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# Protection of Mice against Shiga Toxin 2 (Stx2)-Associated Damage by Maternal Immunization with a *Brucella* Lumazine Synthase-Stx2 B Subunit Chimera

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**Hemolytic-uremic syndrome (HUS) is defined as the triad of anemia, thrombocytopenia, and acute kidney injury. Enterohemorrhagic Shiga toxin (Stx)-producing *Escherichia coli* (EHEC), which causes a prodromal hemorrhagic enteritis, remains the most common etiology of the typical or epidemic form of HUS. Because no licensed vaccine or effective therapy is presently available for human use, we recently developed a novel immunogen based on the B subunit of Shiga toxin 2 (Stx2B) and the enzyme lumazine synthase from *Brucella* spp. (BLS) (BLS-Stx2B). The aim of this study was to analyze maternal immunization with BLS-Stx2B as a possible approach for transferring anti-Stx2 protection to the offspring. BALB/c female mice were immunized with BLS-Stx2B before mating. Both dams and pups presented comparable titers of anti-Stx2B antibodies in sera and fecal extracts. Moreover, pups were totally protected against a lethal dose of systemic Stx2 injection up to 2 to 3 months postpartum. In addition, pups were resistant to an oral challenge with an Stx2-producing EHEC strain at weaning and did not develop any symptomatology associated with Stx2 toxicity. Fostering experiments demonstrated that anti-Stx2B neutralizing IgG antibodies were transmitted through breast-feeding. Pups that survived the EHEC infection due to maternally transferred immunity prolonged an active and specific immune response that protected them against a subsequent challenge with intravenous Stx2. Our study shows that maternal immunization with BLS-Stx2B was very effective at promoting the transfer of specific antibodies, and suggests that preexposure of adult females to this immunogen could protect their offspring during the early phase of life.**

Enterohemorrhagic *Escherichia coli* (EHEC) strains are foodborne pathogens. EHEC infections may develop into bloody diarrhea or hemolytic-uremic syndrome (HUS), which usually causes kidney failure or even death (1, 2). Outbreaks and sporadic cases of HUS secondary to infections with EHEC O157 and non-O157 strains are increasing worldwide (3–6), but in Argentina it is an endemic disease, with certain regions presenting incidence rates as high as 55/100,000 HUS cases (7). This epidemiological situation places typical HUS as the leading cause of acute renal failure in children (8, 9).

The essential element of virulence of EHEC is the production of Shiga toxins (Stx) (10). Shiga toxin family members have an $AB_5$ structure (11). The A subunit is the toxin's active component, and the five identical B monomers are the binding subunit that binds the specific receptor glycosphingolipid globotriaosylceramide (Gb3) on the host cell surface. After binding of the B pentamer to Gb3, Stx enters the host cell, where the A subunit acts as a highly specific RNA *N*-glycosidase that inhibits protein synthesis, causing cell death (12, 13). Among the Stx family, Stx type II (Stx2) is more toxic than Stx type I (Stx1) and more related to HUS development (14, 15). Shiga toxins are one of the targets for protection against the major systemic complications of *E. coli* O157:H7 infection. In particular, the B subunit of Stx 2 (Stx2B) has been suggested as a possible antigen because of its nontoxicity and immunoprophylactic potential (16, 17). The enzyme lumazine synthase from *Brucella* spp. (BLS) is a highly immunogenic protein with adjuvant properties that has been proposed as an effective protein carrier for vaccine development (18). It assembles as a remarkably stable dimer of pentamers, with 10 N-terminal sites of linkage that allow the insertion of small protein domains without disturbing its conformation (19). We have recently developed a fusion protein between BLS and Stx2B (BLS-Stx2B) that provides high titers of neutralizing antibodies and protective capacity against intravenous challenge with Stx2 in adult immunized mice (20).

While there is indirect evidence that human vaccination against *E. coli* O157:H7 may be effective in preventing EHEC infections in humans, at present there are no human vaccines or specific therapies against Stx2-associated illness (21). A successful human vaccine should elicit antibodies aimed at preventing EHEC colonization in the intestinal tract and/or neutralizing Stx to prevent the development of the main systemic complications,

**Received** 7 January 2014 **Accepted** 9 January 2014
**Published ahead of print** 13 January 2014
**Editor:** S. R. Blanke
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doi:10.1128/IAI.00027-14

such as HUS. In addition, since infants are the population most susceptible to develop HUS after EHEC infections, it was interesting to analyze whether vaccination of adult females could confer protection to their offspring.

Thus, the aim of this work was to analyze if immunization of mouse dams with BLS-Stx2B could confer protection against Stx2-associated disease in their offspring. We demonstrated that female mice immunized with BLS-Stx2B before pregnancy were able to passively transfer anti-Stx2B antibodies to their pups. This immune response was highly protective, since pups from immunized dams were completely resistant to a lethal dose of intravenous (i.v.) Stx2. In addition, pups at weaning were completely protected against an oral challenge with an Stx2-producing EHEC strain isolated from a human case of HUS (22). Our results suggest that vaccination of females with BLS-Stx2B is a practical approach for the prevention or reduction of Stx-induced pathology during the first phase of life.

## MATERIALS AND METHODS

**Bacterial strain and growth.** The enterohemorrhagic Stx2-producing *Escherichia coli* O157:H7 (EHEC) strain was isolated from a fecal specimen of a child with HUS and was previously characterized by Brando et al. (22). Bacterial cultures were performed as previously described (22). Briefly, strains were cultured overnight at 37°C in tryptic soy broth (TSB) (Difco, Le Point de Claix, France). A 250-μl volume was inoculated into five Erlenmeyer flasks (125 ml) containing 25 ml TSB and incubated at 37°C for 18 h. Cultures were centrifuged, and bacterial pellets were washed twice in phosphate-buffered saline (PBS) and then resuspended in 1 ml of PBS. Aliquots were diluted ($10^2$ to $10^4$), plated onto plate count agar, and incubated overnight (ON) at 37°C. ON cultures reached a final concentration of $1 \times 10^{10}$ to $1.8 \times 10^{10}$ CFU $ml^{-1}$. The strain was maintained at −70°C in TSB supplemented with 20% glycerol.

**Mice.** BALB/c mice were bred in the animal facilities of the Instituto de Medicina Experimental (IMEX), Buenos Aires. The experiments performed here were approved by the IMEX Care Committee in accordance with the principles set forth in the *Guide for the Care and Use of Laboratory Animals* (23). Throughout the studies, the health and behavior of mice were assessed three times a day. Any unnecessary pain, discomfort, or injury to animals was avoided.

**Proteins used in this study. (i) BLS-Stx2B.** BLS-Stx2B was expressed and purified as previously described (20). Briefly, inclusion bodies containing BLS-Stx2B were solubilized by overnight incubation in 8 M urea–50 mM Tris-HCl–5 mM EDTA (pH 8) buffer and dialyzed against 1 M urea–50 mM Tris-HCl–5 mM EDTA (pH 8.5) buffer. The solubilized proteins were purified by anion-exchange chromatography in a Q-Sepharose (GE Healthcare Life Sciences, Pittsburgh, PA) column using a high-pressure liquid chromatography (HPLC) apparatus (model 320; Gilson, Middleton, WI). Elution was performed using a linear gradient between 0 and 1 M NaCl in a 1 M urea, 50 mM Tris/HCl, pH 8.5 buffer. Protein was dialyzed against phosphate-buffered saline (PBS) before to each immunization.

**(ii) rStx2.** The plasmid pGEM-Stx2 for expression of recombinant Stx2 (rStx2) was generated previously (24, 25). The culture of recombinant *E. coli* strain JM109 transformed with pGEM-Stx2 was obtained by overnight incubation in LB broth supplemented with ampicillin. Bacterial cells were centrifuged, and the resultant pellet was resuspended in PBS and lysed by sonication. After centrifugation (14,000 rpm, 20 min, 4°C), the supernatant from JM109/pGEM-Stx2 was precipitated with ammonium sulfate solution (75%). The pellet was resuspended in PBS, dialyzed against the same buffer for 24 h, and stored at −20°C until use. The total protein concentration was determined using standard methods. The Stx2 concentration was determined with a Ridascreen Verotoxin kit (R-Biopharm, Darmstadt, Germany) (20).

**(iii) Stx2B.** Stx2B was expressed and purified as previously described (20). Briefly, Stx2B was purified by affinity chromatography under native conditions with Ni-nitrilotriacetic acid (NTA) resin (Qiagen, Valencia, CA), following the manufacturer's instructions. The purity of the preparation was determined on SDS-15% (wt/vol) polyacrylamide gels.

**Dam immunization protocol.** Adult BALB/c female mice were immunized with 3 doses of BLS-Stx2B with aluminum hydroxide (subcutaneously [s.c.]) on days 0, 15, and 30. The dose of BLS-Stx2B was equivalent to 20 μg of Stx2B, as previously described (20). Ten days after the last immunization, females were mated with nonimmunized male BALB/c mice. For fostering experiments, pups were interchanged at day 1 postpartum (so that half the pups remained with their dam and half were fostered onto another dam) and left to suckle until weaning.

***In vitro* and *in vivo* neutralizing activities.** Stx2-neutralizing activity in sera was determined as previously described (20). Briefly, 1 50% cytotoxic dose ($CD_{50}$) (for Vero cells) of rStx2 (670 pg of Stx2) and experimental serum samples were preincubated for 1 h at 37°C followed by 1 h at 4°C. The mixtures were overlaid to each well containing $10^4$ Vero cells and incubated for 48 h at 37°C in 5% $CO_2$. Cells were washed with PBS, stained with crystal violet dye, and read on a microtiter plate reader (Asys UVM340; Biochrom Ltd., Cambridge, England) with a 570-nm filter. The percentage of cell survival, which is a measurement of the toxin neutralization, was calculated by the formula $[(OD_{\text{toxin + antibody}} - OD_{\text{toxin only}})/(OD_{\text{no toxin}} - OD_{\text{toxin only}})] \times 100$, where OD is optical density.

For the *in vivo* assay, mice were challenged by i.v. inoculation with 1 100% lethal dose ($LD_{100}$) of Stx2 (2.2 ng/mouse) at 2 months postpartum (m.p.p.) or 3.5 m.p.p.

**Oral infection of weaned mice with EHEC.** Pups born from nonimmunized or immunized dams were orally challenged with Stx2-producing EHEC at weaning (17 to 19 days of age), as previously described (22). Briefly, after 8 h of starvation, animals were intragastrically (i.g.) inoculated via a stainless steel cannula (model 7.7.1; 0.38 mm, 22 gauge) (Harvard Apparatus, Holliston, MA) with 0.1 ml of the bacterial suspension at the $LD_{100}$ ($4 \times 10^{11}$ CFU/kg) or $LD_{50}$ ($6 \times 10^9$ CFU/kg). Food and water were provided to mice *ad libitum* 4 h after the ingestion of the bacterial suspension.

**Bacterial shedding.** Rectal swabs were taken at 48 and 72 h after infection to determine the excretion of *E. coli* O157:H7 bacteria. These samples were cultured onto sorbitol-MacConkey agar (SMAC) (Difco) at 37°C for 18 h. The sorbitol-nonfermenting colonies in confluent growth zones were screened for the $stx_1$, $stx_2$, and *rfbo157* genes by a multiplex PCR using the primers described by Leotta et al. (26), Ziebell et al. (27) and Paton and Paton (28), respectively. The reference *E. coli* strains EDL933 O157:H7 ($stx_1$ $stx_2$) and ATCC 25922 were used as positive and negative controls of gene expression, respectively.

**Hematological and histological studies.** Blood samples were obtained at 48 and 72 h after bacterial feeding for laboratory analyses that included total and differential blood cell counts in a Neubauer chamber and blood urea nitrogen determination. Kidneys and intestinal samples, in which stool was removed, were excised from euthanized mice at 72 h for histological evaluation. Each sample was placed in 5 ml of fixing solution containing formol-PBS (10%) and processed routinely. Sections of paraffin-embedded tissue were stained with hematoxylin and eosin and examined by light microscopy.

Biochemical determinations of urea in mouse plasma were performed in an CCX spectrum autoanalyzer (Abbot Diagnostics Systems, Buenos Aires, Argentina) following standardized instructions.

**Specific antibody determination in sera and fecal extracts (FE). (i) Serum Stx2B-specific IgG.** Stx2B-specific IgG in serum samples was analyzed by enzyme-linked immunosorbent assay (ELISA) as previously described (20). Briefly, 96-well MaxiSorp plates (Greiner Bio-One, Frickenhausen, Germany) were coated with 0.5 μg of purified Stx2B and blocked with 0.4% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO) in PBS-0.05% Tween (PBS-T 0.05%) for 2 h at 37°C. The plates were then incubated for 2 h at 37°C with serially diluted mouse sera. After washing,

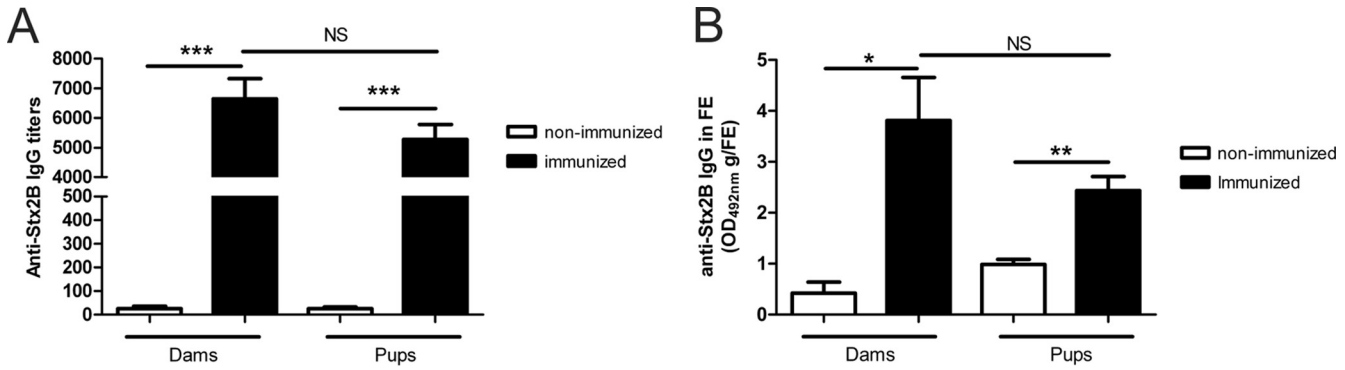


**FIG 1** Anti-Stx2B antibody response in dams and their offspring. (A) Determination of anti-Stx2B IgG in sera from nonimmunized or BLS-Stx2B-immunized dams or their offspring. Dams were bled 10 days after the last immunization, and their pups were bled at weaning. Anti-Stx2B IgG titers in sera were determined by ELISA as detailed in Materials and Methods. Each bar represents the mean ± SEM for 6 to 14 mice/group. ***, $P < 0.001$. (B) Determination of anti-Stx2B IgG in fecal extracts (FE) from nonimmunized or BLS-Stx2B-immunized dams or their offspring. Fecal anti-Stx2B IgG was determined as detailed in Materials and Methods at the same time points as for panel A. Each bar represents the mean ± SEM for 6 mice/group. *, $P < 0.05$; **, $P < 0.005$.

the plates were incubated with peroxidase-conjugated goat anti-mouse IgG (Zymed, Invitrogen, Carlsbad, CA, USA) diluted (1:3,000) in PBS-T 0.05% for 1.5 h at 37°C. Plates were washed, and the reaction was developed with 2 mg/ml *o*-phenylenediamine (Sigma-Aldrich) and 0.3% $H_2O_2$ in citrate-phosphate buffer. The reaction was stopped with 2 M $H_2SO_4$, and absorbance at 492 nm was measured on an Asys UVM340 (microtiter plate reader Biochrom Ltd.). Results were expressed as endpoint titers, calculated as the reciprocal values of the last dilution with an optical density (OD) higher than that of the preimmune serum samples ± 2 standard deviations (SD).

**(ii) Specific antibodies in fecal extracts.** Stools were collected, weighed, and diluted to 1 g/ml with PBS-1 mM phenylmethylsulfonyl fluoride (PMSF). After vigorous homogenization with vortexing, feces were incubated for 1 h on ice and centrifuged (4,000 rpm for 30 min at 4°C). Supernatants were stored at −80°C until IgA/IgG determination by ELISA.

Determination of EHEC-specific IgA/IgG levels in fecal extracts was performed as previously described (29, 30). Intact formalin-killed EHEC cells were prepared as follows. Bacteria were grown ON in TSB, washed twice with PBS by centrifugation, suspended in PBS containing 0.5% neutralized formalin, stored at room temperature for 3 days, and then washed three times with PBS to remove free Stx. Then, 96-well MaxiSorp plates were coated ON at 4°C with the whole-cell suspension of EHEC diluted to an $OD_{600}$ of 0.05 with 15 mM carbonate–25 mM bicarbonate (pH 9.6). Wells were blocked with PBS-0.01% Tween supplemented with 1% BSA at 37°C for 1 h. Plates were then incubated with sequentially diluted fecal samples (1/2 dilution), washed, and incubated with 1/3,000 peroxidase-conjugated goat anti-mouse IgA (Millipore, Billerica, MA) or 1/3,000 peroxidase-conjugated goat anti-mouse IgG. The reaction was developed as described above. Results were expressed as $OD_{492}$ units per gram of feces.

For determination of Stx2B-specific IgA/IgG in fecal extracts, 96-well MaxiSorp plates were coated with 0.5 μg of purified Stx2B, and the assay was carried out as described for the EHEC-specific ELISA.

**Statistical analysis.** Data are presented as the mean ± standard error of the mean (SEM) for each group of mice. Statistical differences were determined using one-way multiple-comparison analysis of variance (ANOVA) with the Newman-Keuls test, and a *P* value of <0.05 was considered significant. Comparisons between two groups were performed with the Student *t* test. Frequency data were analyzed for significance using Fisher's exact test. The log rank test was used to compare survival curves. *P* values of <0.05 were considered to be significant.

## RESULTS

**Antibody response against Stx2B in dams and offspring.** BALB/c female mice were immunized with BLS-Stx2B in aluminum hydroxide adjuvant subcutaneously (s.c.) and were mated 10 days after the last dose. Immunized mice developed high specific antibody titers, similar to those previously reported (Fig. 1A) (20).

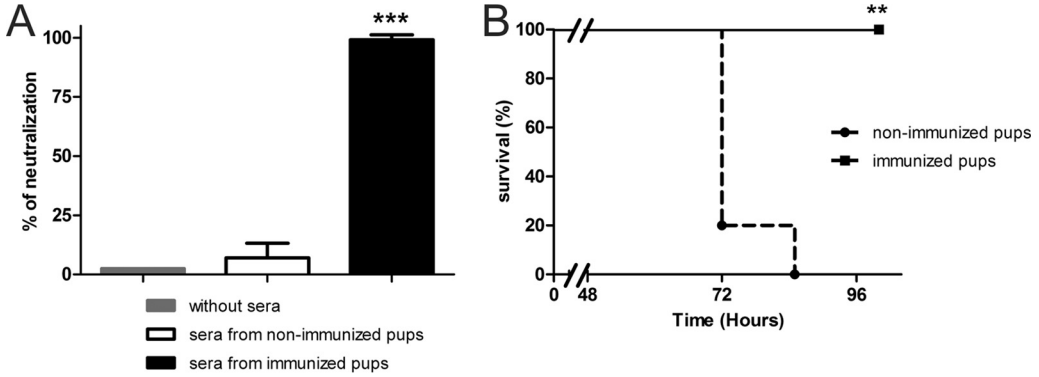


**FIG 2** Anti-Stx2 neutralizing activity in offspring. (A) Neutralizing capacity against rStx2 in sera from pups. Sera (dilution, 1/25) from pups from immunized or nonimmunized dams (at weaning) were incubated *in vitro* with 1 $CD_{50}$ of rStx2. Vero cytotoxicity was assayed as detailed in Materials and Methods. Each bar represents the mean ± SEM for 21 pups (from 3 immunized dams) or 11 pups (from 3 nonimmunized dams). ***, $P < 0.0001$. (B) Protection of pups against a lethal challenge with rStx2. Pups from 3 immunized or nonimmunized dams (4 or 5 pups/group) were challenged at 2 months postpartum (m.p.p.) with 1 $LD_{100}$ of rStx2 (i.v.). **, $P < 0.001$.

The presence of specific antibodies in feces was also tested at 10 days after the last immunization dose. Figure 1B shows that immunized dams also presented specific IgG against Stx2B in fecal extracts (FE). Specific IgA was not detected (data not shown).

In addition, pups from immunized and nonimmunized dams were analyzed at weaning. Figure 1A and B show that pups born from immunized dams presented anti-Stx2B specific IgG antibodies in both serum and FE, at levels similar to those in their mothers, while both samples, serum and FE, obtained from pups from nonimmunized mice were negative for Stx2 antibodies.

**Neutralizing capacity against rStx2.** In order to confirm that maternally transferred antibodies were biologically relevant, the Stx2-neutralizing capacity of offspring sera (at weaning) was tested *in vitro* by the Vero cell cytotoxic assay. Sera from pups born from BLS-Stx2B-immunized dams showed neutralizing activity ($P < 0.001$), while sera from those born from nonimmunized dams did not (Fig. 2A).

To further analyze the protective capacity and the durability of maternally transferred antibodies, we challenged pups with 1 $LD_{100}$ of rStx2 at 2 months postpartum (m.p.p.). Figure 2B shows that 100% of pups born from BLS-Stx2B-immunized dams survived the rStx2 lethal challenge, while 0% of pups from nonimmunized dams did.

**Vertical transfer of immunity.** In order to analyze the main route of antibody transmission, pups were fostered between immune and nonimmune dams in a variation of the experimental protocol and assayed for serum-specific anti-Stx2B antibodies and protection against challenge with rStx2 at 2 m.p.p. For this study, two female mice were immunized and mated. Each resulting litter was divided at day 1 postpartum so that half the pups remained with the immune dam and half were fostered onto a nonimmune dam matched for parturition date. Pups from each corresponding nonimmune dam were reciprocally fostered in the same manner onto the immune dams so that each of the four dams (two immune and nonimmune each) suckled a mixture of biological and adopted offspring. As shown in Fig. 3A, similar levels of specific antibodies were detected in all pups suckled by immunized dams (born either from them or from nonimmunized dams). In contrast, pups suckled by nonimmunized dams (born either from them or from immunized dams) did not show significant levels of anti-Stx2B antibodies. The Stx2-neutralizing capacity of offspring sera (at 2 m.p.p.) was tested *in vitro* by the Vero cell cytotoxic assay. Although a lower *in vitro* neutralizing capacity was observed in sera from pups from immunized dams at 2 m.p.p. compared to at weaning (Fig. 2A), no statistical difference was observed between pups suckled by immunized dams born either from them or from nonimmunized dams (Fig. 3B). In addition, Fig. 3C shows that all pups suckled by immunized dams were protected against an i.v. rStx2 lethal challenge. In contrast, none of the pups suckled by nonimmunized dams survived the rStx2 challenge. Altogether these results suggest that the specific IgG antibodies transmitted during breast-feeding are necessary and sufficient for protection of mice against systemically delivered Stx2, demonstrating that vertical transmission of immunity by this route was very efficient.

**Intragastric EHEC challenge of offspring at weaning.** As a more physiologic indicator of the protective anti-Stx2 immune response, pups were orally challenged with an inoculum of an Stx2-producing EHEC strain (isolated from a HUS patient) at weaning (22). Pups from nonimmunized dams died prior to 96 h post-EHEC challenge (Fig. 4A). It is worth noting that all pups

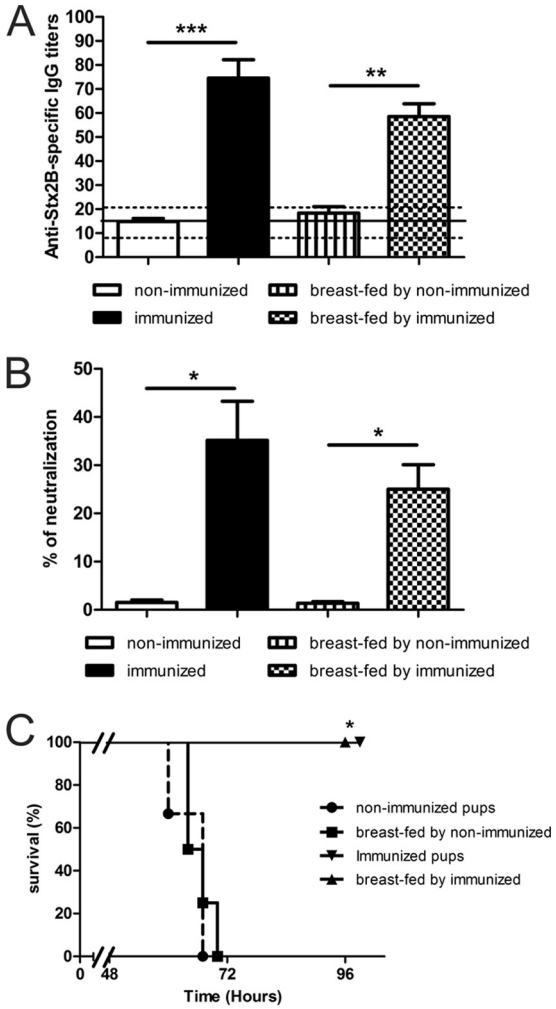


**FIG 3** Vertical transfer of immunity. Two female mice were immunized and mated. Each resulting litter was divided at day 1 postpartum so that half the pups remained with the immune dam (black) and half were fostered onto a nonimmune dam matched for parturition date (vertical lines). Pups from each corresponding nonimmune dam (white) were reciprocally fostered in the same manner onto the immune dam (squares). Pups were bled at 2 months postpartum (m.p.p.), and sera were analyzed. (A) Anti-Stx2B IgG antibodies in sera. Specific anti-Stx2B IgG titers in sera were determined by ELISA as detailed in Materials and Methods. Each bar represents the mean ± SEM for 6 pups/group. Solid and dashed horizontal lines represent the mean ± 2 SD for negative serum samples. **, $P < 0.005$; ***, $P < 0.001$. (B) Neutralizing capacity against rStx2 in sera. Sera from the same experimental groups (dilution, 1/25) were incubated *in vitro* with 1 $CD_{50}$ of rStx2. Vero cell cytotoxicity was assayed as detailed in Materials and Methods. Each bar represents the mean ± SEM for 3 or 4 pups/group. *, $P < 0.05$. (C) Survival rates in response to rStx2 challenge. Pups from the same experimental groups (3 or 4 mice/group) were challenged with 1 $LD_{100}$ of i.v. rStx2 at 2 months postpartum (m.p.p.). *, $P < 0.05$ relative to pups from nonimmunized dams or breast-fed by nonimmunized dams.

delivered from BLS-Stx2B-immunized dams survived the challenge. In agreement with Stx2-associated renal toxicity, only pups from nonimmunized dams had significantly increased plasma urea levels (Fig. 4B). In addition, and as has been previously reported for the weaning mouse model of HUS (22), pups from nonimmunized dams developed systemic disease signs, including a significant decrease in total white cell count simultaneously with

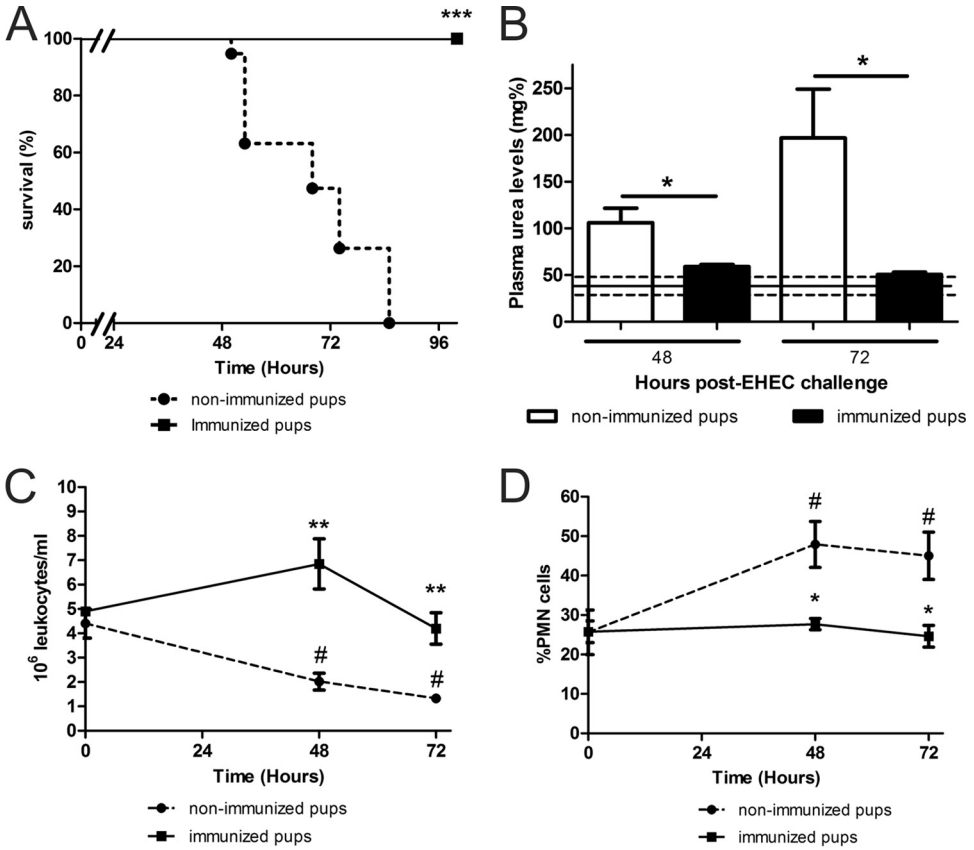


**FIG 4** Intragastric EHEC challenge of offspring at weaning. (A) Survival rates in response to a lethal i.g. challenge with EHEC. Fourteen to 19 pups from 3 dams/group were i.g. inoculated with 1 $LD_{100}$ of Stx2-producing EHEC at weaning. ***, $P < 0.0001$ versus pups from nonimmunized dams. (B) Renal Stx2-induced toxicity. Plasma urea levels at 48 and 72 h post-EHEC challenge were measured as a biochemical parameter of renal damage. Each bar represents the mean ± SEM for 10 to 12 mice/group. Solid and dashed horizontal lines represent the mean ± 2 SD of normal plasma urea values. *, $P < 0.05$. (C and D) Systemic signs of Stx2-associated toxicity. Mice were bled at 48 and 72 h post-EHEC challenge, and total and differential counts of leukocytes were assayed. Each time point represents the mean ± SEM for 3 to 6 mice/group. (C) Absolute numbers of total leukocytes. (D) Relative numbers of polymorphonuclear (PMN) cells. *, $P < 0.05$; **, $P < 0.005$ (relative to pups from nonimmunized dams at the same time point). #, significantly different from nonimmunized pups previous to challenge ($P < 0.05$).

a significant increase in the percentage of circulating neutrophils (Fig. 4C and D). Renal and intestinal histopathological studies confirmed the damage in the main tissues associated with Stx2 toxicity in mice (Fig. 5). Pups from nonimmunized dams showed renal alterations, including glomeruli with mesangial hypercellularity and lack of Bowman space and altered tubular epithelia. In addition, pups from nonimmunized dams presented more severe damage in the colon than pups from immunized dams, as evidenced by the higher score of mucosa thinning, depletion of goblet cells, and both mucosa and submucosa cell infiltration (Table 1).

Altogether these data confirm that pups from nonimmunized dams died as consequence of Stx2-associated toxicity secondary to EHEC ingestion. In contrast, pups from immunized dams were protected from death, disease signs, and tissue pathology.

**Bacterial shedding.** The fecal bacterial shedding was observed at days 2 and 3 post-EHEC challenge. Bacteria were recovered from stool samples and analyzed by PCR for the $stx_2$ and *rfbo157* genes. PCR amplification using primers derived from the $stx_2$ and *eae* genes of recovered *E. coli* O157 organisms yielded PCR amplicons identical to those seen in the control strain, indicating that these organisms corresponded to the challenged EHEC O157 strains. Fifty percent (3 out of 6 pups) and 69% (9 out of 13 pups) of pups born from 3 nonimmunized dams shed bacteria in stool samples at 48 and 72 h postinoculation, respectively. In sharp contrast, 0% (0 out of 6 pups) and 7% (1 out of 14 pups) of pups born from 3 immunized dams shed bacteria at the same time points, indicating that colonization was significantly reduced in this last group (at 72 h, 69% nonimmunized pups versus 7% immunized pups; $P < 0.005$). These results suggest that passively transferred Stx2-protective antibodies also contributed to reduce EHEC colonization.

**Antibody-specific response after EHEC challenge.** Since a fundamental role of maternal antibodies is to attenuate infections to allow physiological vaccination of the offspring, we studied the specific antibody response after EHEC infection in both groups of mice, those born from immunized or nonimmunized dams, at 2 m.p.p. For this experiment, 1 $LD_{50}$ of EHEC was used to achieve survival in 50% of the weaning nonimmunized pups (see Materials and Methods). We assayed anti-EHEC IgA/IgG in FE. Figure 6A shows that one oral EHEC challenge was necessary and sufficient to trigger specific anti-total EHEC IgA antibodies in intestinal mucosa from both groups of mice. Anti-EHEC IgG was not detected in any experimental group (data not shown).

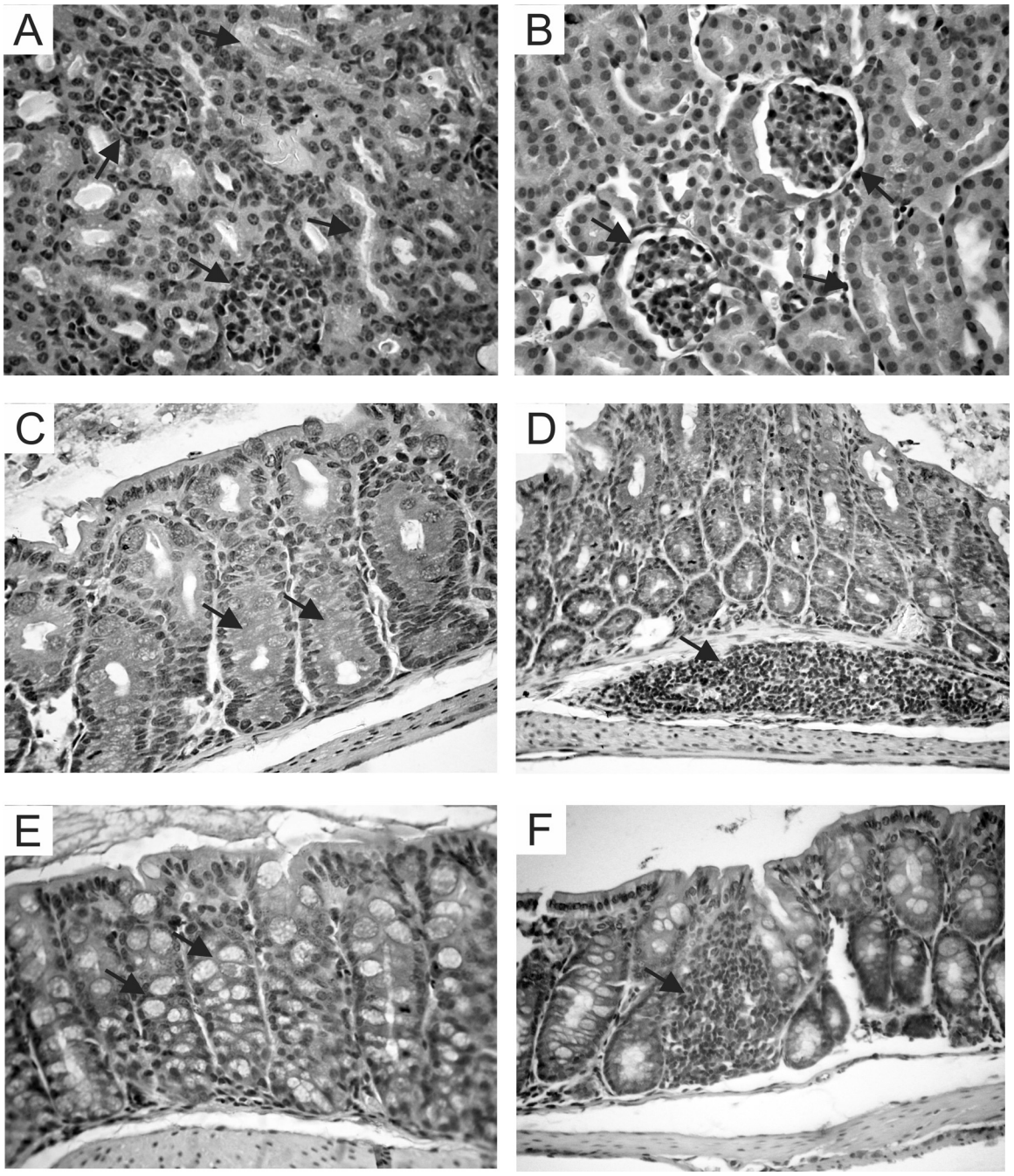


**FIG 5** Histological studies of kidney and colon. Pups from BLS-Stx2B-immunized or from nonimmunized dams were challenged i.g. with 1 $LD_{100}$ of Stx2-producing EHEC. Seventy-two hours after challenge, 3 animals of each experimental group were euthanized, and tissues were fixed and stained with hematoxylin and eosin. Images were acquired using a C. Zeiss III photomicroscope (Oberkochen, Germany). (A and B) Histological study of kidneys. Original magnification, ×400. (A) Representative image of kidney from pups from nonimmunized dams, showing glomeruli with mesangial hypercellularity and lack of Bowman space and proximal and distal tubular epithelia with scant pale cytoplasm. (B) Representative image of kidney from pups from immunized dams, showing slightly retracted glomeruli and tubular epithelia preserved. (C to F) Histological study of colon. (C and D) Representative image of colon from pups from nonimmunized dams, showing absence of goblet cells (C) and presence of submucosal lymphocyte infiltrate (D). (E and F) Representative image of colon from pups from immunized dams, showing normal number of goblet cells (E) and intramucosal lymphocyte infiltrate (F). Original magnifications, ×250 (C and E) and ×100 (D and F).

To evaluate the effect of EHEC challenge on the long-lasting anti-Stx2 humoral immune response, passively immunized mice, challenged or not with EHEC, were bled at 3.5 m.p.p. and anti-Stx2B antibodies in sera were assayed by ELISA. As shown in Fig. 6B only 25% of pups from immunized dams not EHEC challenged presented circulating anti-Stx2B antibodies. In contrast, approximately 70% of passively immunized mice that had received a previous EHEC challenge showed anti-Stx2B antibodies

**TABLE 1** Histological score of colon alterations[a]

| Alteration | Score[b] in pups from: | |
|---|---|---|
| | Nonimmunized dams | BLS-Stx2B-immunized dams |
| Mucosa thinning | ++ | + |
| Goblet cells depletion | ++/+++ | + |
| Mucosal inflammatory cell infiltration | ++ | + |
| Submucosal inflammatory cell infiltration | +++ | + |

[a] Pups from BLS-Stx2B-immunized or nonimmunized dams were challenged i.g. with 1 $LD_{100}$ of Stx2-producing EHEC at weaning. Seventy-two hours after challenge, 3 animals of each experimental group were euthanized, and tissues were fixed and stained with hematoxylin and eosin.

[b] Histological scoring of colon alterations was performed in tissues from 3 mice/group, analyzing 20 high-power fields for each sample. Score: 0, none; +, mild; ++, moderate; +++, intense.

in sera. Next, passively immunized mice, challenged or not with EHEC, were evaluated for protection against injection with 1 $LD_{100}$ of rStx2 at 3.5 m.p.p. At this time point, protection against rStx2 in pups from immunized dams decreased from 100% (observed at 2 m.p.p.) (Fig. 2B) to 28.6% (Fig. 6C). However, those pups from immunized dams receiving a previous EHEC challenge were significantly more resistant to rStx2 toxicity than the pups from immunized dams that did not receive the EHEC challenge (71.4% versus 28.6%) (Fig. 6C). These survival rates are in close correlation with the presence of anti-Stx2B antibodies in sera (Fig. 6B). None of the control pups from nonimmunized dams (that did not receive a previous EHEC challenge) survived 1 $LD_{100}$ of rStx2 (Fig. 6C). This result shows that the EHEC challenge contributed to sustain a specific and protective immune response against Stx2.

## DISCUSSION

In the present study, we found IgG antibodies reactive to Stx2B in sera from female mice immunized with BLS-Stx2B, as was previously reported for adult male BALB/c mice (20). The levels of anti-Stx2B IgG antibodies in sera and feces from pups at weaning were similar to those found in the sera and feces from dams, demonstrating an effective vertical transfer of specific antibodies from dams to their offspring. Most importantly, Stx2-neutralizing antibodies were able to confer protection against oral EHEC challenge at weaning or against rStx2 intravenous injection up to 2 m.p.p. Thus, maternal transfer of neutralizing anti-Stx2B IgG conferred protection against infection during the first months of life.

Since maternal antibodies can be transferred via the placenta and by lactation in mammals (31), we analyzed the route of passive transfer of anti-Stx2B antibodies from immunized dams to offspring. Our results indicate that transmission of immunity throughout lactation was very efficient: all pups nursed by immunized dams, born either from them or from nonimmunized ones, were protected against Stx2 intoxication, indicating that specific antibodies transferred by breast-feeding were enough to confer protection to offspring.

Interestingly, the titer of specific anti-Stx2B antibodies gradually declined over time (from 5,000 to 70 at weaning and 2 m.p.p., respectively), which was reflected in a decrease in the *in vitro* serum Stx2-neutralizing capacity from 100% to 35%, respectively. However, low antibody titers are still able to completely protect mice against systemic inoculation of Stx2, probably due to the low concentration of Stx2 used (≤2 ng/mouse) and the high affinity of the antibodies. However, it is reasonable to think that there would be a threshold of antibody concentration, below which they are not able to neutralize enough toxin to avoid death in mice.

Although we found that Stx2 protection was dependent on lactation, we cannot rule out that antibodies transferred through

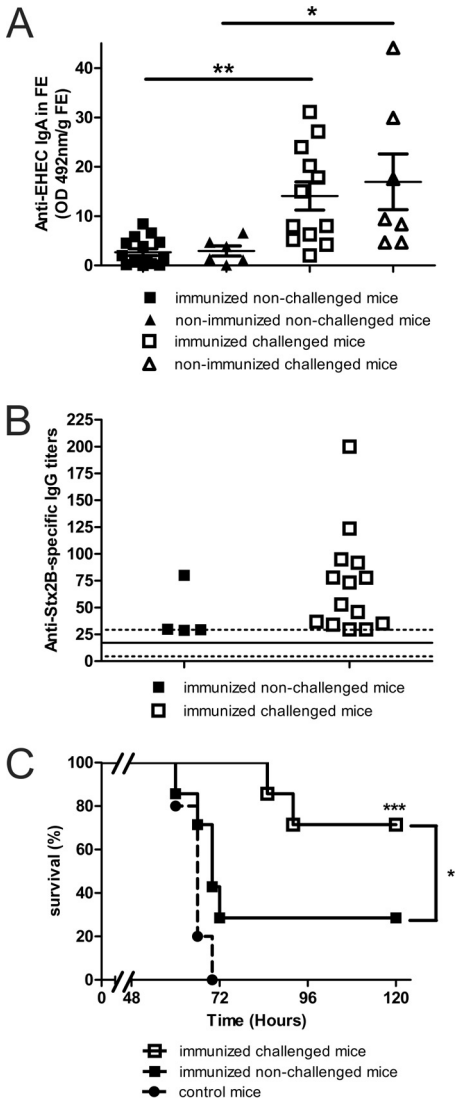


**FIG 6** Immunological response after EHEC challenge. (A) Anti-EHEC antibodies in fecal extracts (FE) at 2 months postpartum (m.p.p.). FE were obtained from immunized or nonimmunized pups (from 3 dams each group) that were not challenged with EHEC (black squares and triangles, respectively) or that survived a previous EHEC challenge (white squares and triangles, respectively). Fecal anti-EHEC IgA was determined as detailed in Materials and Methods. *, $P < 0.05$; **, $P < 0.005$. (B) Anti-Stx2B antibodies in sera. Immunized pups that were not challenged with EHEC (black squares) or that survived a previous EHEC challenge (white squares) were bled at 3.5 months postpartum (m.p.p). Anti-Stx2B IgG titers in sera were determined by ELISA as detailed in Materials and Methods. Solid and dashed horizontal lines represent the mean ± 2 SD for negative serum samples. (C) Survival rates in response to 1 $LD_{100}$ of rStx2. Surviving mice (5 to 7 mice/group) were challenged with 1 $LD_{100}$ of i.v. rStx2 at 3.5 m.p.p. *, $P < 0.05$; ***, $P < 0.0001$ (relative to control mice).

the placenta before birth could be effective during the first days postpartum. Without further IgG transfer via lactation, antibodies transferred during pregnancy could be lost at 2 m.p.p., when we assayed protection against intravenous Stx2 intoxication. Previous reports have shown that the titer of antibodies transferred through the transplacental route declines from day 9 after birth (32), but lactation prolongs the period of passive protection to 5 to 10 weeks in rodents (33, 34) and to approximately 9 months in humans (35). In humans, passive transfer of IgG antibodies is an important mechanism that provides protection to the newborn while the humoral response is inefficient, contributes to the maturation of the immune system, and influences the gut flora (36). In this regard, the early acquisition of maternal antibodies reactive to EHEC virulence factors by children may be associated with the reduced susceptibility to this infection observed during the first year of life in areas of endemicity (37).

As a more relevant mouse model of Stx2 intoxication, intragastric EHEC administration simulates the route of the natural infection of *E. coli* O157:H7 (38). Previous reports by Brando et al. have shown that weaned mice are a suitable model, since obvious pathological changes in the intestines, kidneys, and inflammatory response were observed, including death in a relative high percentage of the mice (22). All these signs, described in this model as indicators of Stx2 cytotoxicity, were dampened in pups born from BLS-Stx2B-immunized dams, demonstrating the efficacy of anti-Stx2B antibodies against Stx2-associated complications after *E. coli* O157:H7 infection. Moreover, pups from immunized dams had a lower rate of EHEC colonization, assayed as a lower percentage of bacterial shedding in feces, in coincidence with lower intestinal damage. The substantially less severe illness in the intestines of these animals may consequently contribute to the improved survival by decreasing the overall systemic absorption of Stx2. Whether protection against intestinal damage is directly linked to mucosal or systemic anti-Stx2B immunity is not clear at this time. Although the precise sequence of events leading from the ingestion of EHEC to the development of HUS is still unknown, it is well accepted that the intimate attachment of EHEC to the epithelial intestinal cells and the production of Stx play critical roles. Thus, it has been demonstrated that blocking the adherence and colonization of EHEC strains *in vivo* leads to a lower rate of Stx2-dependent complications (39, 40), and anti-Stx antibodies also are able to inhibit EHEC colonization (41). Among the different mechanisms proposed, it has been shown that Stx2 may contribute positively to EHEC adherence by enhancing the surface expression of nucleolin, which serves as a eukaryotic receptor for intimin (42, 43).

There is evidence that the immune response to EHEC colonization factors acquired after a first infection may protect against diarrhea in a subsequent infection. This is believed to be so because in a study of HUS cases over a period of 20 years in Utah, HUS occurred twice in the same patient in only 2.6% of all cases. Interestingly, although prodromal diarrhea is normal in typical HUS, diarrhea is very uncommon among the small subset of recurrent HUS cases (44). This finding would suggest that the bacterial factors responsible for diarrhea raise a protective immune response against subsequent diarrheal disease due to EHEC (45). Our experimental results confirmed these epidemiological observations, since pups from nonimmunized and immunized dams that survived a previous EHEC challenge presented IgA antibodies against EHEC in fecal extracts.

In addition, the protection by maternal antibody transfer decreased at 3.5 m.p.p., when immunized offspring were significantly less protected (28%). At this time, the booster effect of EHEC challenge was evident, because protection rose to 78%. Interestingly, this percentage of protection correlated with the percentage of pups that presented positive titers for anti-Stx2B antibodies in sera by ELISA.

In the present work, we show that vaccination of adult females with BLS-Stx2B protects offspring against mortality triggered by both challenges: a systemic i.v. Stx2 injection or oral administration of an Stx2-producing EHEC strain. Because vaccination of pregnant women has been demonstrated to be a good approach to control endemic diseases (46, 47), and considering that in certain regions of Argentina incidences are as high as 55/100,000 HUS cases and EHEC infections are endemic (7, 48), we suggest that BLS-Stx2B could be useful to control disease in areas of endemicity through vaccination strategies in female adults.

## ACKNOWLEDGMENTS

This work was supported by Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) grants 417-08 and 427-11, Argentina.

We thank Héctor Costa and Gabriela Camerano for their excellent technical assistance.

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