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A DNA Vaccine Encoding the Enterohemorragic *Escherichia coli* Shiga-Like Toxin 2 A_2 and B Subunits Confers Protective Immunity to Shiga Toxin Challenge in the Murine Model[∇]

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Production of verocytotoxin or Shiga-like toxin (Stx), particularly Stx2, is the basis of hemolytic uremic syndrome, a frequently lethal outcome for subjects infected with Stx2-producing enterohemorrhagic *Escherichia coli* (EHEC) strains. The toxin is formed by a single A subunit, which promotes protein synthesis inhibition in eukaryotic cells, and five B subunits, which bind to globotriaosylceramide at the surface of host cells. Host enzymes cleave the A subunit into the A_1 peptide, endowed with N-glycosidase activity to the 28S rRNA, and the A_2 peptide, which confers stability to the B pentamer. We report the construction of a DNA vaccine (pStx2ΔAB) that expresses a nontoxic Stx2 mutated form consisting of the last 32 amino acids of the A_2 sequence and the complete B subunit as two nonfused polypeptides. Immunization trials carried out with the DNA vaccine in BALB/c mice, alone or in combination with another DNA vaccine encoding granulocyte-macrophage colony-stimulating factor, resulted in systemic Stx-specific antibody responses targeting both A and B subunits of the native Stx2. Moreover, anti-Stx2 antibodies raised in mice immunized with pStx2ΔAB showed toxin neutralization activity in vitro and, more importantly, conferred partial protection to Stx2 challenge in vivo. The present vector represents the second DNA vaccine so far reported to induce protective immunity to Stx2 and may contribute, either alone or in combination with other procedures, to the development of prophylactic or therapeutic interventions aiming to ameliorate EHEC infection-associated sequelae.

Shiga toxin (Stx)-producing enterohemorrhagic *Escherichia coli* (EHEC) strains are important food-borne pathogens representing the major etiological agents of hemorrhagic colitis and hemolytic uremic syndrome (HUS), a life-threatening disease characterized by hemolytic anemia, thrombocytopenia, and renal failure (19). The infection correlates with ingestion of contaminated meat or vegetables but is also transmitted by water or even person-to-person contact (8, 14, 44). Sporadic or massive outbreaks have been reported in several developed countries but, in Argentina, HUS is endemic and represents a serious public health problem with high morbidity and mortality rates (29, 40). Production of verocytotoxin or Shiga-like toxin (Stx) is the basis of EHEC pathogenesis (18, 20). The toxin is formed by a single A subunit, which possesses N-glycosidase activity to the 28S rRNA and promotes protein synthesis inhibition in eukaryotic cells, and five B subunits, which bind to globotriaosylceramide at the surface of host cells (9, 28). Although two major types (Stx1 and Stx2) and several subtypes have been described, Stx2 and Stx2c are the most frequently found toxins in severe HUS cases among EHEC-infected subjects (12, 41). The degree of antigenic cross-reactivity between Stx2 and Stx1 is low, and several authors have reported that the two toxins do not provide heterologous protection, particularly concerning the B subunits (45, 47). On the other hand, Stx2c and Stx2d variants are readily neutralized by antibodies against Stx2 (27).

Despite the magnitude of the social and economic impacts caused by EHEC infections, no licensed vaccine or effective therapy is presently available for human use. So far, attempts to develop vaccine formulations against EHEC-associated sequelae have relied mainly on induction of serum anti-Stx antibody responses. Several approaches have been pursed to generate immunogenic anti-Stx vaccine formulations and include the use of live attenuated bacterial strains (2, 32), protein-conjugated polysaccharides (21), purified B subunit (33, 48), B-subunit-derived synthetic peptides (15), and mutated Stx1 and Stx2 nontoxic derivatives (5, 6, 13, 16, 37, 39, 42, 45).

In a previous report we described anti-Stx2 DNA vaccines encoding either the B subunit or a fusion protein between the B subunit and the first N-terminal amino acid of the A_1 subunit (8). The DNA vaccine encoding the hybrid protein elicited Stx-specific immune responses in mice and partial protection to Stx2 challenge (1, 33). Recent data have indicated that epitopes leading to generation of Stx-neutralizing antibodies are present on both the B as well as the A subunit (34, 45, 46). In addition, further evidence indicates that the A_2 subunit contains some of the most immunogenic epitopes of the Stx2 toxin (4). Thus, in line with such evidence, we attempted the construction of a new DNA vaccine encoding the last 32 amino acids from the A_2 subunit, in addition to the complete B

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subunit of Stx2, as separated polypeptides which could enhance the immunogenicity and protective effects of the vaccine formulation. In the present report, we describe the generation of a new DNA vaccine encoding both Stx2 A2 and B subunits as an approach to elicit protective antibody responses to Stx2. The results obtained demonstrate that immunization with this vaccine formulation results in systemic antibody responses to Stx2 A and B subunits and toxin neutralization activity both in vitro and in vivo.

MATERIALS AND METHODS

Plasmid constructions. The plasmids were constructed by standard cloning techniques, according to the NIH policy manual on *Working Safely with Hazardous Biological Materials* (35). All restriction enzymes were purchased from Promega, Inc. (Madison, WI). Plasmids were isolated from transformed bacteria by previously described procedures (10). Large-scale purification of recombinant plasmids for immunization trials was carried in chromatographic prepacked columns (Qiagen Inc., Chatsworth, CA). The pGEMT Easy vector carrying the sequence of Stx2, containing downstream and upstream EcoRI restriction sites (7), was digested with AvaI and StuI and religated, generating the plasmid pGEMTStx2ΔAB (Fig. 1A). pGEMTStx2ΔAB was cut with EcoRI and the fragment was isolated and ligated to pcDNA 3.1 previously digested with the same enzyme, giving rise to the pStx2ΔAB vector. This plasmid was replicated in competent *E. coli* DH5α cells (Bethesda Research Laboratories), while *E. coli* JM109 cells were electroporated with pStx2 or pGEMTStx2ΔAB for production of the corresponding recombinant proteins. The pGM-CSF plasmid (a gift from E. A. Scodeller, Centro de Virología Animal, Buenos Aires, Argentina) consists of the same pcDNA 3.1 vector carrying the gene for murine granulocyte-macrophage colony-stimulating factor (GM-CSF). The constructed recombinant plasmids were screened by restriction mapping, PCR, and sequencing by the dideoxy chain termination method.

Stx2ΔAB protein production and purification. *E. coli* JM109 transformed with plasmid pGEMT-Stx2ΔAB was used to express the Stx2ΔAB protein. Bacteria were grown in Luria broth overnight in the presence of ampicillin (50 μg/ml). Cells were broken by ultrasonic treatment. The lysate was centrifuged at 13,200 × *g* for 20 min at 4°C and the supernatant was treated with ammonium sulfate to 70% saturation. The precipitate was collected by centrifugation (13,200 × *g* for 20 min at 4°C), resuspended in 3 ml of phosphate-buffered saline, and dialyzed with the same buffer for 24 h. Total protein concentration was determined by standard methods (Bradford). Specific Stx2ΔAB protein concentration was estimated by polyacrylamide gel electrophoresis of known quantities of ovalbumin. Identity of detected protein bands was confirmed by Western blotting (WB) with specific monoclonal antibodies (MAbs) against the Stx2 B subunit (Biodesign) and against the Stx2 A subunit (Toxin Technology, Sarasota, FL).

Stx2 preparation. *E. coli* JM109 transformed with plasmid pGEMT-Stx2 was used to express Stx2 protein following the same protocol described above for Stx2ΔAB production. The same batch of Stx2 preparation was used throughout the experiments with WB and enzyme-linked immunosorbent assays (ELISAs), as well as and in vitro and in vivo toxicity tests.

In vitro Stx2ΔAB expression in eukaryotic cells. BHK-21 cells (Syrian hamster kidney fibroblast) from the American Type Culture Collection were grown in six-well culture plates (Nalge-Nunc Int., Rochester, NY) at 37°C in 5% CO_2. Plasmid DNA (1 μg) was mixed in Lipofectin reagent (Invitrogen) following the manufacturer's instructions. Cells were washed twice with serum-free medium and incubated with the mixture during 3 hours at 37°C in 5% CO_2. Then, the transfection mix (DNA-Lipofectin) was washed away, and the cells were incubated for 48 h more in complete medium at 37°C in 5% CO_2. For WB analysis cells were removed from plates and lysed. Protein was quantified from clarified lysates and 10 μg of total protein was mixed with nonreducing Laemmli buffer (24), boiled for 5 minutes, and seeded in a 17.5% gradient polyacrylamide minigel. Specific bands were revealed with specific MAbs against the Stx2 B subunit and the Stx2 A subunit.

In vitro and in vivo evaluation of Stx2ΔAB toxicity. Toxicity of the Stx2 and Stx2ΔAB proteins was assessed in vitro by the Vero cell cytotoxic assay. Vero cells were grown overnight to confluence in 96-well plates and challenged with increasing concentrations of Stx2 or Stx2ΔAB up to 25 μg/ml. The 50% cytotoxic dose (CD_{50}) for our Stx2 preparation was 4.5×10^{-3} μg/ml, calculated as previously described (11). In vivo toxicity was determined after intraperitoneal (i.p.) injection of different amounts of Stx2 or Stx2ΔAB. All animals inoculated with Stx2 died between 120 and 144 h postinoculation. Renal damage was also monitored in mice receiving three i.p. doses of the purified protein Stx2ΔAB (0.5 μg) or three intramuscular (i.m.) doses of pStx2ΔAB (50 μg). Blood samples were taken at 15 days and 1, 2,4, and 6 months after the last i.m. injection of pStx2ΔAB and tested for urea levels. In addition, liver and kidneys were excised and histologically examined at different times up to 6 months after the last dose of Stx2ΔAB or encoding plasmid.

Immunization protocols and sample collection. BALB/c mice were bred in the animal facility at the Department of Experimental Medicine, Academia Nacional de Medicina, Buenos Aires, Argentina. They were maintained under a 12-h light-dark cycle at 22 ± 2°C and fed with standard diet and water ad libitum. The experiments performed herein were conducted according to principles set forth in the National Research Council's *Guide for the Care and Use of Laboratory Animals* (36). Adult mice were immunized by i.m. injection on days 0, 14, and 21 into the rear leg with 50 μg of purified plasmids (pStx2ΔAB or pcDNA3.1 in control groups). Three days before each dose a recombinant plasmid encoding murine GM-CSF (pGM-CSF) was injected (50 μg i.m.) in the pStx2ΔAB- or pcDNA3.1-immunized experimental groups in order to enhance the induced Stx-specific immune responses (23). Serum samples were collected 1 day before administration of each vaccine dose and 15 days after the last dose. Serum samples were individually tested for reactivity with Stx2 antigen by immunoblots and ELISA.

Analysis of antibody responses. Stx2-specific immunoglobulin G (IgG) in serum samples of vaccinated mice was determined by WB and ELISAs. Purified wild-type Stx2 was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose. The blots were then probed with mouse serum diluted 1:100 followed by horseradish peroxidase-conjugated secondary rabbit antibodies against mouse IgG (Bio-Rad Laboratories, Hercules, CA). The ELISA was carried out with recombinant Stx2 as the solid-phase bound antigen. Briefly, 96-well MaxiSorp plates (Nunc) were coated with 1 μg of purified Stx2 and after blocking, the plates were incubated for 1 h with serially diluted mouse sera. After washing, the plates were incubated with diluted (1: 3,000) peroxidase-conjugated rabbit anti-mouse IgG (Sigma) for an additional hour. The plates were developed with *O*-phenylenediamine (0.4 mg/ml; Sigma) and H_2O_2. Absorbance at 492 nm was measured with a microtiter plate reader (LabSystem). All tested samples were assayed in duplicate wells. Results were expressed as endpoint titers, calculated as the reciprocal values of the last dilution with an optical density higher than the medium of preimmune serum samples ± 2 standard deviations (SD) and are presented as means ± standard errors of the means (SEM).

In vitro and in vivo neutralizing activity. To determine in vitro Stx2-neutralizing antibody titers, equal volumes of Stx2 corresponding to 2× the CD_{50} and serial dilutions of the experimental serum samples were preincubated for 1 h at 37°C followed by 1 h at 4°C. The mixtures were overlaid onto each well containing 10^4 Vero cells and incubated for 2 days at 37°C in 5% CO_2. Cells were washed, stained with crystal violet dye, and read on a Microwell System reader 230S (Organon, Teknika, OR) with a 550-nm filter. The neutralizing activity was expressed as the reciprocal value of the highest dilution that blocked Stx2 toxicity to Vero cells. For in vivo lethality studies, Stx2 was injected intravenously (i.v.) at doses of 1.6×10^{-4} μg/mouse (1× the 100% lethal dose [LD_{100}]), which is known to lead to 100% mortality 120 to 144 h after injection, as previously described (38). In vivo Stx2 neutralization activity was determined after incubation of the Stx2 aliquots (1.6×10^{-4} μg) with serum samples collected from mice submitted to the different immunization procedures during 1 hour at 37°C and for another hour at 4°C. After the incubation period, the treated Stx2 aliquots were i.v. inoculated into naive mice. In addition, control (nonimmunized) and immunized mice were injected with Stx2 (1.6×10^{-4} μg/mouse), in order to further evaluate in vivo protection capacity of plasmid vaccines.

Statistical analysis. All data correspond to the means ± SEM for individual mice. Statistical differences were determined using a one-way multiple comparison analysis of variance by an analysis of variance (ANOVA)–Newman-Keuls test, and P values of <0.05 were considered significant. Individual groups were compared using the unpaired Student's t test. The χ^2 test or Fisher's exact test was used for class variables in two independent samples.

RESULTS

Construction of the pStx2ΔAB vector encoding both Stx2 A_2 and B subunits. The plasmid vector (pStx2ΔAB) encoded the entire Stx2 B subunit plus the last 32 amino acid residues of A_2, including the signal peptides for the A and B subunits. Based on the known Stx2 sequence data (http://www. expasy.org/uniprot

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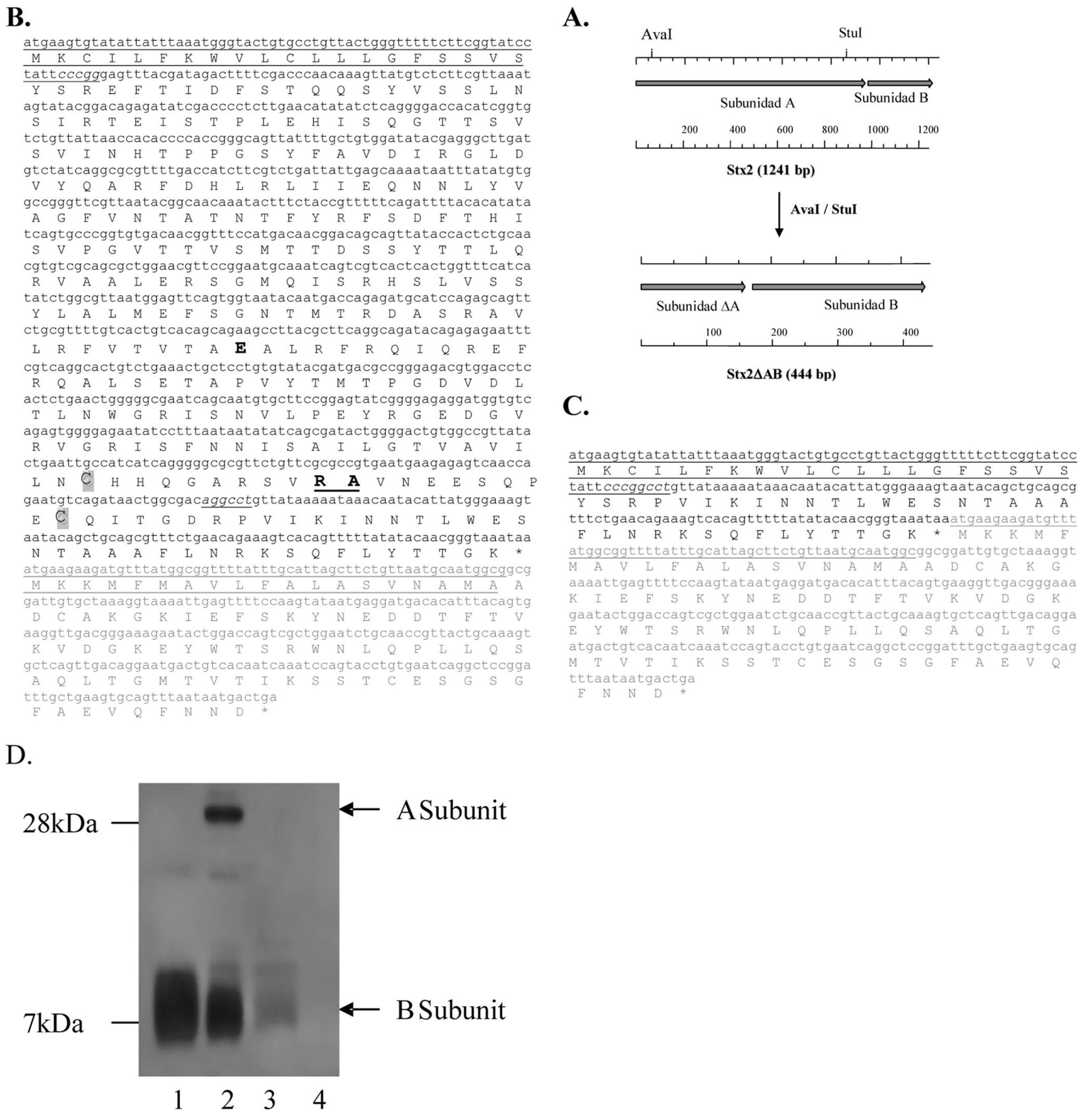

FIG. 1. Construction of pStx2ΔAB and expression of the encoded peptides. (A) Schematic representation of the restriction step (digestion with both AvaI and StuI restriction enzymes) leading to deletion of most the A_1 subunit. (B) Nucleotide and amino acid sequences of native Stx2, showing the A subunit (black) and B subunit (gray) and their corresponding signal sequences (underlined). The active site in bold (E, at 189), the cysteine residues involved in disulfide bond (shaded; Cs), and the protease cleavage site in the A subunit (in bold and underlined) are also shown. (C) Nucleotide and amino acid sequences of Stx2ΔAB, showing the absence of the active site, cysteine residues, and cleavage site. (D) Expression of Stx2 cross-reactive peptides in protein extracts of pStx2ΔAB-transfected BHK-21 cells and pGEMT-Stx2ΔAB-transformed JM109 cells. Western blots were developed with both anti-Stx2 A and B subunit MAbs. Lanes: 1, whole-cell extract of pStx2ΔAB-transformed JM109 cells; 2, purified Stx2; 3, whole-cell extract of pStx2ΔAB-transfected BHK-21 cells; 4, whole-cell extract of pcDNA3.1-transfected BHK-21 cells. Molecular mass markers are indicated on the left side of the figure.

/P09385) and the restriction enzymes used, the mutated toxin (Stx2ΔAB) lacks the active site, the cysteine residues involved in disulfide bond formation, and the protease cleavage site required for dissociation of A_1 and A_2 peptides (Fig. 1B and C). pStx2ΔAB was introduced into BHK-21 cells and the encoded peptides were detected in immunoblots with anti-Stx2B/Stx2A MAbs. As shown in Fig. 1D, whole-cell extracts of pStx2ΔAB-transfected BHK-21 cells contained a single anti-

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Stx2 serum-reactive protein band, corresponding to the Stx2 B subunit (7.7 kDa), similar to the protein pattern observed with whole-cell extracts of pGEMT-Stx2-transformed bacteria. The absence of the A_2 peptide in immunoblots of pStx2ΔAB-transfected BHK-21 cells was not surprising due to the reduced molecular mass (3.6 kDa) of the encoded peptide.

The Stx2ΔAB protein, purified from recombinant *E. coli* cells, did not show any in vitro toxicity to Vero cells even at a concentration of 25 μg/ml, equivalent to 5.5×10^3 CD_{50} of native Stx2 toxin (4.5×10^{-3} μg/ml). Moreover, lethality assays carried out with i.p. injection of Stx2ΔAB (up to 1 μg/mouse) did not result in any detected mortality, while all mice injected with native Stx2 (1.6×10^{-4} μg/mouse) died between 120 and 144 h postinoculation. In another set of experiments, mice were given three i.p. doses of 0.5 μg Stx2ΔAB or three i.m. injections of 50 μg pStx2ΔAB. None of these mice showed histological or biochemical alterations of renal function even 6 months after the protein or plasmid inoculation (data not shown). Collectively, these data suggest that Stx2ΔAB, either as purified protein or encoded by the DNA vaccine, does not express any residual toxicity to eukaryotic cells.

Generation of specific anti-Stx2 antibody responses in mice immunized with pStx2ΔAB. The immunogenicity of the DNA vaccine encoding both Stx2 A_2 and B subunits was evaluated in BALB/c mice i.m. immunized with three doses (50 μg of DNA/dose) of pStx2ΔAB. Sera collected 2 weeks after the last immunization recognized both A and B subunits of Stx2-enriched preparations in a WB assay (Fig. 2A). Furthermore, sera harvested from mice immunized with pStx2ΔAB reacted with native Stx2 when probed in an ELISA (Fig. 2B). In order to enhance the final anti-Stx2 serum IgG titers measured in pStx2ΔAB-immunized mice, a GM-CSF-encoding plasmid was administered to mice 3 days before each pStx2ΔAB dose. As shown in Fig. 2B, previous administration of the pGM-CSF plasmid enhanced the final anti-Stx2 IgG titer and reduced the individual anti-Stx2 titer variation among pStx2ΔAB-vaccinated mice. In addition, sera harvested from mice immunized with pStx2ΔAB plus pGM-CSF or pGM-CSF alone showed nonspecific reactive bands against the Stx2 preparation in the WB assay (Fig. 2A). These results suggest the presence of antibodies that cross-reacted with nonspecific antigens present in the Stx2 preparation, probably due to a pGM-CSF adjuvant effect. Meanwhile, no reactive bands were detected when Stx2 was probed with sera harvested from mice immunized with pcDNA3.1.

In vitro and in vivo toxin neutralization activity of anti-Stx2 antibodies raised in mice immunized with pStx2ΔAB. As a protection correlate for the anti-Stx2 DNA vaccine, Stx2 neutralization tests on Vero cells were carried out with sera harvested from mice immunized either with pStx2ΔAB or with pGM-CSF/pStx2ΔAB. Stx2-enriched preparations were efficiently neutralized by hyperimmune sera collected from mice vaccinated either with pStx2ΔAB alone or with pGM-CSF/pStx2ΔAB (Fig. 3A). An additional correlate of the anti-Stx2 neutralization activity was based on the ex vivo Stx2 neutralization activity of hyperimmune sera harvested from vaccinated mice. As indicated in Fig. 3B, preincubation of Stx2 aliquots, at a concentration corresponding to 1 LD_{100}, with sera harvested from mice immunized with pStx2ΔAB or pGM-CSF/pStx2ΔAB conferred 30% and 50% survival, respectively.

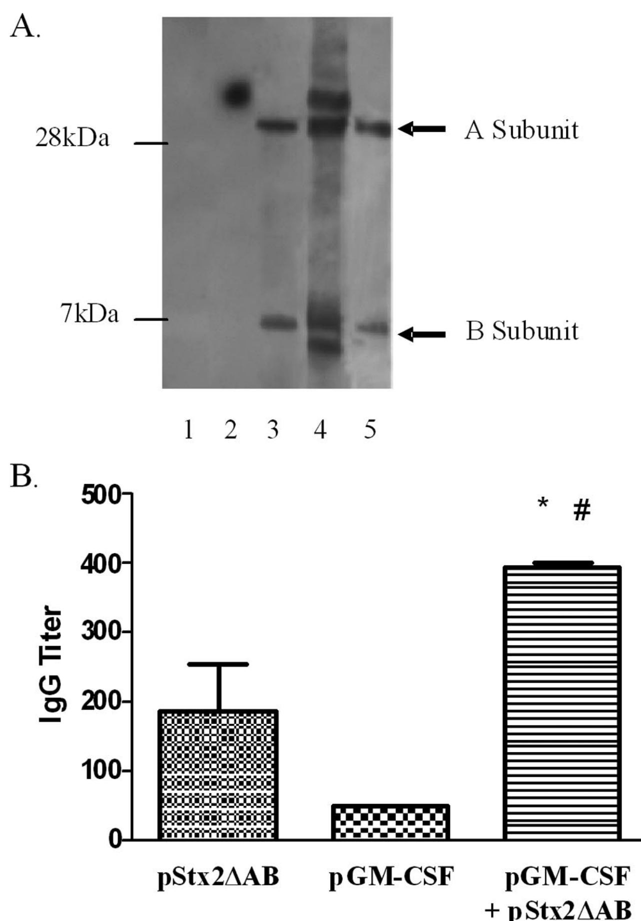

FIG. 2. Induction of anti-Stx2 serum antibodies in mice immunized with pStx2ΔAB. (A) Detection of Stx2 cross-reacting peptides with anti-Stx2 antibodies raised in mice immunized with the DNA vaccine formulations. All serum samples were probed with purified Stx2 preparations sorted in 17.5% polyacrylamide gels. Lanes: 1, serum pool harvested from mice immunized with pcDNA3.1; 2, serum pool harvested from mice immunized only with pGM-CSF; 3, serum pool harvested from mice immunized with pStx2ΔAB; 4, serum pool harvested from mice immunized with pGM-CSF/pStx2ΔAB; 5, Stx2-specific (A and B subunits) monoclonal antibodies. Molecular mass markers are indicated on the left side of the figure. (B) Determination of specific anti-Stx2 IgG antibody titers in mice immunized with the DNA vaccines. Sera harvested from mice immunized with pStx2ΔAB (pStx2ΔAB), pGM-CSF (pGM-CSF), or pGM-CSF/pStx2ΔAB (pGM-CSF + pStx2ΔAB) were assayed by Stx2-specific ELISA. The tested serum samples were harvested 2 weeks after the last DNA dose. The cutoff value for positive reactions was established as the mean absorbance value plus two times the SD detected in mice immunized with two doses of pcDNA3.1. Each bar corresponds to the mean ± SEM from at least six mice individually tested, per group. *, $P < 0.05$, significantly different from the pStx2ΔAB-immunized group; #, $P < 0.05$, significantly different from the pGM-CSF-immunized group, by ANOVA–Newman-Keuls multiple comparison test.

Sera collected from mice immunized with pcDNA3.1 or pGM-CSF did not confer any protection to Stx2 challenge.

As a final demonstration of the protective efficacy of the tested DNA vaccine, we performed Stx2 challenge experiments in mice immunized with pStx2ΔAB or with pGM-CSF/pStx2ΔAB following i.v. administration of a lethal Stx2 load (1.6×10^{-4} μg) 2 weeks after the last vaccine dose. Under this

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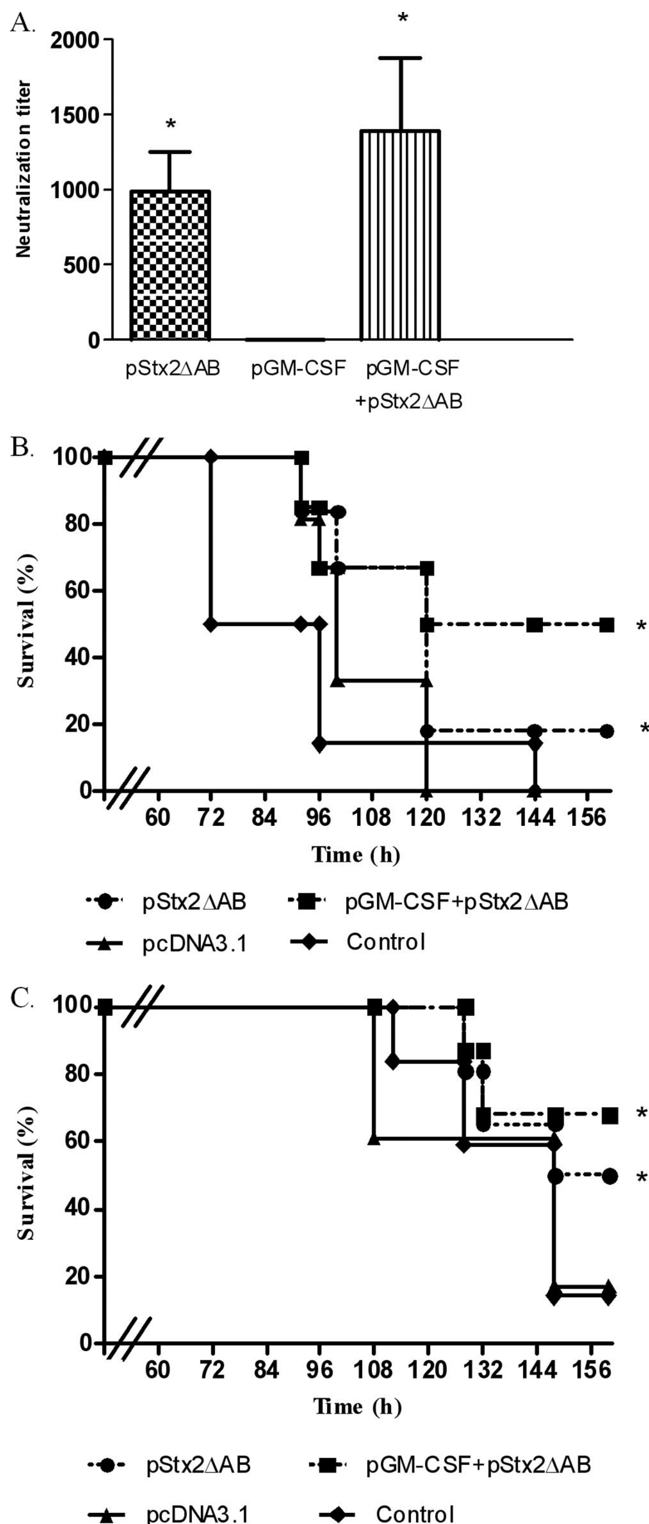

FIG. 3. In vitro, ex vivo, and in vivo anti-Stx2 activities of antibodies raised in mice immunized with the tested DNA vaccine formulations. (A) Neutralization of Stx2 cytotoxicity on in vitro-cultured Vero cells. Stx2-neutralizing activities of individual serum samples harvested from mice immunized with pStx2ΔAB, pGM-CSF, or pGM-CSF/pStx2ΔAB (pStx2ΔAB + pGM-CSF) were determined as described in Materials and Methods. The cutoff value for positive reactions was established as the mean value (plus 2 SD) detected in mice immunized with two doses of pcDNA3.1. Each bar corresponds to the mean ± SEM of the last dilution giving a positive reaction per 1 ml ($n = 6$ per mouse group). *, statistically different ($P < 0.05$) from mice immunized with pcDNA3.1 by ANOVA–Newman-Keuls multiple comparison test. (B) Ex vivo neutralization activity of Stx2 toxicity with serum samples collected from immunized mice with the different vaccine formulations. Survival rates of mice challenged with a Stx2 load corresponding to 1× LD_{100} and previously incubated with serum pools of mice immunized with pcDNA3.1, pStx2ΔAB, or pGM-CSF/pStx2ΔAB or nonimmunized mice (control). *, statistically different ($P < 0.05$) from mice immunized with pcDNA3.1 by χ^2 test. (C) In vivo neutralization of Stx2 toxicity in mice immunized with pStx2ΔAB. Survival rates were determined in mice challenged with Stx2 at a final concentration corresponding to 1× LD_{100} after vaccination with pcDNA3.1, pStx2ΔAB, or pGM-CSF/pStx2ΔAB or nonimmunized mice (controls). Symbols and lanes are as indicated in panel B. *, statistically different ($P < 0.05$) from mice immunized with pcDNA3.1, based on the chi-square test.

protocol, 50% of the pStx2ΔAB-vaccinated mice and 70% of the pGM-CSF/pStx2ΔAB-vaccinated mice survived the Stx2 lethal challenge. Under the same experimental conditions, only 10% of the animals immunized with pcDNA3.1 or pGM-CSF survived the parenteral challenge with Stx2 (Fig. 3C).

DISCUSSION

The major contribution of the present report is the description of a Stx2-derived antigen (Stx2ΔAB), representing the Stx2 A_2 peptide plus the B subunit, devoid of detectable toxicity, both for cultured Stx2-sensitive Vero cells and BALB/c mice either as purified protein or as a DNA vaccine. Moreover, the DNA vaccine encoding the Stx2-derived toxoid induced systemic IgG antibodies that specifically recognized both the A and B subunits and partially neutralized the native Stx2.

The inclusion of the A_2 sequence into the new construct was based on different reasoning. First, monoclonal antibodies directed to the Stx2 A subunit have been proved to be equal or more protective than those directed to the B subunit (17, 43, 45). Although the mechanisms involved in toxin neutralization are still a matter of debate, it has been recently reported that anti-A subunit antibodies interfere with retrograde transport of the toxin and may interact with Stx2 when still bound to membrane receptors (22). In addition, antibodies directed to the Stx2 A subunit, as opposed to those directed against the B subunit, have broader cross-reactivity that includes other Stx2 variants, such as Stx2c (27, 43). The data presented herein demonstrate that the antibody response detected in mice vaccinated with pStx2ΔAB conferred in vitro and in vivo protection to Stx2 toxic effects.

Second, previous attempts to generate toxoids with the whole or a part of the A_1 subunit, generated by active site-directed mutagenesis, showed that the resulting proteins retained significant toxicity for both Vero cells and mice (45). Thus, we reasoned that removal of all A_1 peptides, except for the signal peptide, would significantly reduce the toxicity and, therefore, increase the safety of the resulting peptide. In fact, Stx2ΔAB or the Stx2ΔAB-encoding plasmid proved to be nontoxic under both in vitro and in vivo conditions.

Finally, recent evidence has shown that the combination of monoclonal antibodies specific to A and B Stx2 subunits results

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in maximal toxin neutralization activity (22). In addition, the demonstration that the A_2 sequence encompasses the most immunogenic epitopes, as suggested by an epitope prediction program based on the Stx2 structure (4), further supported our decision to include the A_2 peptide into the vaccine formulation. The present findings indicated that immunogenic epitopes important for Stx2 protection are present on both A_2 and B Stx2 subunits, and antibodies directed against both peptides synergistically act to enhance neutralization of the native Stx2 toxin. Accordingly, the new construction proved to work better than our previous DNA vaccine encoding the B subunit alone or the B subunit fused to an A_1-deleted form (7). It is also important to highlight that the absence of antigenic cross-reactivity between B subunits of Stx1 and Stx2 led us to work with Stx2, since Stx2, or its derivatives, are the most frequently HUS-associated toxins.

While a 7.7-kDa protein band corresponding to the Stx2 B subunit was identified in pStx2ΔAB-transfected bacteria and eukaryotic cell lysates, no cross-reactive band corresponding to the A_2 fragment was detected, probably reflecting the expected low molecular mass (3.6 kDa) of the encoded peptide. On the other hand, the band corresponding to the Stx2 A subunit (32.7 kDa) was clearly identified in immunoblots prepared with native Stx2 after probing with antibodies raised in mice immunized with pStx2ΔAB (Fig. 3A). This result indicates that both A_2 and B sequences were transcribed and translated in vivo following the i.m. inoculation of pStx2ΔAB into BALB/c mice. The expression of the two nonfused peptides by the eukaryotic expression vector most probably reflects the presence of putative promoter-like sequences located upstream of Stx2 A_2 (bp 175 to 225) and B (bp 374 to 424) sequences, as demonstrated with a promoter sequence search tool (www.fruitfly.org/cgi-bin/seq_tools/promoteer.pl). Although indirect, the generation of Stx2-specific antibodies able to react with A and B subunits and to neutralize Stx2 activity in pStx2ΔAB-immunized mice is strong evidence that both A_2 and B subunits are expressed by in vivo pStx2ΔAB-transfected cells.

DNA vaccines have several features favoring their use in immunization regimens, including a good safety record and rather easy and low-cost large-scale production in comparison with conventional subunit vaccines. However, systemic immune responses elicited in mammalian hosts immunized with DNA vaccines may have a low magnitude and experience a sharp decline following administration to human hosts (26, 31). Several experimental alternatives have been proposed to enhance the immunogenicity of DNA vaccines, including the use of encoded cytokines as adjuvant (23). Indeed, in our hands the immunogenicity of pStx2ΔAB has been significantly improved by injection with GM-CSF 3 days before administration of the DNA vaccine, as previously reported (23). However, even under this vaccination protocol it should be taken into account that Stx2 neutralization was not complete. This effect could be due to the low affinity of Stx2-specific antibodies that leads free Stx2 to interact with its specific receptor in the endothelial cells. In this context increasing the amounts, affinity, and/or avidity of Stx2 antibodies for the toxin may be decisive.

Another successful approach to improve the immunogenicity of DNA vaccines may rely on prime-boost immunization regimens in which DNA-primed animals are boosted via mucosal or parenteral routes with live bacterial or viral vectors encoding the same target antigen (3, 25, 30). Future studies aimed at the development of new vaccine approaches against Stx2-associated diseases might consider the generation of live bacterial vectors expressing the Stx2ΔAB sequence that, in combination with DNA vaccines, would generate enhanced systemic and secreted anti-Stx2 immunity.

In conclusion, we report the generation of a DNA construct encoding a nontoxic antigenic Stx2 toxoid which induces both specific humoral responses to Stx2 and confers in vitro as well as in vivo Stx2 neutralization activity. Given that Stx2 is the main EHEC virulence factor associated with and necessary for HUS development, the rational design of effective anti-HUS vaccine formulations should, in our view, include nontoxic Stx2 derivates. We believe that the herein-reported DNA vaccine represents a relevant alternative for vaccine formulations conferring protection against Stx2.

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