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The intranasal vaccination of pregnant dams with Intimin and EspB confers protection in neonatal mice from *Escherichia coli* (EHEC) O157:H7 infection

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ABSTRACT

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 is responsible for intestinal disease and hemolytic uremic syndrome (HUS), a serious systemic complication which particularly affects children. In this study, we evaluated whether passive immunization protects from EHEC O157:H7 colonization and renal damage, by using a weaned BALB/c mouse model of infection. Recombinant proteins EspB and the carboxyl-terminal fragment of 280 amino acids of γ-intimin (γ-Int C_{280}) were used in combination with a macrophage-activating lipopeptide-2 (MALP) adjuvant to immunize pregnant mice by the intranasal route. Neonatal mice were allowed to suckle vaccinated or sham-vaccinated dams until weaning when they were challenged by the oral route with a suspension of an *E. coli* O157:H7 Stx2+ strain. The excretion of the inoculated strain was followed for 72 h. All vaccinated dams exhibited elevated serum IgG response against both γ-Int C_{280} and EspB. Passive immunization of newborn mice resulted in a significant increase in serum IgG titers against γ-Int C_{280} and a slight increase in EspB-specific antibodies. The neonates from vaccinated dams showed a significant reduction in EHEC O157:H7 colonization 48 h post challenge. In addition, the level of plasma urea concentration, a marker of renal failure, was significantly higher in offsprings of sham-vaccinated mice. In conclusion, vaccination of pregnant dams with γ-Int C_{280} and EspB could reduce colonization and systemic toxicity of EHEC O157:H7 in their suckling offsprings.

1. Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 is a major etiologic agent of diseases in humans, whose clinical spectrum includes diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome, the leading cause of chronic renal failure in children in Argentina and several other countries [1,2]. 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 [3,4]. This bacterium produces Shiga toxins (Stx1 or Stx2), which are responsible for systemic damage [5]. Current treatment is largely limited to supportive care, as no specific regimen against an *E. coli* O157:H7 infection exists and because the use of antibiotics is not recommended. One reason for not using antibiotics is that the release of Stx from the bacterium following antibiotic treatment can worsen the clinical course [6–8].

In addition to Shiga toxins, EHEC O157:H7 is characterized by other virulence-associated traits, which enable it to colonize the intestinal mucosa of humans and animals with characteristic attaching and effacing lesions due to intimately attached bacteria and effacement of the absorptive microvillar brush [9]. The attaching and effacing activity is mediated by the bacterial adhesin intimin, its translocated receptor Tir, and several effector proteins. These effector proteins are translocated into the epithelial cell by a type 3 secretion system (TTSS) encoded by the locus of enterocyte effacement chromosomal pathogenicity island [10]. EspB is a TTSS translocator protein required for the transit of effector proteins across the host cell membrane, and in turn is translocated into the host cell contributing to the creation of a pore in the eukaryotic cell membrane [11]. Our group successfully assayed a vaccine comprising both proteins in an experimental bovine model [12].

Furthermore, the development of prophylactic approaches, such as vaccines, requires animal models of EHEC infection. Cattle or conventional mice do not manifest key features of disease, such as kidney failure, intestinal damage, and systemic illness. In order

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to have an infection model that better reflects the pathogenesis of EHEC O157:H7, Brando et al. [13] have developed an animal model in weaned mice after oral infection with Shiga toxin 2-producing EHEC O157:H7. They demonstrated that human Stx2-producing EHEC strains can damage the intestinal epithelium of weaned BALB/c mice, followed by a local injury that can lead to renal disease and death in infected mice.

In most mammals, maternal antibodies can be transferred via the placenta and by lactation [14]. Previous reports have shown that the titer of antibodies transferred through the transplacental route declines from day 9 after birth [15], but lactation prolongs the period of passive protection to 5 to 10 weeks in rodents [16,17] and to approximately 9 months in humans [18]. In humans, passive transfer of IgG antibodies through the placenta and during lactation is an important mechanism that provides protection to the newborn, contributes to the maturation of the immune system, and influences the gut microbiota [19]. 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 during the first year of life in areas of endemicity [20]. As bovine does not transfer antibodies from placenta, maternal antibodies are mainly transferred via colostrum during the first 72 h of lactation [21,22].

The aim of this study was to evaluate whether immunization of pregnant dams with a vaccine based on both intimin and EspB proteins protects the suckling newborns from EHEC O157:H7 colonization and renal damage. For this purpose, we used weaned BALB/c mice as a model of infection.

2. Materials and methods

2.1. Bacterial strain and culture media

Escherichia coli O157:H7 strain 125/99, isolated from a human HUS case, was kindly provided by Marta Rivas, ANLIS - Instituto Nacional de Microbiología Carlos G. Malbrán, Buenos Aires. The strain contains the stx_2 gene, the *eae* gene encoding adhesin intimin subtype gamma, and the espB gene encoding a type III secretion system protein. The strain was routinely grown aerobically in Luria Bertani (LB) medium at 37 °C. For the challenge, the inoculum was prepared starting from a 1:100 dilution of an overnight culture in fresh LB broth, which was grown with shaking for 16 h at 37 °C until reaching an OD_{600}∼1. The culture was then centrifuged and the bacteria re-suspended at a concentration of 1×10^7 CFU in 200 μl of phosphate buffered saline pH 7.4 (PBS).

2.2. Animals

Four-week-old specific pathogen free (SPF) female BALB/c mice were obtained from the animal facility of the Facultad de Ciencias Veterinarias–Universidad Nacional de La Plata, Argentina. All animals were housed in individually ventilated cages with HEPA filtered air and free access to food and sterile water. All experiments involving animals were performed following the guidelines of the Animal Welfare Committee of the Instituto Nacional de Tecnología Agropecuaria.

2.3. Immunization schedule and experimental infection

The recombinant protein EspB and the carboxyl-terminal 280 amino acid fragment of γ-intimin ($_{(}$γ-Int $C_{280)}$ were used as immunogens in combination with the macrophage-activating lipopeptide-2 (MALP-2) adjuvant [23,24]. Fig. 1 shows a schema of procedures. Prior synchronization by chemical communication through pheromones [25] each male was placed in a container with three females for mating. After the females were fecundated (this was determined by the presence of a vaginal plug post-coital), they were divided in three groups of six pregnant mice.

Then, these mice were immunized intranasally three times (days 0, 15, and 30) with a vaccine composed of 20 μg of a recombinant protein (γ-Int C280 or EspB, respectively), formulated in 10 μl of PBS and homogenized with 5 μg/dose of MALP-2 adjuvant [29]. A control group received PBS mixed with adjuvant.

The infant mice were allowed to suckle from vaccinated mothers or controls until weaning (17–21 days of age and 8–11 g body weight). The offspring was formed by 14 infant mice per group of adult female mice. Subsequently they were challenged directly into the stomach after 8 h of starvation for food [26], via a 5 French pediatric feeding tube, with a suspension of 1×10^7 CFU of *E. coli* O157:H7 strain 125/99. Rectal swabs were taken at 48 and 72 h after infection to determine the excretion of *E. coli* O157:H7 by bacterial count. The collected stool was re-suspended in 200 μl of PBS and mixed vigorously by vortexing. Following the Miles and Misra method [27], serial dilutions were performed and plated on Sorbitol MacConkey agar medium (Oxoid, Basingstoke, UK) supplemented with cefixime-tellurite (SMAC) and incubated at 37 °C for 18 h. Non-sorbitol-fermenting colonies were evaluated for *E. coli* O157 LPS by a latex agglutination test (Oxoid). Selected latex-positive colonies were confirmed by a multiplex PCR for the stx_1, stx_2, *eae*, and rfb_{O157} genes as described previously [12]. Briefly, assays were carried out in a 25 μl volume containing 2.5 μl of nucleic acid template, 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$; 0.6 μM concentrations of each primer, 0.2 mM concentrations of each deoxynucleoside triphosphate, and 2 U of Taq DNA polymerase (Inbio Highway, Argentina). Temperature conditions consisted of an initial 94 °C-denaturation step for 2 min followed by 30 cycles of 94 °C for 1 min, 57 °C for 1 min, and 72 °C for 1 min. Amplified DNA fragments were resolved by gel electrophoresis using 1% (w/v) agarose. The gels were stained with ethidium bromide and visualized with UV illumination.

Blood samples were collected from dams and weaned mice from the lateral tail vein to determine the titer of specific IgG antibodies against γ-Int_{280} and EspB. After clotting, blood was left at 4 °C for 16 h and then was centrifuged for 10 min at 2700 × *g* and 4 °C. Serum was stored in aliquots at −20 °C until use. The newborn mice were euthanized in CO_2 chamber five days after challenge and immediately after samples from intestine and kidneys were taken for histopathological analysis.

2.4. Serum urea measurement

A specific enzymatic method for quantitative determination of urea in blood was used according to the manufacturer's instructions (Uremia kit, Wiener Lab, Argentina). The urea concentration was expressed as gram of urea by liter of serum.

2.5. Detection of mouse IgG antibodies by ELISA

The serum samples were analyzed for the presence of IgG antibodies against γ-Int-C_{280} and EspB. Microtiter plates (Nunc Immuno MaxiSorp; Nunc A/S, Roskilde, Denmark) were coated overnight at 4 °C with 10 μg/mL of purified protein (100 μL per well, 96-well plates) in PBS. The antigen-coated plates were washed three times with PBS 0.05% Tween 20 (PBS-T), and then blocked with PBS-T containing 5% skim milk for 1 h at 37 °C. The blocked wells were washed three times with PBS. Pools of sera were serially diluted in PBS-T. Positive and negative controls were included. The samples (100 μl) were added to each well and incubated at least for 1 h. The plates were incubated at 37 °C for 1 h, washed three times with PBS-T, and HRP-conjugated rabbit anti-mice IgG (Cayman Chemical Company, USA) (1:3,000) was added to each well. The plates were incubated at 37 °C for 1 h, and washed three

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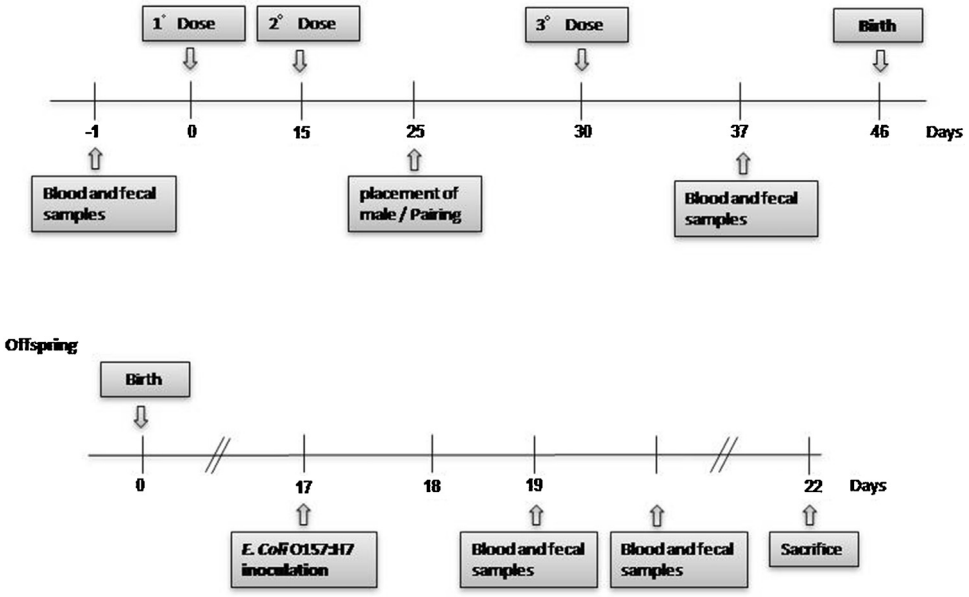

Fig. 1. Immunization schedule and sampling.

times with PBS-T. After three washes, 100 μL of 2,2′-azino-di (3-ethyl-6-sulfonic benztiazolin) (ABTS, Amresco Inc, USA) peroxidase substrate was added and incubated for 15 min at 37 °C. Reactions were stopped by the addition of 5% sodium dodecyl sulfate, and the optical density was read at 450 nm (OD_{450}) on a BioTek ELx808 reader (BioTek Instruments, USA). ELISA was repeated twice (with two technical replica per sera dilution).

2.6. Western blot

Western blotting was performed on pre- and post-immunization samples, from each group to confirm the specificity of the antibody response. One dimension sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in a 12.0% polyacrylamide gel under reducing conditions [28] with 2.5 μg γ-Int C_{280} or EspB proteins loaded per lane, transferred to a nitrocellulose membrane (Amersham Pharmacia, Germany) [29] and blocked with 5% nonfat dry milk and then incubated with 1:100 dilution of dams serum. Sera obtained from the offsprings were pooled by group and 1:50 and 1:20 dilutions were assayed. Finally, the membranes were incubated with HRP-conjugated anti-mouse IgG diluted 1:1,000 in PBS-T. The blots were revealed with 4-Cl-1-naphthol (Pierce, Rockford, USA).

2.7. Statistical analysis

ELISA data of sera from vaccinated pregnant mice and control mice were compared, before and after having been immunized by the Student's t-test. The significance of differences between the control group and treated in the results of the measurement of plasmatic urea and enumeration of *E. coli* O157:H7 in feces was determined by the Fisher's exact test. Statistical analysis was performed using Graph Pad Prism Software (San Diego, CA, USA). In all cases, $p \leq 0.05$ values were considered significant.

3. Results

Overall, vaccinated dams developed high serum IgG titers against both γ-Int C_{280} ($p \leq 0.5$) and EspB proteins (Fig. 2) compared to the pre-immune level and to non-vaccinated dams. The offsprings from vaccinated dams showed a significant increase in serum IgG titers against γ-Int C_{280} and a lower increase in EspB-specific IgG antibodies (Fig. 3) when they were compared with suckling mice delivered from non-immunized dams. In all cases, the specificity of serum antibodies against EspB and γ-Int C_{280} was

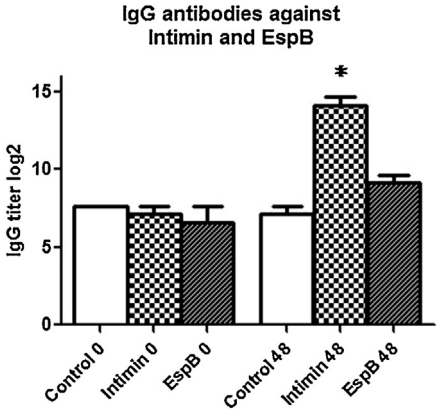

Fig. 2. IgG responses in pregnant mice vaccinated with γ-Int C_{280} and EspB and non-vaccinated controls measured by ELISA. 0 and 48 represent sera sampled at pre-immune and at 48 h post-vaccination, respectively. The results are presented as $\log_2$. * Indicates statistical significance compared to the control ($p < 0.05$).

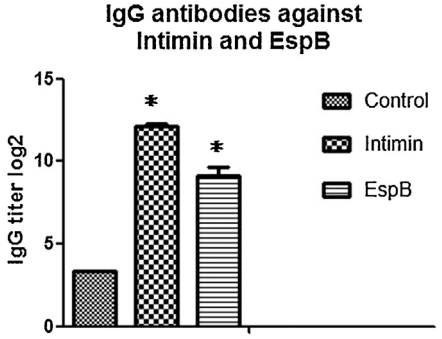

Fig. 3. IgG responses in the offspring of pregnant mice vaccinated with Intimin and EspB (measured by ELISA. The results are presented as $\log_2$. * Indicates statistical significance compared to the control ($p < 0.05$).

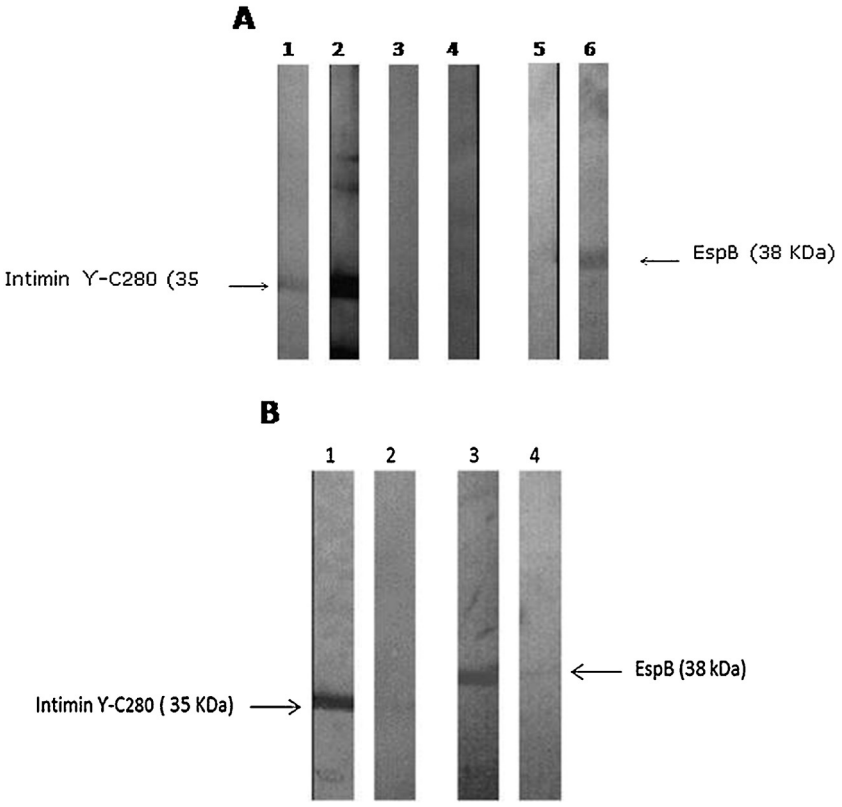

Fig. 4. (A) Reactivity against γ-Int C_{280}, serum from immunized dams (dil 1: 100.) lane 1, pre-immunization; lane 2, post-vaccination. Reactivity against γ-Int C_{280} and EspB, placebo dams serum (Dil 1:20) lane 3, pre-immunization; lane 4, post-vaccination. Reactivity against EspB, serum from immunized dams (dil 1:20) lane 5, pre-immunization; lane 6, post-vaccination (B) Reactivity of sera from weaned mice from immunized dams lane 1 sera from weaned mice from γ-Int C_{280} (dil. 1:50). immunized dams; lane 2, sera from weaned mice from placebo mothers. EspB (dil. 1:20). Lane 3, the reactivity of serum from weaned mice from immunized mothers. Lane 4, sera from weaned mice from placebo dams.

Table 1
Bacterial recovery in feces and uremia in weaned mice at 48 h and 72 h post-inoculation.

Mice group	O157:H7 recovery in feces		High uremia	
	48 h	72 h	48 h	72 h
Control	8/14	5/14	7/14	7/14
Intimin-vaccinated	1/14*	0/14*	0/14*	1/14*
EspB-vaccinated	2/14*	1/14	2/14*	2/14

* Statistically significant ($p < 0{,}005$) by Fisher's exact test.

confirmed by Western blot (Fig. 4). Weak intensity bands observed in pre-immunized mice (lane 1) may be due to an infection of mice with EPEC carrying cross-reacting intimin. At 48 h after challenge, neonates nursing from dams vaccinated with either Intimin or EspB showed a significant reduction ($p < 0{,}005$) in *E. coli* O157:H7 colonization compared to the control group (Table 1). At 72 h, the total colonization lowered in all groups but the difference between suckling mice from intimin-vaccinated and non-vaccinated group remains significant ($p < 0{,}005$).

Throughout the assays, all the weaned mice gained weight normally and experienced no clinical signs of illness following inoculation with *E. coli* O157:H7.

The uremia serum level, as a clinical parameter of the systemic effect of Shiga toxins (Table 1), was significantly lower ($p < 0{,}005$) in the offsprings born from γ-Int C_{280} and EspB-vaccinated dams at 48 h. At 72 h, the uremia level comparison was significant only in the case of suckling mice from intimin-vaccinated group.

At necropsy, on day 5 after challenge, no renal or intestinal lesions were observed by histopathological examination.

4. Discussion

In this work, we assessed the potential of antibodies directed to selected proteins of *E. coli* O157:H7 to prevent infection in a mouse model. Very few studies assessed the impact of mother immunization on the susceptibility of offspring to *E. coli* O157:H7 pathogenicity [30]. γ-Int C_{280} and EspB-induced high levels of specific IgG antibodies in the serum of mice intranasally vaccinated in accordance with previous results from our group [24]. In both works, γ-Int C_{280} consistently appears as more antigenic than EspB. Likewise, IgG antibodies against γ-Int C_{280} and EspB were efficiently transferred to the offspring of immunized dams. Even though maternal antibodies can be transferred via the placenta and by lactation in most mammals [14], our model did not allow distinguishing between both processes.

Here, we demonstrated that intranasal vaccination of mouse dams with γ-Int C_{280} and EspB effectively reduced the total bacterial shedding of *E. coli* O157:H7 after experimental challenge of their offsprings. These results suggest that passive immunization with antibodies against γ-Int C_{280} or EspB may reduce intestinal colonization by *E. coli* O157:H7 and prevent kidney toxicity effects of Shiga toxins, and even inhibit the development of HUS.

Systemic damage in the mouse model of infection is believed to be produced by Shiga toxins [24]. Previous reports suggest that Stx2-specific IgG antibodies transmitted during breast-feeding protect weaned pups against both systemically delivered Stx2 and oral challenge with a lethal doses of *E. coli* O157:H7 [30]. Although the precise sequence of events leading from the ingestion of *E. coli* O157:H7 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 Stxs play critical roles. In this

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line, blocking the adherence and colonization of EHEC strains *in vivo* leads to a lower rate of Stx2-dependent complications [31,32]. The fact that antibodies directed to intimin confer protection against colonization is expected, as intimin is a major bacterial virulence factor which leads to strong intimate attachment to intestinal surface. Indeed, an *E. coli* O157:H7 mutant deficient in intimin is less effective in colonizing mice [33,34]. Although the vaccine formulation used in our study does not contain Shiga toxin, we may assume that a reduced attachment of *E. coli* O157:H7 due to the presence of anti γ-Int C_{280} or anti-EspB antibodies leads to a less efficient delivery of Shiga toxin to the intestinal tissues.

Vaccination of pregnant women has been demonstrated to be a good approach to control endemic diseases [35,36]. According to our results, we suggest that the vaccination of with γ-Int C_{280} and EspB proteins could be useful to control disease in areas where infections with *E. coli* O157:H7 are endemic.

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Conflict of interest statement: I declare no conflict of interest.

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