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Immunization with BLS-Stx2B chimera totally protects dams from early pregnancy loss induced by Shiga toxin type 2 (Stx2) and confers anti-Stx2 immunity to the offspring

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ABSTRACT

Shiga toxin producing *Escherichia coli* (STEC) are bacterial pathogens involved in food-borne diseases. Shiga toxin (Stx) is the main virulence factor of STEC and is responsible for systemic complications including Hemolytic Uremic Syndrome (HUS). It has been previously demonstrated that Shiga toxin type 2 (Stx2) induces pregnancy loss in rats in early stage of pregnancy. The main purpose of this study was to determine if an active immunization prevents Stx2 mediated pregnancy loss and confers passive protective immunity to the offspring. For that purpose Sprague Dawley female rats were immunized with the chimera based on the enzyme lumazine synthase from *Brucella* spp. (BLS) and the B subunit of Shiga toxin 2 (Stx2B) named BLS-Stx2B. After immunization females were mated with males. At day 8 of gestation, dams were challenged intraperitoneally with a sublethal and abortifacient dose of Stx2. The immunization induced high anti-Stx2B-specific antibody titers in sera and most important, prevented pregnancy loss. Pups born and breastfeed by immunized dams had high anti-Stx2B-specific antibody titers in sera. Cross-fostering experiments indicated that passive protective immunity against Stx2 was transmitted through lactation. These results indicate that immunization of adult female rats with BLS-Stx2B prevents Stx2-induced pregnancy loss and confers anti Stx2 protective immunity to the offspring.

1. Introduction

Shiga toxin (Stx) is the main virulence factor of Shiga toxin producing *Escherichia coli* (STEC), a bacterial pathogen involved in food-borne diseases. Two types of Stx (Stx1 and Stx2) and their variants can be expressed by STEC. However, Stx2 producing strains are more virulent and epidemiologically more relevant than those producing only Stx1 or both [1,2]. Stx2 is encoded by bacteriophages and its production is the major risk factor for the development of Hemolytic Uremic Syndrome (HUS). It is well known that STEC infections comprise mostly children [1,3], but adults can also be involved [4,5]. Since Stx is a transposable virulence factor gene new STEC profiles can emerge [6,7] and susceptible populations not previously considered could be affected. In humans, early pregnancy loss due to infections comprises almost 15% of all recognized pregnancy losses [8,9], and most of the causes of spontaneous miscarriage often remains unexplained [10]. Previous reports support the hypothesis that symptomatic or asymptomatic STEC infections during pregnancy may cause maternal or fetal damage mediated by Stx2 [11–14]. In addition, we have previously demonstrated that Stx2 intraperitoneally (i.p.) injected in rats in the early stage of pregnancy, causes spontaneous abortion by a direct cytotoxic effect in the highly perfused feto-uteroplacental unit [13,14].

Abbreviations: ANOVA, analysis of variance; BLS, lumazine synthase from *Brucella* spp.; CD_{50}, 50% cytotoxic dose; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; gd, gestation day; Gb3, globotriaosylceramide; HUS, Hemolytic Uremic Syndrome; i.p., intraperitoneally; IgG, immunoglobulin G; IgA, immunoglobulin A; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; Stx, Shiga toxin; Stx1, Shiga toxin type 1; Stx2, Shiga toxin type 2; Stx2B, B subunit of Stx2; Stx2A, A subunit of Stx2; STEC, Stx producing *Escherichia coli*; SD, standard deviation.

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One of the mechanisms of the immune system to fight against toxins or pathogens is the development of neutralizing antibodies. In this regard, studies of two STEC outbreaks associated the seropositivity of anti-Stx2 antibodies with protection to the development of systemic complications [15]. These findings together with the extremely rare occurrence of a second episode of HUS in the same patient, suggest a protective role for anti-Stx antibodies against HUS development [16]. Epidemiological studies demonstrated that people between 20 and 50 years old have high titers of anti-Stx2 [17]. This pattern could reflect the age-related incidence of HUS. Taking these data together, adults without Stx2 antibodies serum titers could be exposed to an increased risk for developing STEC complications mediated by Stx.

It is well known that meanwhile building up its immune system, the infant is supported by the transplacental IgG [18] and by IgA antibodies during breastfeeding [19]. Thus passive transmission of specific immunoglobulins provides the same protection for pathogens that the mother is being protected.

Nowadays no licensed vaccine or effective therapy is available for human use against Stx2. Taking into account this fact a novel immunogen was recently developed by inserting the B subunit of Stx2 at the amino termini of enzyme lumazine synthase from *Brucella* spp. (BLS-Stx2B). Active immunization with this chimera induced high titers of anti-Stx2 neutralizing protective antibodies in mice [20]. In addition, passive immunization of the offspring conferred protection against a lethal challenge with STEC [21]. The main purpose of the present study was to determine if circulating anti-Stx2 neutralizing antibodies generated by an active immunization with BLS-Stx2B in female rats prevent early pregnancy loss mediated by Stx2. We also evaluated if pups breastfeed by immunized mothers were able to acquire passive immunity against Stx2 and obtain protection against a lethal dose of Stx2.

2. Materials and methods

2.1. Drugs and chemicals

Purified Stx2 was purchased from Phoenix Laboratory, Tufts Medical Center, Boston, MA, USA and it was checked for LPS contamination by *Limulus amoebocyte lysate assay* (Biowhittaker Inc. Maryland, USA). Toxin was diluted with sterile phosphate-buffered saline (PBS) before injection. The final solution contained <10 pg LPS/ng of pure Stx2.

2.2. Animals

Sprague Dawley female and male rats (200–280 g; 2–3 months of age) were acquired from the Animal Facility of the School of Pharmacy and Biochemistry. Timed pregnant rats were obtained as previously described [13]. Day 1 of gestation (gd 1) was determined when sperm was observed in the vaginal smear. Animals received food and water *ad libitum* and were housed under controlled conditions of light (12-h light, 12-h dark) and temperature (23–25 °C). This study was carried out in strict accordance with the recommendations detailed in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Protocols were approved by the Committee for the Care and Use of Laboratory Animals of the University of Buenos Aires (CICUAL, Permit Number 2954/10 and 1494/2013).

2.3. Experimental protocol

2.3.1. Immunization

Female rats ($n = 7$) were subcutaneously immunized with three doses of 200 μg of BLS-Stx2B chimera [20] with aluminium hydroxide (1:1) at days 0, 15 and 30. Nonimmunized rats received PBS (Control $n = 4$). One day before every immunization, blood samples were collected by venopuncture of the tail vein and serum was obtained allowing clotting for 1 h at 37 °C and then centrifuged for 10 min at 2500 rpm. Serum samples were collected and stored at −20 °C until used. This experimental protocol was repeated twice (total n/group = 9–15).

2.3.2. Serum Stx2B-specific immunoglobulin determination

Serum Stx2B specific antibodies were analyzed as previously described [20]. Briefly, ELISA plates were coated with 0.5 μg/well with Stx2B. For total specific IgG determination, peroxidase-conjugated goat anti-rat IgG (H + L, 1:5000; Pierce) was used as a secondary antibody. The antigen-antibody reaction was detected with O-phenylendiamine (Sigma, St Louis, MO), and absorbance was read at 492 nm. Results were expressed as end point titers, calculated as the reciprocal values of the last dilution with an absorbance higher than that of the preimmune serum samples +2 standard deviation (SD).

2.3.3. Stx2-neutralization assay

In vitro Stx2 neutralizing activity of sera was analyzed on Vero cells. For that purpose, a 50% cytotoxic dose of Stx2 (CD_{50}: 0.5 ng/ml) for Vero cells was pre incubated with serial dilutions (1:50 to 1:1600) of serum samples for 90 min at 37 °C and 250 rpm. The mixtures were then added to Vero cells. Culture of cells was performed as previously described with modifications [22]. Briefly, 18.000 Vero cells/well were plated in 96-well plates and grown to confluence in complete Dulbecco's Modified Eagle's Medium (DMEM) containing 10% of fetal bovine serum (FBS), glutamine 2 mM, penicillin 100 UI and streptomycin 100 μg/ml. Then, cells were incubated under growth-arrested conditions (DMEM medium without FBS) either with Stx2 alone or with the mixture for 72 h at 37 °C and 5% CO_2. After treatment, cells were incubated with 0.05 mg/ml of neutral red solution for 2 h at 37 °C and 5% of CO_2. After neutral red incorporation, cells were fixed with 4% formaldehyde and 1% $CaCl_2$ and then lysed with 50% ethanol and 1% acetic acid solution. Absorbance was read in a microplate reader (RT-6000, Rayto Life and Analytical Sciences Co. Ltd. China) at 540 nm. Absorbance values from cells incubated under identical conditions but without treatment were considered as 100% of viability. The neutralizing titer was calculated as the last dilution of the serum that was able to completely inhibit Stx2-cytotoxicity.

2.3.4. Pregnancy

After the immunization protocol, Immunized (Imm) and Control dams were mated with males and then i.p. challenged at gd 8 with a sublethal dose of purified Stx2 (0.5 ng Stx2/g of body weight (bwt), 250 μl). Therefore, two groups were performed: Imm + Stx2 ($n = 6$) and Stx2 ($n = 4$), respectively. An additional group of control dams were injected with PBS (Control, $n = 5$). After challenge, dams were housed individually, weighed and controlled until the expected day for delivery. The experiment was repeated twice (total n/group = 9–15).

2.3.5. Body weight, pregnancy progression and fostering experiments

After challenge, Control, Stx2 and Imm + Stx2 dams groups were weighed daily for 9 days. Delivery, litter size and pup body weight were registered. To analyze anti-Stx2B antibody titers, serum from pups born from Imm + Stx2 dams were obtained at weaning (21 day-old) and one month after weaning (51 day-old). For cross-fostering experiments, Control ($n = 5$) and Imm + Stx2 dams ($n = 5$) groups remained with 10 pups each one after delivery with the purpose to equal the litters and stimulate similarly the mammary glands. Then, half of the pups born from Imm + Stx2 dams were fostered to Control dams (Imm pups-Control dam,

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