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Immunization with a Chimera Consisting of the B Subunit of Shiga Toxin Type 2 and Brucella Lumazine Synthase Confers Total Protection against Shiga Toxins in Mice

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The striking feature of enterohemorrhagic Escherichia coli (EHEC) infections is the production of Shiga toxins (Stx) implicated in the development of the life-threatening hemolytic uremic syndrome. Despite the magnitude of the social impact of EHEC infections, no licensed vaccine or effective therapy is available for human use. One of the biggest challenges is to develop an effective and safe immunogen to ensure nontoxicity, as well as a strong input to the immune system to induce long-lasting, high-affinity Abs with anti-Stx–neutralizing capacity. The enzyme lumazine synthase from Brucella spp. (BLS) is a highly stable dimer of pentamers and a scaffold with enormous plasticity on which to display foreign Ags. Taking into account the advantages of BLS and the potential capacity of the B subunit of Stx2 to induce Abs that prevent Stx2 toxicity by blocking its entrance into the host cells, we engineered a new immunogen by inserting the B subunit of Stx2 at the amino termini of BLS. The resulting chimera demonstrated a strong capacity to induce a long-lasting humoral immune response in mice. The chimera induced Abs with high neutralizing capacity for Stx2 and its variants. Moreover, immunized mice were completely protected against i.v. Stx2 challenge, and weaned mice receiving an oral challenge with EHEC were completely protected by the transference of immune sera. We conclude that this novel immunogen represents a promising candidate for vaccine or Ab development with preventive or therapeutic ends, for use in hemolytic uremic syndrome–endemic areas or during future outbreaks caused by pathogenic strains of Stx-producing E. coli. The Journal of Immunology, 2013, 191: 000–000.
BLS-Stx2B DNA vaccine

To develop a DNA vaccine coding for BLS-Stx2B (pCI-BLS-Stx2B), the BLS-Stx2B DNA sequence was amplified by PCR, using pET-BLS-Stx2B as template, and subcloned in the pCI-neo vector (Promega, Madison, WI), with primers pCI Forward 5'-GTTAAGAATTCGAGAACACGATCCAC-ATGCATGC(G/A)-3' (EcORI site underlined; ROZAK consensus sequence in bold type) and pCI Reverse 5'-TGCTACGATCTAGATGGCATCA-CAAGCGCGATGC(G/C)-3' (NheI site underlined). To ensure that the plasmid construct was intact and functional, it was sequenced across the gene insert.

Plasmid was amplified in E. coli DH5α cells and isolated using Maxiprep plasmid isolation columns (QIAGEN, Valencia, CA), following the manufacturer’s instructions.

Proteins used in this study

BLS and Stx2B. BLS and Stx2B were expressed and purified, as previously described (26, 27). Briefly, BLS was purified in a Mono-Q column in an HPLC apparatus using a linear gradient of NaCl between 0 and 1 M in 50 mM Tris (pH 8.5) buffer. The peak enriched with BLS was further purified on a Superdex-200 column with PBS and 1 mM DTT. Stx2B was purified by chromatography on a Ni-NTA column (QIAGEN), following the manufacturer’s instructions. The purity of the preparation was determined on SDS-PAGE 15% (w/v) polyacrylamide gels.

Crude preparation of recombinant Stx2 and purified recombinant Stx1. Recombinant plasmids for expression of recombinant Stx2 (rStx2) and recombinant Stx1 (rStx1) were generated previously (28, 29). The culture supernatant from E. coli JM109 strain, transformed with the recombinant plasmid pGEM-Stx2 or pGEM-Stx1, was obtained by overnight incubation in Luria–Bertani broth supplemented with ampicillin. Bacterial cells were centrifuged, and the resultant pellet was resuspended in PBS with 1 mM PMSF and lysed by sonication. To obtain the crude preparation of rStx2, the lysate from JM109/pGEM-Stx2 was centrifuged (14,000 rpm, 20 min at 4˚C), and the supernatant was precipitated with ammonium sulfate solution (75%). The pellet was resuspended in and dialyzed against the same buffer for 24 h, and stored at −20˚C until use. Total protein concentration was determined using standard methods. Stx2 concentration was determined with a RIDASCREEN Verotoxin kit (R-BIOPHARM, Darmstadt, Germany) (30, 31). rStx1 was purified by affinity chromatography with commercially available Gibrotroise Fractogel (IsoSep, Tullinge, Sweden), as previously described (30). Briefly, the supernatant from JM109/pGEM-Stx1 was applied to the Gibrotroise Fractogel column and incubated at 4˚C for 2 h. rStx1 was eluted with PBS containing 4 M MgCl2. The eluted material was pooled, dialyzed against PBS, and concentrated by centrifugation filtration with a 5000 MWCO Amicon Ultra concentration system (Millipore). The purity of the preparation was determined by SDS-PAGE (15%, w/v) and quantified by standard methods.

Stx2-producing EHEC. Stx2-producing EHEC and Stx2 variants were generously provided by Dr. Marta Rivas (Administración Nacional de Laboratorios e Institutos de Salud “Dr. Carlos G. Malbrán”). E. coli 680/01 (O157:H7, produces Stx2), E. coli 242/01 (O117:H21, produces Stx2c), and E. coli 265/02 (O117:H2, produces Stx2d) were grown overnight. Cultures were centrifuged at 15,000 × g for 20 min at 4˚C, and supernatants were collected and stored at −20˚C until used.

Structural analysis of BLS-Stx2B

Circular dichroism. The circular dichroism (CD) spectra of BLS-Stx2B, BLS, and Stx2B in the far UV region (255–200 nm) were measured on a JASCO J-810 spectropolarimeter in PBS (pH 7) buffer at 25°C, using quartz cuvettes of either 1- or 5-mm path length. Data were converted to molar ellipticity ([θ]dpsmol protein (deg cm2 dmolprot−1)).

Static light scattering. The average molecular mass of BLS-Stx2B was determined on a Precision Detectors PD2010 light scattering instrument connected in tandem to an HPLC system, including a Waters 486 UV detector and an LKB 2142 differential refractometer. Proteins were purified by anion exchange chromatography in a Q-Sepharose column (Pharmacia, GE Healthcare Life Sciences) using an HPLC apparatus (Gilson model 320). Elution was performed using a linear gradient between 0 and 1 M NaCl in buffer consisting of 1 M urea and 50 mM Tris/HCl (pH 8.5).

Protein was dialyzed against PBS prior to each immunization.

Materials and Methods

Ethics statement

Experiments were approved by the Instituto de Medicina Experimental Care Committee in accordance with the principles set forth in the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals.

Molecular modeling

The theoretical structure of the BLS-Stx2B chimera was modeled with the program PyMOL 1.5 (http://www.pymol.org), fusing the C-terminal end of the pentamer of B subunits as long as a flexible linker long enough to avoid steric hindrance is used to connect the monomers of both proteins. The pentamer of B subunits folded on top of each of the BLS pentamers would benefit from the high local concentration of the subunits dictated by the covalent attachment to the scaffold, as well as the high thermodynamic stability of BLS. Effectively, the resulting chimera (BLS-Stx2B) showed a remarkable stability and demonstrated a strong capacity to induce long-lasting humoral immune responses. The chimera administered to mice under different formulations and regimens induced Abs with a high neutralizing capacity for Stx2 and its variants. Moreover, mice immunized with BLS-Stx2B were completely protected against challenges with high lethal doses of Stx2 up to 10 mo after the last immunization. Most importantly, sera from immunized mice also protected mice during a relevant model of EHEC infection, demonstrating that the transferred Abs were capable of neutralizing the toxin as it is delivered by EHEC. We conclude that this novel immunogen represents a promising candidate for vaccine or Ab development, with preventive or therapeutic purposes to be used in HUS-endemic areas or during future outbreaks caused by pathogenic strains of Shiga toxin–producing E. coli.
O-phenylenediamine (Sigma, St Louis, MO), and absorbance was read at 492 nm. BLS and E. coli BL21 extracts were used as negative controls for nonspecific binding.

**Immunization protocols and sample collection**

Adult BALB/c mice (2 mo old) were immunized with three doses of BLS-Stx2B: i.p. with Freund’s adjuvant (FA; DIFCO-BO, Detroit, MI), s.c. with aluminum hydroxide (AH), or i.p. with no adjuvant (N/A) on days 0, 15, and 30. Groups of mice also were immunized i.p. with BLS and Stx2B in FA. The doses of BLS and BLS-Stx2B were corrected by their molecular weights to inoculate equimolar amounts of each protein compared with that of Stx2B (the dose was equivalent to 20 μg Stx2B).

For prime boost immunization, mice were injected i.m. with 100 μg pCI-BLS-Stx2B on days 0, 14, and 28 into the rear legs, followed by a final i.p. booster of BLS-Stx2B in incomplete FA at day 40.

**Analysis of Ab responses**

Stx2B-specific IgG determination. ELISA plates were coated with 0.5 μg/well Stx2B. For total specific IgG determination or IgG subtyping, peroxidase-conjugated goat anti-mouse IgG or IgG1 and IgG2a (1:1000; BD Pharmingen, Franklin Lakes, NJ) were used as secondary Abs. The Ag–Ab reaction was detected as described for the Gb3Cer-binding assay. Results were expressed as end point titers, calculated as the reciprocal values of the last dilution with an OD higher than that of the preimmune serum samples + 2 SD.

Ab-affinity determination. The Stx2B affinities of Abs raised in immunized mice were determined by the thionocyanate elution–based ELISA, as previously described (33). The concentration of ammonium thiocyanate required to dissociate 50% of the bound Ab was determined. The percentage of binding was calculated as follows: OD100nm - (OD0min) / (OD100nm), in the absence of ammonium thiocyanate.

**In vitro and in vivo neutralizing activity.** Stx2-neutralizing Ab titers were determined as previously described (18), with 1 CD50 of rStx2 (670 pg Stx2). The neutralizing activity was expressed as the reciprocal value of the highest dilution that blocked 50% of Stx2 toxicity to Vero cells. The neutralizing activity was expressed as the reciprocal value of the highest dilution that blocked 50% of Stx2 toxicity to Vero cells. The neutralizing activity was expressed as the reciprocal value of the highest dilution that blocked 50% of Stx2 toxicity to Vero cells.

**Results**

**Engineering of the chimeric protein BLS-Stx2B**

The construction of the chimera was carried out using the strategy described by Laplagne et al. (26). The coding sequence of the first eight residues of the N-terminal end of BLS was replaced with the coding sequence of Stx2B and a flexible G/S decapeptide linker (GSGSGSGGS) that connects both proteins. Both Stx2B and BLS form pentameric structures of the same C5 symmetry in which each protomer interacts with the other two. Molecular modeling of the chimera (Fig. 1) suggests that the linker used would be long enough to allow assembly of the Stx2B pentamers onto the pentameric modules of BLS without any steric hindrance. In the decameric BLS particle, two Stx2B pentamers are displayed in opposite directions at the top and bottom of the structure. It is worth noting that, in the BLS-Stx2B chimera, the Gb3Cer-binding surface of Stx2B is totally exposed to the solvent and is distal to the BLS scaffold.

**Expression and characterization of recombinant BLS-Stx2B protein**

The plasmid pET-BLS-Stx2B was transformed into competent E. coli BL21 (DE3) cells. The inclusion bodies, containing the majority of the BLS-Stx2B protein, were dissolved in 8 M urea. Because wild-type BLS remains folded in those conditions (23,
Ab in sera from vaccinated mice for chimera or Stx2B in the presence of FA. We examined specific IgG compared with the isolated recombinant purified Stx2B subunit, ability of BLS-Stx2B to improve the humoral immune response.

**FIGURE 2.** Analysis of the immune response of BLS. From these findings, we conclude that the B subunits on was mediated through Stx2B and not through unspecific binding allowed us to confirm that the binding of BLS-Stx2B to Gb3Cer the avidity for Gb3Cer between the different ligands. However, it values did not allow us to draw conclusions about differences in ELISA was a pool of sera from BLS-Stx2B–immunized mice, OD but not with BLS (Fig. 2C). Because the primary Ab used in the ELISA was a pool of sera from BLS-Stx2B–immunized mice, OD values did not allow us to draw conclusions about differences in the avidity for Gb3Cer between the different ligands. However, it allowed us to confirm that the binding of BLS-Stx2B to Gb3Cer was mediated through Stx2B and not through unspecific binding of BLS. From these findings, we conclude that the B subunits on top of the BLS decamer are correctly assembled and maintain their Gb3Cer-binding capacity.

**Analysis of the immune response**

**Time course of the Ab titers during the immunization.** To assess the ability of BLS-Stx2B to improve the humoral immune response compared with the isolated recombinant purified Stx2B subunit, groups of mice were injected i.p. with equimolar amounts of the chimera or Stx2B in the presence of FA. We examined specific IgG Ab in sera from vaccinated mice for >2 mo. IgG Abs specific to Stx2B were elicited in both vaccinated groups (Fig. 3). ELISA titers of the BLS-Stx2B group increased during the first three vaccinations and peaked at 14 d after the third vaccination. However, Stx2B-immunized mice did not show a specific Ab response before 45 d after the first vaccination, with high titer variability between individuals. At all analyzed times, the Ab titers generated by BLS-Stx2B were significantly higher than those of the Stx2B group (p < 0.005) (Fig. 3). No Stx2B-specific Abs were detected in the BLS-immunized group.

**Ab titers under different immunization protocols.** To evaluate the capacity of BLS-Stx2B to stimulate the immune system and to generate Abs under different formulations or vaccine regimens, we immunized groups of mice with BLS-Stx2B protein with FA or AH, without any adjuvant, or following a DNA–protein prime-boost schedule. Serum samples were collected at different times after vaccination, and titers of specific IgG were determined by ELISA. The results indicate that BLS-Stx2B stimulates the immune response under all protocols tested, even without any exogenous adjuvant (Fig. 3). However, differences in the intensity and/or kinetics of the humoral response among different protocols were observed. In particular, immunization with BLS-Stx2B formulated in AH or FA was kinetically similar in the development of specific Ab, but mice immunized with FA reached significantly higher titers (Fig. 3). In contrast, the DNA–protein prime-boost regimen induced Stx2B-specific Abs later (35 d after the protein boost), but at the same time reached similar titers than BLS-Stx2B with AH. The protein in the absence of adjuvant induced the lowest humoral response, in terms of maximal titers and time needed to reach them. In fact, mice from this group showed anti-Stx2B–specific Abs after the third immunization, and the Ab titer was 10-fold lower than in mice immunized with BLS-Stx2B in AH.

**Ab subtyping.** IgG1 is associated with the Th2-mediated humoral immune response, and IgG2a is associated with the Th1-mediated cellular response (42). To determine which type dominates the immune response, we examined the titers of the IgG subtypes (IgG1 and IgG2a) in mouse sera from all BLS-Stx2B groups at 45 d after the last immunization. BLS-Stx2B Ag induced higher anti-Stx2B–specific IgG1 titers than IgG2a titers (p < 0.001) for all protocols analyzed, with the exception of the prime-boost schedule, in which there was no significant difference between the IgG subclasses (Supplemental Fig. 2).

**Ab affinity.** Because the neutralizing and protective capacity of sera are closely dependent on the titer, as well as on the affinity of Abs for Stx2, we evaluated sera affinity of the different vaccinated groups by ELISA upon dissociation with ammonium thiocyanate. Results shown in Table I indicate that sera from mice immunized with BLS-Stx2B formulated with FA or AH displayed Abs with higher affinity for Stx2B than did sera from mice immunized with purified Stx2B in FA. Similar affinity results were observed in sera collected from mice immunized with BLS-Stx2B without adjuvant or in prime-boost regimens.
Neutralization titers against rStx2. A more correlative in vitro indicator of the protective immune response is the neutralizing capacity in the Vero cells’ cytotoxicity assay. Sera from mice immunized with BLS-Stx2B formulated with FA showed the highest neutralizing titer in the neutralization test against rStx2 ($p < 0.001$). In contrast, sera from mice immunized with Stx2B, even when formulated in FA, showed the lowest neutralization activity (Table I). Sera from nonimmunized or BLS-immunized mice did not show neutralization activity.

Cross-reactivity of mouse sera. Additionally, we evaluated neutralizing activity against wild-type toxins produced by pathogenic EHEC strains. For this purpose, supernatants from human-isolated EHEC strains producing Stx2 or the Stx2c or Stx2d variant were incubated with sera from immunized mice, and toxicity on Vero cells was evaluated. We also evaluated neutralization capacity against rStx1. Sera from mice immunized with BLS-Stx2B strongly neutralized wild-type Stx2 and its variants, as well as rStx1. In sharp contrast, the only serum sample harvested from one of six mice immunized with Stx2B was able to weakly neutralize wild-type Stx2, and none of them neutralized the Stx2 variants or rStx1 (Table II).

Protection of immunized mice against rStx2 challenge

Ex vivo rStx2-neutralization activity. In addition, anti-Stx2–neutralization activity was evaluated ex vivo in mortality curves by preincubating 1 LD$_{50}$ of rStx2 with one neutralizing unit of each pool of sera from different experimental groups. As indicated in Fig. 4A, preincubation of rStx2 with sera harvested from mice immunized with BLS-Stx2B under all protocols fully abrogated rStx2 toxicity. In contrast, sera collected from mice immunized with Stx2B or BLS did not prevent rStx2 toxicity.

In vivo Stx2-neutralization activity. As a final demonstration of the protective efficacy of the novel BLS-Stx2B vaccine, we challenged all groups of immunized mice with 1 LD$_{50}$ of rStx2 50 d after the last immunization. Fig. 4B shows that 100% of the BLS-Stx2B–vaccinated mice and 33% of the Stx2B-vaccinated mice survived the lethal rStx2 challenge. As expected for 1LD$_{50}$ of rStx2, 0% of the animals immunized with BLS or that were not immunized survived the challenge (Fig. 4B).

Protection against EHEC pathogenicity

The weaned mouse model is an effective and reliable animal model for studying the pathology of EHEC infection (34, 43). We demonstrated that weaned mice infected with Stx2-producing *E. coli* O157:H7 strains, isolated from HUS patients, experience renal dysfunction and death during the 3 d postinfection. In the same study, we also reported that a similar infection with an O157:H7 strain that does not produce Stx2 did not result in any of the pathological changes seen with the Stx2-producing strain, including a lower intestinal colonization rate. Hence, in this animal model, all of the described pathological changes result from the action of Stx2. For these reasons, we chose the weaned mouse model to assay the capacity of immune sera to protect mice from lethality induced by Stx2 released by EHEC infection in vivo. Naive weaned mice were divided into two groups receiving 50 μl of nonimmune or immune sera (BLS-Stx2B + FA) by i.p. injection immediately before challenge with 4 × 10$^{11}$ CFU/kg of Stx2-producing *E. coli* O157:H7. Animals were observed daily, and blood and stool samples were collected until death. Fig. 5A shows that mice receiving immune sera were totally protected against death by EHEC infection. Mice receiving nonimmune sera that died after 72 h exhibited high levels of plasmatic urea, indicating renal dysfunction (Fig. 5B), and an increased percentage of circulating neutrophils, as previously described (Fig. 5C) (34). Interestingly, the frequency of O157:H7-positive stool samples, characterized as described in Materials and Methods, was higher in mice receiving nonimmune sera compared with those receiving immune sera (Fig. 5D).

### Table I. Ab affinity and rStx2-neutralization capacity of sera from immunized mice

<table>
<thead>
<tr>
<th>Immunization Protocols</th>
<th>Ab Affinity$^a$</th>
<th>Neutralization Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLS-Stx2B + FA</td>
<td>0.87 ± 0.17$^*$</td>
<td>1508 ± 336$^*$</td>
</tr>
<tr>
<td>BLS-Stx2B + AH$^1$</td>
<td>1.01 ± 0.11$^*$</td>
<td>178 ± 86</td>
</tr>
<tr>
<td>BLS-Stx2B N/A</td>
<td>0.52 ± 0.10</td>
<td>104 ± 66</td>
</tr>
<tr>
<td>Prime boost</td>
<td>0.62 ± 0.14</td>
<td>203 ± 92</td>
</tr>
<tr>
<td>Stx2B + FA</td>
<td>0.41 ± 0.05</td>
<td>60 ± 48</td>
</tr>
</tbody>
</table>

$^a$Ab affinity is represented as the molar concentration of ammonium thiocyanate required to dissociate 50% of the bound Abs (in ELISA assay).

### Table II. Neutralization titers against purified rStx1, wild-type Stx2, and Stx2 variants

<table>
<thead>
<tr>
<th>Toxins</th>
<th>BLS-Stx2B + FA</th>
<th>Stx2B + FA</th>
</tr>
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<tbody>
<tr>
<td>Stx2</td>
<td>2461 ± 522</td>
<td>42 ± 42</td>
</tr>
<tr>
<td>Stx2c</td>
<td>1731 ± 346</td>
<td>0</td>
</tr>
<tr>
<td>Stx2d</td>
<td>1740 ± 386</td>
<td>0</td>
</tr>
<tr>
<td>rStx1</td>
<td>966 ± 486</td>
<td>0</td>
</tr>
</tbody>
</table>

Sera from mice immunized with Stx2B or BLS-Stx2B, both formulated with FA (45 d post last immunization), were incubated in vitro with 1 CD$_{50}$ of rStx1 and EHEC-produced Stx2 variants. Vero cytotoxicity was assayed as detailed in Materials and Methods.

Data are mean ± SEM ($n = 6$ mice/group).
Discussion

The major finding in this study is that a chimera consisting of BLS and Stx2B is highly efficacious as a vaccine against the potent cytotoxins Stx2, Stx2 variants, and Stx1. The use of genetic toxoids of Stx-type toxins is not a novel vaccine strategy (15, 16, 30, 44). However, the genetic construction of a chimeric toxoid engineered with the scaffold protein BLS decorated with 10 units of the B subunit of Stx2 is a new approach to circumvent the lack of immunogenicity of this binding subunit (19).

Both components of the chimeric toxoid were selected based on their particular properties. The B subunit of Stx2 was selected because, among the Stx family, Stx2 is the most pathogenic toxin. Because cross-reactivity between B subunits is controversial (15–17), an Stx2B-based immunogen would protect against the Stx most related to HUS development. In addition, the B subunit represents the binding unit of the toxin and is nontoxic for mammalian cells (9, 10). Abs able to block binding to the specific receptor (Gb3Cer) in mammalian cells should prevent the first step of the toxicity cascade (45). In addition, an Stx-based vaccine against HUS would not only protect against known EHEC strains, typically O157 and non-O157 serotypes, it would also be useful against new or rare pathogenic strains of Stx-producing E. coli, such as

![FIGURE 4](http://www.jimmunol.org/)

**FIGURE 4.** Protection of mice against rStx2 toxic activity. (A) Ex vivo neutralization of rStx2 toxicity (1 LD_{100}) with sera from immunized mice. Pools of sera from each immunized group (n = 4–6 mice/pool) (45 d post last immunization) or nonimmunized mice were diluted according to the in vitro neutralization titers, as indicated in the key. (B) Protection of immunized mice against a lethal challenge of purified rStx2. Mice immunized with Stx2B + FA, different formulations of BLS-Stx2B, or BLS + FA (n = 4–6 mice/group) were challenged i.v. with 1 LD_{100} rStx2 50 d after the last immunization. *p < 0.05, versus Stx2B + FA group, and p < 0.005, versus nonimmunized or BLS + FA group, **p < 0.005, versus all other groups.

![FIGURE 5](http://www.jimmunol.org/)

**FIGURE 5.** Protection against EHEC-induced pathogenicity. (A) Protection of weaned mice injected i.p. with immune sera against a lethal intragastric challenge with EHEC. Seventeen- to nineteen-day-old mice (n = 4–10 mice/group) were injected i.p. with 50 μl of nonimmune or immune sera (group BLS-Stx2B + FA, at 45 d post last immunization) diluted 1:100 in PBS. Immediately after injection, mice were infected orally with 4 × 10^{11} CFU/kg of Stx2-producing E. coli O157:H7. Results are representative of two separate experiments. (B) Renal Stx2-induced toxicity. Plasmatic urea levels at 48 and 72 h post-EHEC challenge were measured as a correlate of renal damage. Each bar represents the mean ± SEM of 5–10 mice/group. (C) Systemic symptoms of HUS-like illness. Mice were bled at 72 h post-EHEC challenge, and total and differential counts of leukocytes were assayed. Each bar represents the mean ± SEM of the relative numbers of PMN cells from four or five mice/group. (D) Bacterial shedding in EHEC-infected mice. Fecal samples were obtained at 72 h after challenge (n = 4–13 mice/group). Excretion of E. coli O157:H7 was determined by PCR against Stx2 and rfbO157 genes in nonsorbitol-fermenting colonies. *p < 0.05, versus nonimmune sera group from the same time point, **p < 0.005, versus nonimmune sera group.
The recent large outbreak of HUS caused by the O104:H4 strain (46, 47).

The other component of the chimera, BLS, was chosen because of its advantages as a carrier for Ag delivery (24–26). BLS is especially useful because it folds as a dimer of pentamers that share the same C5 symmetry as the pentameric Stx2B Ag. The N termini of the pentameric modules of BLS are located at opposite sites: at the top and bottom of the decameric particle. These are suitable insertion sites for the display of target proteins through recombinant fusion. However, the display of nonmonomeric proteins in the context of BLS may be problematic because of interparticle cross-linking and aggregation through homomeric interactions of the target subunits. This phenomenon may be critical when pentamers remain free because of uneven stoichiometry between the target oligomer and BLS. In contrast, we reasoned that BLS would represent a perfect scaffold for the assembly and stabilization of a pentameric protein if flexible peptide linkers of convenient length are used for the attachment of both proteins to avoid steric hindrance. The results presented in this article indicate that attachment of the Stx2B subunits to the BLS scaffold promotes the pentamerization of the toxin and stabilizes its structure as a result of the high local concentration of the subunits and the influence of the high thermodynamic stability of BLS.

The strong B cell response elicited when mice were immunized with BLS-Stx2B could be explained by this stabilization, as well as by the ability of BLS to target and activate dendritic cells (48). Specific ELISA titers and neutralization activity of the Abs elicited by BLS-Stx2B were improved significantly compared with those elicited by Stx2B when both immunogens were administered in FA. The most likely explanation is that the Stx2B pentamer is only marginally stable in the absence of the A subunit; thus, when used as an immunogen, is not able to raise specific Abs against conformational epitopes that are located primarily at the interfaces between monomers of the pentamer. In contrast, the fusion to BLS strongly increases the stability of Stx2B, allowing the development of Abs against the native conformation of the pentamer.

More significantly, the Abs induced by the chimera showed a similar neutralizing capacity against wild-type Stx2, its variants, and rStx1. It was demonstrated that all Gb3Cer binding sites in this family of toxins are located on the same face of the B pentamer, opposite the A subunit. Two of the three binding sites are formed by residues contributed by neighboring monomers and require correct assembly of the pentamer (45). Thus, the serum’s capacity to neutralize different Stx family members primarily would be due to Abs recognizing these binding sites that are conserved in all members of the Stx family (49), which are only present when the B subunit adopts the spatial pentameric conformation (50). This fact is of great importance for prophylaxis or therapeutics of HUS, because Abs showing broad reactivity against Gb3Cer binding sites should be highly effective at preventing the damage caused by the entire Stx family.

Effective vaccines rely on two key factors: an Ag against which adaptive immune responses are generated and an immune stimulus or adjuvant to signal the innate immune system to potentiate the Ag-specific response (51). Highly purified Ags offer potential advantages over traditional vaccines, including their safety and the capacity to elicit highly specific immune responses (52); however, in general, they need to be coadministered with immunostimulant substances (adjuvants) because they are poorly immunogenic (51, 53, 54). In the current study, immunization with BLS-Stx2B chimera formulated in FA was the most effective regimen to induce high titers of specific anti-Stx2B Abs. However, because FA is only suitable for experimental approaches, we tested the immune response and protection induced by the chimera formulated in AH and without exogenous adjuvant. Interestingly, all BLS-Stx2B—immunization regimens, even in the absence of adjuvant, induced a clear protection against the toxin in both in vitro and ex vivo experiments, and all mice vaccinated with the BLS-Stx2B chimera were protected against rStx2 challenge, despite the low ELISA Ab titers observed in some cases. Most importantly, sera from immunized mice also protected mice during a relevant model of EHEC infection, demonstrating that the transferred Abs were capable of neutralizing the toxin as it is delivered by EHEC. In fact, mice receiving immune sera failed to exhibit any of the hallmarks of an EHEC infection (e.g., physical signs of illness, renal disease, neutrophilia, and death). Furthermore, the fact that immune sera reduced EHEC colonization in the intestine suggests that anti-Stx2B Abs may have the ability to neutralize Stx2 locally, because accumulating evidence suggests that Stx2 improves EHEC colonization in mice and cattle (55–57). These results encouraged us to propose that this immunogen could be used as a vaccine against HUS.

In line with previous data (58, 59), we found that the DNA–protein prime-boost regimen resulted in a long-lasting production of anti-Stx2B—specific Abs with neutralizing capacity. In fact, 9 mo after the last immunization, mice vaccinated with the DNA–protein prime-boost regimen showed specific Ab ELISA titers that were similar to their highest values, whereas sera titers from mice vaccinated with FA decreased to 25% of their maximum values. It is important to highlight that all vaccinated mice remained healthy

**FIGURE 6.** Long-term specific IgG Ab response and protective immune response. (A) Titers of specific IgG against Stx2B assayed by ELISA up to 10 mo after the last immunization. Each time point represents the mean ± SEM of n = 4–6 mice/group. *p < 0.05, **p < 0.005, compared with BLS-Stx2B N/A- or AH-immunized mice. (B) Mice surviving the first rStx2 challenge received a second challenge with 3 LD100 of rStx2 at the time indicated in (A) (second challenge). **p < 0.005, versus nonimmunized mice.
for 11 mo and were protected even after injection of rStx2 at 3 LD100 (10 mo after the last immunization).

In conclusion, considering that Stx2 is the pathogenic factor associated with HUS development, our results demonstrate that BLS-Stx2B should be a useful candidate for the development of subunit vaccines against HUS or for the development of therapeutic anti-Stx–neutralizing Abs to be used during future outbreaks with EHEC or new strains carrying Stx genes.

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Disclosures

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References


