INFECTION AND IMMUNITY, July 2003, p. 3971–3978 Vol. 71, No. 7
0019-9567/03/$08.00+0 DOI: 10.1128/IAI.71.7.3971–3978.2003

Development of DNA Vaccines against Hemolytic-Uremic Syndrome in a Murine Model

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Received 9 October 2002/Returned for modification 26 November 2002/Accepted 12 March 2003

Shiga toxin type 2 (Stx2) produced by *Escherichia coli* O:157H7 can cause hemolytic-uremic syndrome in children, a disease for which there is neither a vaccine nor an effective treatment. This toxin consists of an enzymatically active A subunit and a pentameric B subunit responsible for the toxin binding to host cells, and also found to be immunogenic in rabbits. In this study we developed eukaryotic plasmids expressing the B subunit gene of Stx2 (pStx2B) and the B subunit plus the gene coding for the A subunit with an active-site deletion (pStx2ΔA). Transfection of eukaryotic cells with these plasmids produced proteins of the expected molecular weight which reacted with specific monoclonal antibodies. Newborn and adult BALB/c mice immunized with two intramuscular injections of each plasmid, either alone or together with the same vector expressing the granulocyte and monocyte colony-stimulating factor (pGM-CSF), elicited a specific Th1-biased humoral response. The effect of pGM-CSF as an adjuvant plasmid was particularly notable in newborn mice and in pStx2B-vaccinated adult mice. Stx2-neutralizing activity, evaluated in vitro on VERO cell monolayers, correlated with in vivo protection. This is the first report using plasmids to induce a neutralizing humoral immune response against the Stx2.

Infection with Shiga toxin (Stx)-producing *Escherichia coli* serotypes that cause hemorrhagic colitis is a serious public health problem. In some cases colitis leads to a complication known as hemolytic-uremic syndrome (HUS), characterized by hemolytic anemia, thrombocytopenia, and renal failure (28). This syndrome can be life-threatening, particularly in children less than 5 years of age. Although peritoneal dialysis has significantly reduced mortality, 30% of the affected children undergo severe chronic renal failure or neurological complications. There is neither a vaccine nor an effective treatment for HUS.

Two antigenically distinct Stx types, Stx1 and Stx2, are the primary pathogenic factors (25; M. A. Karmali, M. Petric, C. Lim, P. C. Fleming, and B. T. Steele, Letter, Lancet **ii:**1299-1300, 1983), but epidemiological and experimental studies have suggested that Stx2 is clinically more relevant than Stx1 (35). Although animals immunized with Stx2 toxoid preparations are protected against Stx2 holotoxin challenge, there are safety concerns associated with using inactivated holotoxins in human vaccines (3, 4, 23, 37).

Shiga toxins consist of a single A and a pentamer of B subunits (16). The A subunit possesses N-glycosidase activity against 28S rRNA and inhibits protein synthesis in eukaryotic cells. The B subunit pentamer binds to globotriaosylceramide receptors on the cell membrane (16). Although the isolated B subunit has biological activities such as the triggering of fluid secretion in the colon (C. Ibarra, unpublished observations), it is nontoxic to VERO cells, HeLa cells, and monocytic THP-1 cells (42) and has immunoprophylactic potential (8, 35).

Large-scale production of the Stx2 B subunit (Stx2B) has not been efficient, probably due to the instability of the B multimers when synthesized without the A subunit (2, 9, 35). An alternative approach could be to express the antigen in vivo, by developing a DNA vaccine (1, 10, 12, 13, 32). These vaccines are typically composed of bacterium-derived plasmid DNA carrying eukaryotic gene regulatory elements that drive the expression of genes encoding antigens.

Delivery of these vaccines can be carried out by a variety of methods, including direct intramuscular (i.m.) injection of the plasmid in saline solution or oral administration (14, 22). The i.m. injection of a standard 50 μg results in the rapid dispersion of DNA throughout the muscle, making this an easy way to elicit potent humoral and cellular immune responses in mice (17).

Genetic vaccines have many features that make them an appropriate strategy to prime an immune response, particularly in early life. CpG unmethylated islands present in bacterial DNA (15) activate neonatal immature antigen-presenting cells (APC) (5). Other advantages of genetic vaccines include their well-known composition and the simplicity of producing and purifying them. Moreover, plasmid DNA is very stable and resistant to extreme temperatures, properties that reduce storage and transport costs (40).

Immune responses elicited by genetic vaccines can be enhanced by the coinoculation of plasmids expressing different cytokines or immune stimulatory factors. The coadministration of a plasmid expressing murine granulocyte-macrophage colony-stimulating factor (pGM-CSF), a growth factor that in-

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creases the production of macrophages and granulocytes and promotes the maturation and activation of APC, improves the protective immunity induced by DNA-based immunization (20, 26, 31, 41).

Genetic vaccines represent a new generation of immunogen delivery being evaluated to develop antigen-specific immune responses in humans. New human immunodeficiency virus DNA vaccines are in phase I trials (7, 34). Other phase I trials of therapeutic vaccines include naked plasmid encoding fibroblast growth factor type 1 (11), as well as naked DNA immunization as immunotherapy for prostate cancer (38).

The aim of this study was to develop a protective genetic vaccine against HUS in a mouse model using either the B subunit gene or the B subunit gene plus the truncated A subunit gene. Both plasmids were tested as DNA vaccines in newborn and adult mice, alone or in combination with pGM-CSF. The induction of specific serum antibodies, and the classes and subclasses of the immunoglobulins produced were determined. Moreover, a relationship between serum Stx2-neutralizing activity determined in vitro and the protection achieved in vivo was established.

MATERIALS AND METHODS

Plasmid constructions. The plasmids were constructed by standard techniques (38a). All restriction enzymes were purchased from Promega Inc. (Madison, Wis.). The plasmids were isolated from competent bacteria by the mini DNA preparation procedure (18). Large-scale purification of recombinant plasmids for immunization trials was performed in special columns (Qiagen Inc., Chatsworth, Calif.).

The complete gene for Stx2 was amplified by PCR from total DNA from *E. coli* O157-H7 C600 (933W) with primers upstream (Ds, 5′ GAA TTC ATT ATG CGT TGT TAG 3′) and downstream (R1, 5′-GAA TTC TCA GTC ATT ATT AAA CTG-3′), both containing an *Eco*RI restriction site. The resulting fragment of about 1,422 bp was cloned in pGEMT easy vector (Invitrogen, San Diego, Calif.), generating the plasmid pGEMTStx2. This plasmid was replicated in competent *E. coli* DH5α cells (Bethesda Research Laboratories).

Stx2B sequence, including the leader sequence, was amplified from pGEMT-Stx2 by PCR with oligonucleotides D1 (5′-AGC CCC ATC CAT GAA GAA GAT GTT T-3′), carrying a *Bam*HI restriction site, and R1 as the reverse primer. The resulting fragment of 282 bp was gel extracted (DNA gel extraction kit; Promega Inc.), treated with *Bam*HI and *Eco*RI, and ligated to pCDNA 3.1+ (Invitrogen) already digested with the same enzymes. This plasmid was called pStx2B.

pGEMTStx2 was cut with *Sma*I and *Stu*I (corresponding to bp 305 and 1102, respectively, withdrawing the information for the active site) and religated. The plasmid obtained (pGEMTStx2ΔA) was introduced in JM109 *E. coli.* Sequence analysis of this construction showed the *stx2a* gene deleted, while the whole *stx2b* gene was left intact. The truncated sequence was then amplified by PCR using primers R1 and D2 (5′-AGC GGG ATC C CAT TAT GCG TTG TTAGC-3′) (which carries a site for *Bam*HI). The resulting fragment (of about 1,100 bp) was isolated from 1.5% agarose gel, treated with *Bam*HI and *Eco*RI, and ligated to pCDNA predigested with the same enzymes, giving pStx2ΔA. pGM-CSF plasmid (a gift from E. A. Scodeller, Centro de Virología Animal, Buenos Aires, Argentina) consists of the same pCDNA vector carrying the gene for murine granulocyte-macrophage colony-stimulating factor.

Animals and immunization trials. 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–12-h-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 elsewhere (38b).

Newborns (2 days old) and adults (2 months old) were immunized by i.m. injection in the rear legs on days 0 and 15. Newborn mice received 10 μg and adult mice received 50 μg of each purified plasmid.

Blood samples were obtained by puncture of the retro-orbital plexus every two weeks within the first two months since the first day of vaccination and were obtained monthly afterwards. Serum samples were stored frozen (−20°C) until use in enzyme-linked immunosorbent assays (ELISAs) and neutralization assays.

Stx2ΔA protein production and purification. *E. coli* JM109 transformed with plasmid pGEM-Stx2ΔA was used to express Stx2ΔA 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 then 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 (PBS), and dialyzed with the same buffer for 24 h. Total protein concentration was determined by standard methods. Specific protein was quantified in polyacrylamide gel electrophoresis (PAGE) by comparing with known quantities of ovalbumin. Identity was confirmed by Western blotting with specific monoclonal antibodies (MAbs) against the Stx2B subunit (Biodesign) and against the Stx2A subunit (Toxin Technology, Sarasota, Fla.). Following the development of the Western blots, the lanes were scanned with an LKB Ultroscan XL enhanced Laser densitometer. The Stx2ΔA peaks were integrated using Beckman system Gold software (Beckman Instruments, Fullerton, Calif.).

In vitro evaluation. BHK-21 cells were grown in six-well culture plates (Nalge-Nunc, Inc.) at 37°C in 5% CO_2. Cells were washed twice with serum-free medium and infected with a recombinant vaccinia virus that expresses T7 polymerase (21). Plasmid DNA (2 μg) was mixed in Lipofectin reagent (Invitrogen) following the manufacturer's instructions. After 3 h, the transfection mix (DNA Lipofectin) was washed away, and the cells were incubated for 3 h more in complete medium (24).

For Western blot 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 (29), boiled for 5 min, and seeded in a 12 to 20% gradient polyacrylamide mini-gel.

SDS-PAGE and Western blotting. Sodium dodecyl sulfate (SDS)-PAGE was performed in 12 to 20% polyacrylamide gradient minigels. Gels were either stained with Coomassie brilliant blue or electroblotted to a nitrocellulose membrane.

Immunoblots. Immunoblot membranes were blocked with PBS–2% skim milk for 1 h at room temperature. MAb against the Stx2B subunit and/or MAb against the A subunit was diluted (1:100) in PBS–1% bovine serum albumin (BSA) and incubated overnight with stirring at 4°C. Reactivity was revealed with anti-mouse hypoxanthine phosphoribosyltransferase peroxidase conjugate (Amersham, Pharmacia, N.J.) using diaminobenzidine (Sigma Aldrich) and 0.05% H_2O_2.

ELISA. Nunc-Maxisorp microtiter plates (Becton Dickinson, Oxnard, Calif.) were coated with recombinant Stx2ΔA protein at a concentration of 1 mg/ml in ELISA coating buffer (0.012 M Na_2CO_3, 0.038 M $NaHCO_3$ [pH 9.6]) overnight at 4°C, washed with PBS–0.05% Tween 20, and blocked with PBS–1.5% BSA (150 μl/well). After washing twice with PBS, serial twofold dilutions of sera (in PBS–1% BSA) were added and left overnight at 4°C (50 μl/well). On the next day, the plates were washed three times with PBS–0.05% Tween 20 and twice with PBS. Anti-mouse hypoxanthine phosphoribosyltransferase (Jackson Laboratories Inc., Bar Harbor, Maine) peroxidase conjugate was added and incubated for 1 h at 37°C. Development of a colored reaction involved the addition of *o*-phenylenediamine (0.35 mg/ml) in Phosphate/Citrate buffer and 0.025% H_2O_2. After 15 min at room temperature the absorbance was determined at 490 nm. Absorbance values from negative control sera were calculated for each serum dilution.

ELISAs to evaluate levels of immunoglobulin G1 (IgG1), IgG2a, IgG2b, IgG3, and IgM isotypes in serum were carried out in a similar fashion, except that after the addition of 1:40 diluted sera, the presence of each isotype was determined with anti-isotype rabbit serum (Bio-Rad Inc., Hercules, Calif.) diluted 1:3 in PBS–1% BSA (50 μl/well), followed by anti-rabbit peroxidase conjugate.

Stx2 preparation. Stx2 (Denka Seiken Co. Ltd., Nigata Japan) was kindly provided by Sugiyama Junichi. Purity was analyzed by the supplier, showing only one peak in high-performance liquid chromatography. Stx2 preparation was evaluated for endotoxin contamination by Limulus amebocyte lysate assay as previously described (19) given a contamination less than 40 pg of lipopolysaccharide/μg of protein. This Stx2 was tested for toxicity activity on VERO cells, and the 50% cytotoxic dose (CD_{50}) was calculated as previously described (19). CD_{50} was approximately 0.063 pg.

In vitro neutralizing activity. To determine Stx2-neutralizing antibody titer, equal volumes of experimental serum (dilution 1:35) and serial dilutions of Stx2-CD_{50} (0.01; 0.05; 0.1; 0.5; 1; 1.5, and 2) were preincubated for 1 h at 37°C and 1 h at 4°C. The mixtures were added to each well containing 10^4 VERO cells and incubated for 3 days at 37°C in 5% CO_2. Cells were washed, stained with crystal violet dye, and read on a Microwell system reader (model 230S; Organon, Teknika) with a 550-nm filter. The neutralizing activity was expressed as the quantity of CD_{50} that was neutralized by 1 ml of serum.

In vivo neutralizing activity. The same batch of Stx2 preparation was used throughout the experiments. In a previous manuscript (39) we evaluated the in vivo lethality of Stx2 by intravenous injection (in the retro-orbital plexus) of serial dilutions in pyrogen-free saline. We chose a dose of 15 ng/kg (approximately 300 pg/mouse), which induced a mortality of ~100% between 3 and 4 days after injection. Control and preimmunized mice were injected in order to evaluate protection capacity of plasmid vaccines. In another trial, the same amount of Stx2 was preincubated with immune sera during one hour at 37°C and for another hour at 4°C before intravenous inoculation in naive mice.

Detection of plasmid DNA and mRNA in mouse tissues. Ten months after DNA vaccination, animals were sacrificed and a portion of the left rear quadriceps muscle at the site of immunization, aortic lymph nodes, kidneys, spleen, and liver were removed from each animal for PCR detection of plasmid DNA. All tissue samples were frozen (−70°C) until DNA extraction. Total DNA was isolated from tissue samples by standard techniques (18) and analyzed for the presence of plasmid DNA using primers matching the ampicillin resistance gene and cytomegalovirus (CMV) promoter. PCR products from tissues were separated by agarose gel electrophoresis and identified by ethidium bromide staining, giving a band of about 450 bp. The sensitivity of the assay was 1 pg of specific DNA/μg of total genomic DNA. Total RNA was extracted from tissue samples using TRIZOL reagent (Life Technologies, Grand Island, N.Y.); 2 μg of RNA was used in each reaction to analyze the presence of specific mRNA by detection of Stx2B sequences using a reverse transcription-PCR (RT-PCR) kit (Access-Quick RT-PCR System; Promega).

Statistical analysis. All data correspond to the means ± standard errors of the means of individual mice. Statistical differences were determined using one-way analysis of variance, and a P value of <0.05 was considered significant. Individual groups were compared using the unpaired Student's t test.

RESULTS

In vitro transfection of BHK-21 cells. Two pCDNA plasmid vectors were constructed, one encoding the entire gene for the B subunit of Stx2 (pStx2B), and another containing the first 85 codons of the gene for Stx2 A subunit plus the entire gene for the B subunit, designated pStx2ΔA (Fig. 1A).

In order to ensure that the plasmid DNA vaccine constructs were intact and functional, expression was analyzed in vitro by transient transfection of eukaryotic cells (BHK-21). Proteins expressed by pStx2B and pStx2ΔA had the expected molecular masses of about 7 and 15 kDa, respectively, and were recognized by a specific MAb against Stx2B (Fig. 1B). The 15 kDa protein was also recognized by a specific MAb against Stx2A (data not shown).

Evaluation of the specific humoral response induced by DNA vaccines. In order to assess these plasmids as DNA vaccines, groups of 6 adults and 6 newborns were injected i.m. with 50 or 10 μg of the plasmids, respectively, and revaccinated 15 days later. The plasmids pStx2B and pStx2ΔA were inoculated alone or in combination with pGM-CSF. A control group was inoculated with the pCDNA vector without insert.

In order to evaluate the antibody response, recombinant Stx2ΔA protein was used as the trapping antigen in ELISA assays for all experimental groups because its production was more efficient than Stx2B. Figure 2 shows that adult mice were able to elicit a specific humoral response after immunization with both plasmids pStx2B and pStx2ΔA, which significantly increased after the second dose. Simultaneous injection of pGM-CSF induced a significant increase in the antibody titer only when adult mice were immunized with pStx2B. This enhancement was noteworthy at 30 and 60 days postvaccination (dpv).

Newborn mice immunized with pStx2B or pStx2ΔA mounted a low-titer specific antibody response. Neonates immunized with pStx2ΔA developed a specific and significant antibody response at 30 dpv, but it was not significantly different from

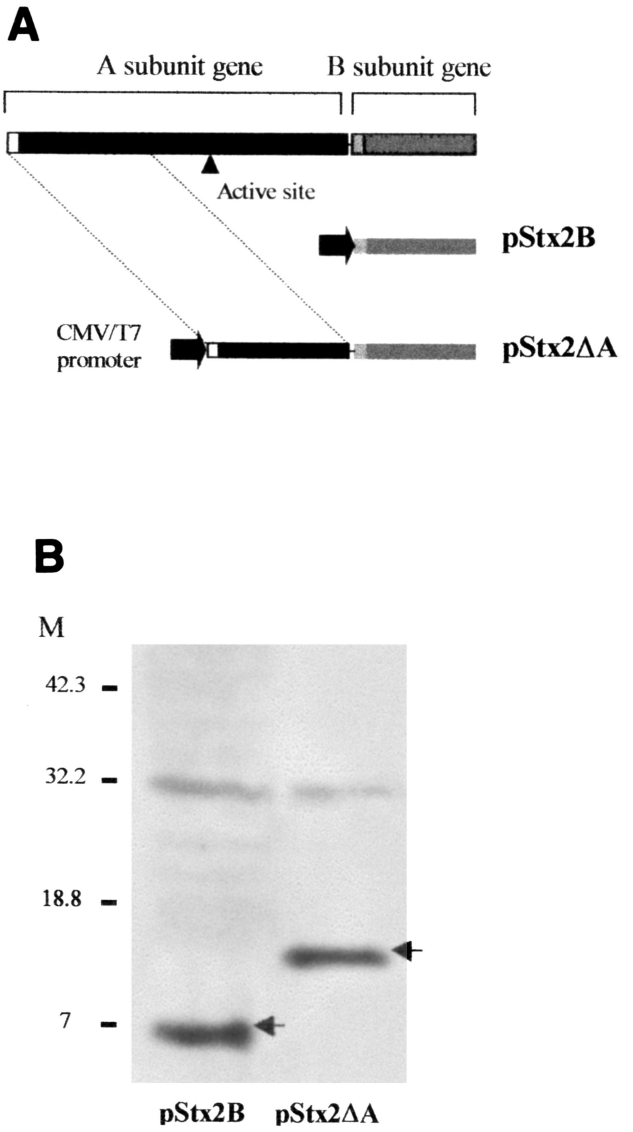

FIG. 1. Development of genetic vaccines. (A) Stick model of the coding sequence for Stx2 and the constructed genes cloned in pCDNA3.1+ expression vector under the CMV and T7 promoter (black arrows). Plasmid pStx2B carries the complete gene for Stx2B subunit. pStx2ΔA comprises the gene for Stx2B and the first 438 bases of Stx2A subunit gene, withdrawing the active site. (B) Expression of Stx2B and Stx2ΔA in eukaryotic cells. BHK-21 monolayer cells were infected with recombinant vaccinia (Vtf7) and transfected with plasmids pStx2B or pStx2ΔA, as detailed in Materials and Methods. Lysates from these cells were resolved by SDS–15% PAGE immunoblot incubated with the MAb against Stx2B. Distance migrated by each prestained calibration standard is indicated by their respective molecular weights (10^3), M lane. Arrows indicate the bands corresponding to proteins expressed from each plasmid.

that observed in control mice at 60 dpv (Fig. 2). However, neonates coinoculated with pGM-CSF showed levels of specific antibodies significantly higher than controls still at 60 dpv, suggesting that pGM-CSF contributed to maintain antibody levels up to 60 dpv. On the other hand, newborns injected with pStx2B mounted a significant antibody response after 60 dpv, and the combination of pGM-CSF plus pStx2B did not induce a significant increase in the antibody response.

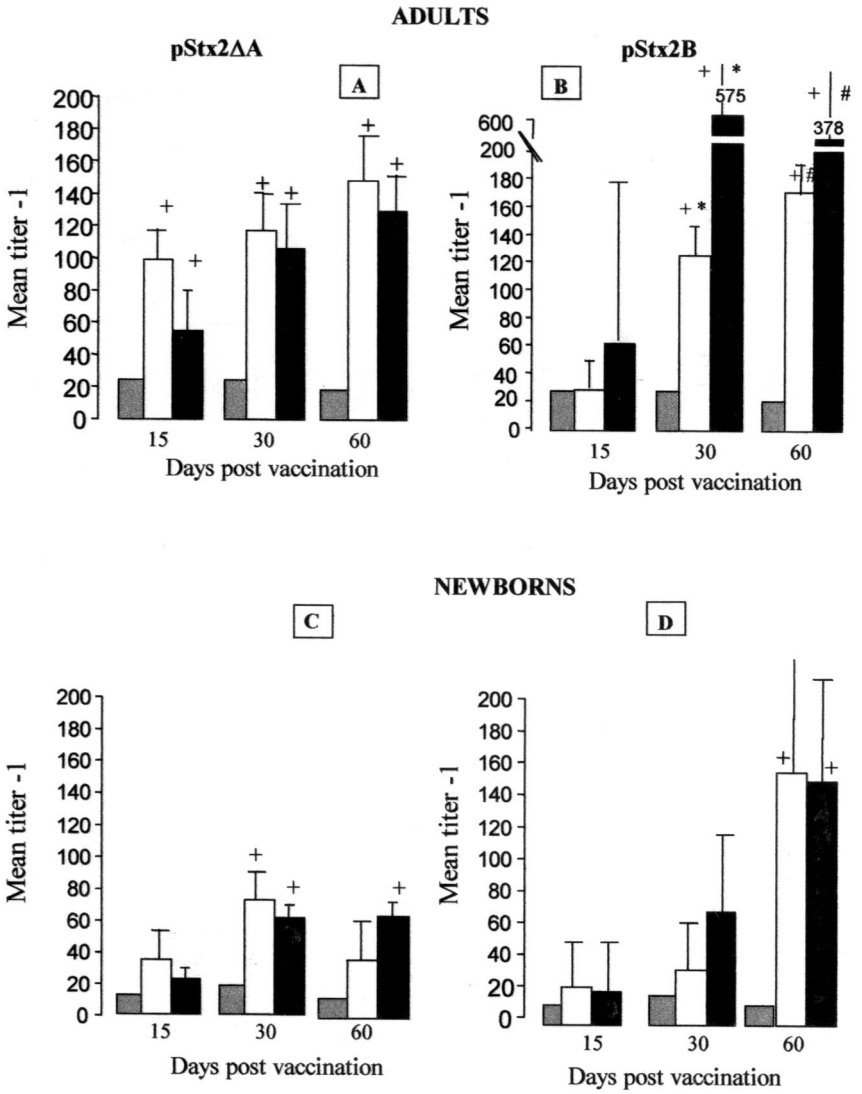

FIG. 2. Specific antibody binding to recombinant Stx2ΔA protein. Serum samples from adult (A and B) and newborn (C and D) mice immunized with 50 or 10 μg, respectively, of the corresponding DNA vaccine were tested by ELISA on recombinant-Stx2ΔA-coated plates. Serum samples from mice vaccinated with pStx2B and pStx2ΔA, alone or together with pGM-CSF, were collected at 15, 30, or 60 days after the initial vaccination dose and tested by serial dilutions for the ability to bind recombinant Stx2ΔA protein captured in microtiter wells. The graph shows mean ELISA titer values ± standard errors of the means (error bars) for each group of at least six animals, calculated as indicated in Materials and Methods. Symbols: ▨, pCDNA; □, genetic vaccine without adjuvant; ■, genetic vaccine plus pGM-CSF; +, differences significant with respect to pCDNA-vaccinated group ($P < 0.05$); # and *, differences significant between the indicated groups ($P < 0.05$). (A and C) pStx2ΔA vaccines; (B and D) pStx2B vaccines.

Serum isotypes in immunized mice. The isotypes of Stx2-specific antibodies present in sera of immunized mice were analyzed using an ELISA. The specific antibodies produced by adult mice inoculated with pStx2ΔA alone or together with pGM-CSF switched from IgM to IgG2a isotype at 30 dpv. In adults vaccinated with pStx2B the antibody conversion from IgM to IgG isotypes was evident at 60 dpv. At that time, the isotypes above control levels were IgG2a, IgG2b, and IgG3, while IgM was also detected. The coinoculation of pStx2B and pGM-CSF accelerated this switch. In fact, sera from mice injected with these plasmids had specific IgG2a levels at the second bleeding (30 dpv), similarly to mice injected with pStx2ΔA.

Newborns vaccinated either with pStx2B or Stx2ΔA produced specific isotypes, but this response was not significantly different from control values at 30 dpv, and both plasmids elicited only a primary response at 60 dpv. When vaccinated with pStx2B or pStx2ΔA plus pGM-CSF, newborn mice switched the response towards IgG2a at 60 dpv.

Noteworthily, anti-Stx2 IgG1 was not detected either in adults or in newborn animals.

In vitro neutralizing activity. Since murine immunization with pStx2ΔA and pStx2B resulted in the induction of antigen-specific antibody responses, it became important to examine whether these sera could also inhibit the holotoxin activity. For this purpose, Stx2 neutralizing activity of immune sera was evaluated in vitro on VERO cells. Individual 1:35 diluted sera obtained at different periods after immunization up to 150 dpv

were incubated with serial dilutions of Stx2 as described in Materials and Methods and then exposed to VERO cell monolayers. Results depicted in Fig. 3 show that immunization with pStx2ΔA caused higher Stx2-neutralizing activity than pStx2B, both in adult and newborn mice. Sera from adults injected with the specific plasmid alone displayed neutralizing titers at 60 dpv, while the coinjection of pGM-CSF induced an enhancement in the blocking activity from 60 up to 150 dpv. Newborn animals inoculated with pStx2ΔA responded with variable neutralizing activity. While some sera showed a high blocking capacity, others did not. The coinjection of pGM-CSF led to 50% of mice with high neutralizing titers as soon as 30 dpv, and there was no significant increase at 60 dpv. On the other hand, sera from adults immunized with pStx2B were not able to block Stx2 activity. However, coinoculation of pStx2B and pGM-CSF caused a high but transient neutralizing activity in serum at 15 dpv. In mice immunized as neonates with pStx2B or pStx2B and pGM-CSF, the antibodies present in the sera did not neutralize Stx2 toxicity. No neutralizing activity was found in sera from control mice injected with pCDNA or pGM-CSF alone (data not shown).

In vivo neutralizing activity. Two different in vivo experiments were performed in order to determine the protective capacity of these vaccines. First, Stx2 was preincubated with a pool of immune sera obtained from 2- to 5-month-vaccinated mice that had high neutralizing titers (titer range = 225 to 2,250), as detailed in Materials and Methods, and inoculated to naive adult mice. Control mice were inoculated with Stx2 preincubated with sera from mice injected with pCDNA. Figure 4A shows that the pool of sera from mice vaccinated with pStx2ΔA plus pGM-CSF (in vitro neutralizing activity titer = 1,000) exerted a significant protection against Stx2 lethality. On the other hand, the pool of sera from mice immunized with pStx2B (in vitro neutralizing activity titer = 350) induced a delay in mortality but was not able to counteract Stx2 lethality.

In a second experiment, when a lethal dose of Stx2 was injected to mice immunized with pStx2ΔA+pGM-CSF at 5 months postvaccination (neutralizing titer range = 450 to 2,250), there was a significant delay in the mortality with respect to the control group, with mean times of death of 234 and 54 h, respectively ($n = 4$; $P < 0.05$). However, differences were not significant for the pStx2B+pGM-CSF vaccinated group (neutralizing titer range = 225 to 450) (data not shown).

Immunized animals were classified according to their serum neutralizing activity on VERO cells and the level of protection achieved in vivo, independently of the plasmid vaccine used. As shown in Fig. 4B, animals that reached 2,250 neutralizing titer in vitro were fully protected. Furthermore, differences in protection against Stx2 toxicity, evaluated as time of death, were significant ($P < 0.05$) when comparing animals that have a neutralizing titer of 45 with those that have 225, 450, and 1,000.

Detection of plasmid DNA and mRNA in mouse tissues. Mouse tissue was examined by PCR and RT-PCR for the persistence of bacterial plasmid or Stx2B specific mRNA, respectively. The plasmid DNA could be detected neither in naive mice nor in mice injected with pCDNA. Some mice injected with pStx2B had plasmid DNA before three months post immunization and only in the site of injection. Adults and newborns injected with pStx2ΔA alone or together with pGM-CSF presented plasmid DNA in the site of injection still 10 months after vaccination ($n = 6$). In addition, all vaccinated adults presented plasmid DNA in aortic lymph nodes and one out of three also in the kidney (data not shown). When tissues from control mice (naive or pCDNA injected mice) were analyzed by RT-PCR no band of the molecular weight corresponding to Stx2B cDNA (approximately 250 bp) was found. On the contrary, all newborn and adult mice injected with pStx2ΔA alone or together with pGM-CSF presented the 250 bp band in RNA isolated from their quadriceps muscles. Liver and spleen of all animals did not present either plasmid DNA or specific mRNA for Stx2B.

DISCUSSION

Systemic administration of anti-Stx2 neutralizing antibodies protects animals from death in experimental HUS (36, 43), emphasizing the importance of the humoral response as a protective mechanism in HUS. The Stx2B has been proposed as a good candidate for immunization protocols. However, the use of this recombinant protein for vaccination trials has been difficult because of its instability and low efficiency system of production (approximately 0.7 mg/liter of bacterial culture), poor antigenicity and lipopolysaccharide contamination in the preparations (8, 35). Therefore, studies on alternative approaches for vaccination are still required. In this context, DNA vaccination has not been previously explored.

Immunization with genes encoding immunogens, rather than with the immunogen itself, has opened new possibilities for vaccine research. Most studies on DNA-based immunization have used pCDNA vectors with the CMV promoter to drive antigen expression (6, 14, 27). Naked DNA immunizations and immunotherapy are now undergoing phase I and phase II studies, encouraging their use for human administration (11, 38).

We constructed two potential DNA vaccines, comprising either the Stx2B subunit gene (pStx2B) or a hybrid gene formed by the complete sequence of the B subunit and the first 85 amino acids of the A subunit of Stx2 (pStx2ΔA). Their immunogenicity was clearly demonstrated by ELISA. Although it was developed using the recombinant Stx2ΔA protein as the antigen, it carries the complete sequence of the B subunit identical to the one found in Stx2B and in the holotoxin. Indeed, both plasmids induced high antibody serum titers as soon as two weeks after the first dose. However, it is interesting to point out that the antibody titer obtained by ELISA did not always correlate with Stx2-neutralizing activity. The pStx2ΔA vaccines induced lower specific antibody levels as measured by ELISA than the pStx2B vaccines, but the maximal neutralizing activity in vitro was observed in adults that had received pStx2ΔA and pStx2ΔA plus pGM-CSF. Several reasons could account for these results: differences in the plasmid DNA and/or in the ability to express the protein. Although we observed the presence of pStx2ΔA in mice tissues later than pStx2B, the complete analysis of this issue needs further investigation. Other possibility would be a higher protein stability of Stx2ΔA than Stx2B. In this regard, it has been suggested that the addition of a short Stx2 A subunit sequence enhances the stability of the B subunit (2). In addition, pStx2ΔA could favor the correct exposure of relevant neutralizing epitopes or

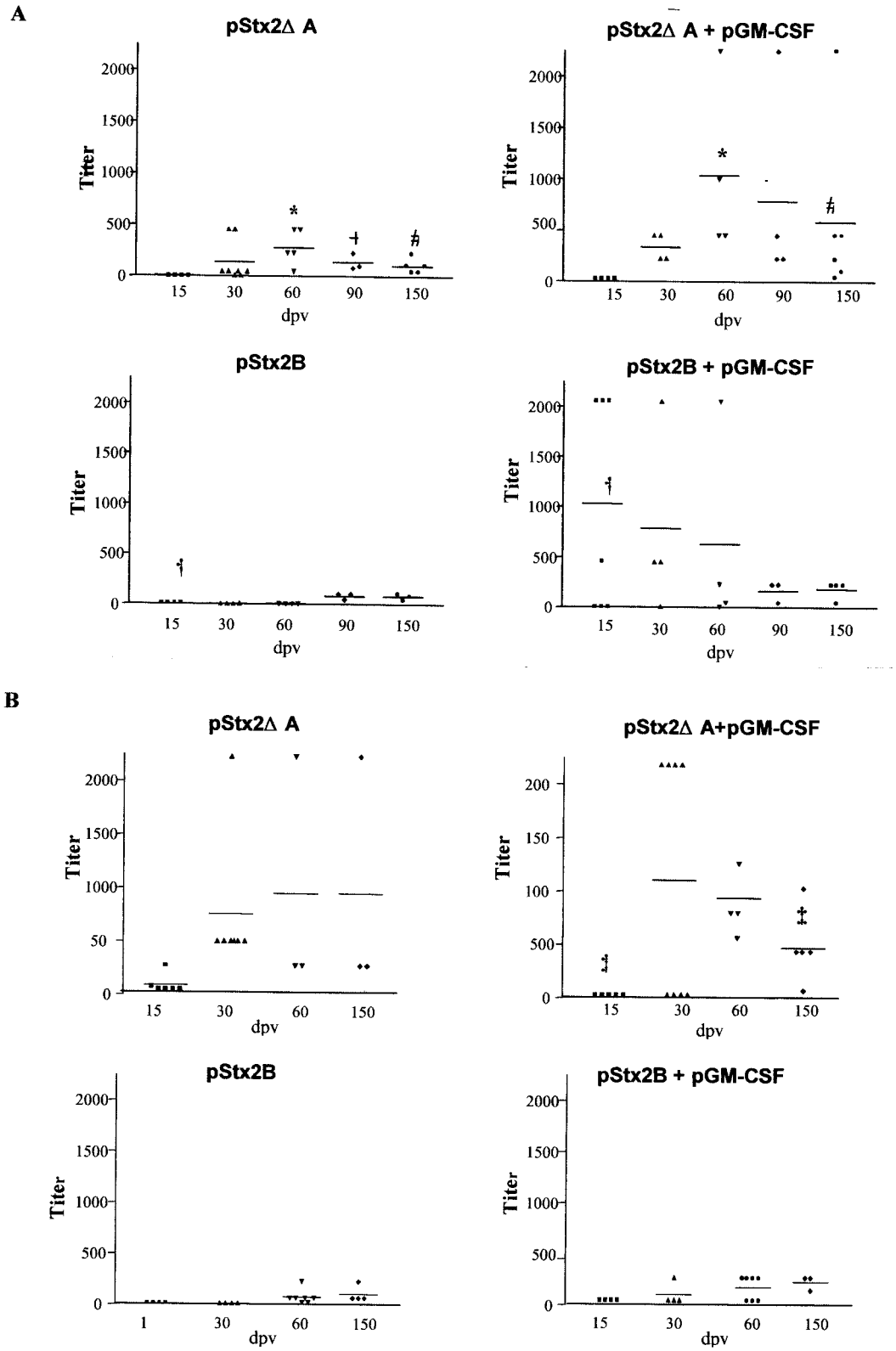

FIG. 3. Serum Stx2-neutralizing activity in VERO assay. Comparative Stx2 neutralization activity by immune sera was evaluated on VERO cells. A dilution (1:35) of each serum was incubated with Stx2 during 60 min at 37°C and another 60 min at 4°C, and toxicity was assayed on VERO cell monolayers, incubated for 72 h at 37°C, as indicated in Materials and Methods. Neutralizing titers were expressed as the quantity of CD_{50} neutralized by 1 ml of serum. The graphs show individual and mean titers of immune sera from adult (A) and newborn mice (B) immunized with the indicated genetic vaccine. Symbols: †, ‡, #, *, +, differences significant between the indicated groups ($P < 0.05$).

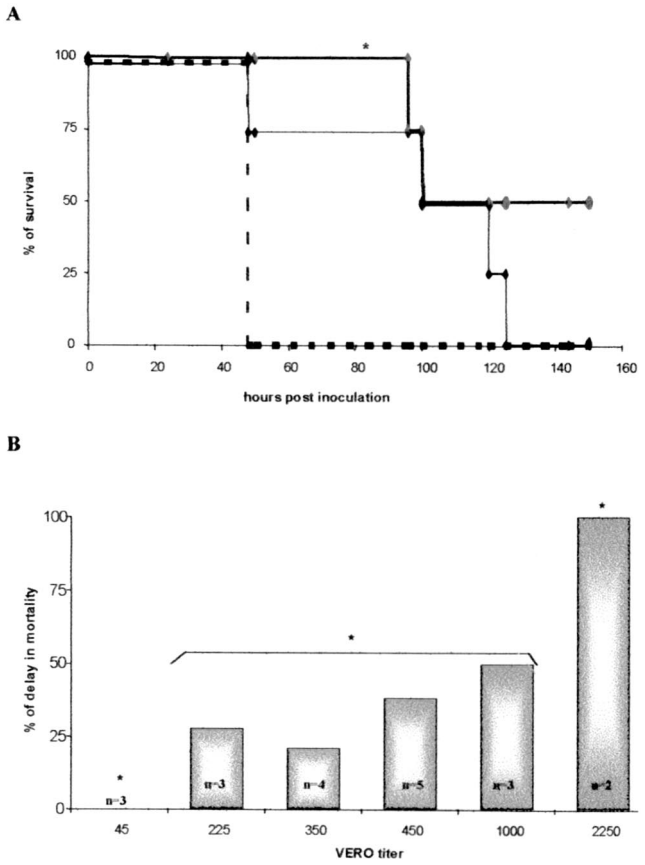

FIG. 4. Protective efficacy of the genetic vaccines. (A) Inoculation of naive mice with Stx2 preincubated with immune sera. A lethal dose of Stx2 was preincubated with immune sera from pStx2ΔA + pGM-CSF (thick line)-, pStx2B + pGM-CSF (thin line)-, or pCDNA (dashed line)-vaccinated mice, before intravenous inoculation to naive adult mice. Lethal effects of Stx2 were evaluated at regular periods of 12 h. The figure represents the percentage of survivors at each time point from a total of four mice per group. This treatment schedule was repeated twice, giving similar results. *, significant difference over control group (inoculated with Stx2+pCDNA immune sera) by Fisher exact test ($P < 0.05$). (B) Relationship between in vitro and in vivo Stx2-neutralizing activity of immune sera. Sera were grouped according to their in vitro Stx2-neutralizing titers on VERO cells, independently of the vaccine used, and survival hours post-Stx2 challenge were recorded for each in vitro neutralizing titer. The graph shows the percentage of death delay expressed as mean hours with respect to the time of death of the pCDNA-vaccinated group. The corresponding sample size is indicated in each column. *, significant difference between the indicated groups ($P < 0.05$).

the induction of antibodies with a higher toxin affinity. Moreover, preliminary results have shown the induction of A subunit antibodies in the sera from pStx2ΔA vaccinated mice (unpublished results).

DNA vaccines mainly favor a Th1-type of T-cell response (30, 33). Th1-response profile is characterized by a preferential production of complement-dependent IgG2a antibody, while Th2 responses produce complement-independent IgG1 antibodies (33). Consistent with previous reports, adult mice under our DNA vaccination protocol displayed a Th1 response for both plasmids. On the contrary, mice immunized as neonates developed an IgM response, independently of the plasmid used. In this regard, it has been reported that neonates present developmental deficiencies in components of the immune response, such as deficiencies in immunoglobulin-12 and gamma interferon production (33) as well as APC immaturity (26) that could explain the absence of an IgG-secondary response.

One frequent approach to increase DNA vaccine efficacy involves the coadministration of plasmids encoding immunostimulatory cytokines that can alter the type and/or magnitude of the immune response (26). In our case, the effect of pGM-CSF was evident in both adult and neonate mice, particularly when it was inoculated together with the pStx2ΔA, either increasing the production of specific antibodies or favoring the switch from IgM towards IgG isotypes, in adults and newborns, respectively.

An important datum presented in this report was that neutralizing levels obtained in vitro closely correlated with those observed in vivo. Moreover, vaccinated animals whose sera have in vitro neutralizing titers of at least 2,250 were fully protected from Stx2 challenge. These results reinforce the concept of the immunoprophylactic potential of inducing a good neutralizing antibody response.

It can be concluded that i.m. immunization with a plasmid encoding the first 85 codons of the gene for Stx2 A subunit together with the entire gene for Stx2 B subunit was both immunogenic and protective in the mouse model of HUS. This is the first report on the development of plasmids inducing a neutralizing humoral immune response against Shiga toxin. This strategy could eventually lead to testing of genetic vaccines against HUS in humans or perhaps to the production of immune sera for therapeutic use during the early phase of this disease.

ACKNOWLEDGMENTS

We are grateful to P. Grigera and E. Scodeller for their generous gift of GM-CSF and pCDNA plasmids and to C. D. Pasqualini and P. Grigera for their advice and critical review of the manuscript.

This work was supported by an AZTIRIA award and an R. Carrillo-A. Oñativia award from the Ministerio de Salud of Argentina.

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Editor: A. D. O'Brien