B subunit-based EHEC vaccine as we effectively protects mice from the lethal effects of Stx2 using an atoxic endotoxin free antigen combined with a human-approval adjuvant and carrier protein.

Figure 4. Ramos cell apoptosis neutralizing activity of sera obtained from individual mice immunized with KLH only or the Stx2 B subunit-KLH conjugate admixed with RAS-TDM or Alhydrogel. The error bars represent the standard error of the mean values from triplicate determinations. The blue bars represent serum samples that significantly (p < 0.05, student’s t-test) reduced Stx2 toxicity relative to the activity of control sera. K106 is a positive control serum sample obtained from a rabbit immunized with the recombinant Stx2 B subunit in the presence of endotoxin as described in our previous article (1).

Figure 5. Survival plots for mice immunized with KLH alone or the Stx2 B subunit-KLH conjugate admixed with RAS-TDM or Alhydrogel (n = 10 mice per group). KLH mixed with Alhydrogel, Stx2 B subunit-KLH mixed with RAS-TDM, and Stx2 B subunit-KLH mixed with RAS-TDM. The differences in the survival rates of mice immunized with the Stx2 B subunit-KLH conjugate preparation were significant (p < 0.05 by Fisher exact test).

References

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