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Pathogenesis and toxins

Effects of *Clostridium perfringens* iota toxin in the small intestine of mice

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ABSTRACT

Iota toxin is a binary toxin solely produced by *Clostridium perfringens* type E strains, and is structurally related to CDT from *C. difficile* and CST from *C. spiroforme*. As type E causes hemorrhagic enteritis in cattle, it is usually assumed that associated diseases are mediated by iota toxin, although evidence in this regard has not been provided. In the present report, iota toxin intestinal effects were evaluated *in vivo* using a mouse model. Histological damage was observed in ileal loops treated with purified iota toxin after 4 h of incubation. Luminal iota toxin induced fluid accumulation in the small intestine in a dose dependent manner, as determined by the enteropooling and the intestinal loop assays. None of these changes were observed in the large intestine. These results suggest that *C. perfringens* iota toxin alters intestinal permeability, predominantly by inducing necrosis and degenerative changes in the mucosal epithelium of the small intestine, as well as changes in intestinal motility. The obtained results suggest a central role for iota toxin in the pathogenesis of *C. perfringens* type E hemorrhagic enteritis, and contribute to remark the importance of clostridial binary toxins in digestive diseases.

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1. Introduction

Clostridium perfringens type E infection in domestic animals was first reported in the late 1940s [1]. *C. perfringens* type E has been described to produce hemorrhagic enteritis in calves [2,3], cows [4], sheep [5] and goats [6]. Although these infections have generally been considered a rare occurrence in ruminants, there are numerous reports suggesting that type E isolates may account for approximately 5% of all *C. perfringens* isolates and that could be also associated with 50% of fatal hemorrhagic enteritis in calves [3,7]. In rabbits, like *C. spiroforme* infection [8], *C. perfringens* type E disease has been clinically characterized by general loss of condition, diarrhea and characteristic hemorrhagic lesions of the cecal serosa and mucosa, and eventually the distal ileum and proximal colon with presence of watery mucoid content [8]. As *C. perfringens* type E strains are defined by the production of iota toxin (ITX) [5], it is usually assumed that associated diseases are mediated by ITX, although no definitive evidence in this regard has been provided. ITX belongs to the family of binary actin ADP-ribosylating toxins [9]. Other members of this toxin family are *C. difficile* transferase (CDT), *C. spiroforme* transferase (CST), *C. botulinum* C2 toxin, and *Bacillus cereus/sphaericus* vegetative insecticidal proteins (VIP) [10]. These actin ADP-ribosylating binary toxins are composed of two unlinked proteins, an enzymatic component with ADP-ribosyltransferase activity and a binding component which binds to the cell surface receptor [11,12] and facilitates the enzymatic

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component entry to the cytosol [9].

Although cellular intoxication by ITX and binary toxins has been extensively studied [9,11–13], information about intestinal alterations induced by ITX are scant and usually limited to descriptions of natural cases of type E diseases [3,4,6]. Therefore, the current work aims to examine the intestinal effects of ITX and to define the role of ITX in *C. perfringens* type E infection, revealing new insights into *C. perfringens* type E enteritis pathogenesis. Also, the results from this study suggest that the mouse is a useful animal model to study type E and ITX pathogenesis *in vivo*.

2. Materials and methods

2.1. Animals

Conventionally reared 20–25 g NHI Swiss outbred male mice were used. Animals were housed in a light cycle, humidity and temperature controlled room. Studies presented here were reviewed and approved by the institutional animal care and use committee (IACUC) from the CICVyA-INTA, protocol 32/2011.

2.2. Iota toxin

Iota toxin (ITX) was purified from a type E culture as described by Stiles [14]. The purity of ITX was >95% as assessed by densitometry on 12% SDS/PAGE followed by Coomassie Blue staining. Activity and synergistic effect of Ia and Ib were tested on Caco-2 cell monolayers [15]. For *in vivo* assays, ITX concentration was expressed based on the activity on cell monolayers as ITX units (U), which are defined as the reciprocal of the highest dilution inducing cytopathic changes on cell monolayers. According to total protein concentration and the results of cell cytotoxicity tests, it was possible to determine that 200 U/ml of ITX corresponded to a concentration of 1 μg/ml of Ia and 2 μg/ml of Ib.

2.3. Lethality of ITX in mice

The intragastric (i. g.) or intravenous (i. v.) lethality of ITX was determined using groups of 4 mice each. Animals received two-fold dilutions of ITX in 1% peptone water. Before i. g. challenge, mice were fasted overnight but allowed access to water until 2 h before the start of the experiment. Groups of mice were inoculated by i. g. gavage with 0.5 ml of 1.5% PBS $NaHCO_3$ containing purified ITX (50, 100, or 200 U/ml) or buffer solution without ITX. Another set of mice were i. v. injected with a total volume of 0.5 ml of PBS containing purified ITX (50, 100, or 200 U/ml) or buffer solution without ITX. All mice were observed for up to 72 h to monitor lethality, which was defined as death or development of significant respiratory or neurological signs. Mice showing significant respiratory or neurological signs were immediately euthanized and included in lethal dosed fifty (LD_{50}/ml) calculations [16].

2.4. Effects of ITX on mice intestinal loops

2.4.1. Mice intestinal loop test

Mice were fasted during 18 h and deprived of water 2 h before the experiments. Anesthesia was then induced by intraperitoneal (i. p.) injection of 100 mg/kg of ketamine and 5 mg/kg of diazepam. The abdomen of each mouse was disinfected with povidone-iodine solution (Pervinox) immediately before surgery. A midline laparotomy was performed, and the ileum or colon was exposed. Only one 2 cm long intestinal loop was prepared in the ileum or the colon of each animal by a double ligation. Care was taken to avoid overdistension of bowel loops and interference with the blood supply, eliminating a possible ischemic component to the toxin-induced damage. During surgery, the serosal surface of the loops was kept wet by frequent soaking with normal saline solution. After injecting the inoculum, the abdominal incision was closed by separate muscle and skin sutures. The surgical procedure lasted approximately 3 min per animal. Mice were kept under anesthesia by periodic administration of ketamine-diazepam mix until the end of the experiments, 4 h after inoculation, and euthanized by cervical dislocation.

2.4.2. Inoculum

In all experiments, a 0.5 ml aliquot of a Ringer's solution containing specified amounts of purified ITX was injected into each intestinal loop. For the dose-response experiments a mixture containing Ringer's solution with 0, 100 or 200 U/ml of purified ITX was injected into each loop. . Additional loops received an injection of Ringer's solution containing 200 U/ml of purified ITX that had been pre-incubated for 30 min at room temperature with neutralizing mice anti-ITX polyclonal antibody or with anti-ITX IgY [17]. The amount of both anti-ITX antibodies (mice and egg yolk) used was the minimum amount that neutralize ITX cytopathic changes on Caco-2 cell monolayers. Control loops were injected with ITX pre-incubated with antibodies obtained from pre-immune sera of mice or laying hens.

2.4.3. Histological analyses

At the end of the experiments, intestinal loops were excised and fixed by immersion in 10% neutral buffered formalin at pH 7.2 for a minimum of 48 h, after which they were dehydrated through graded alcohols to xylene and embedded in paraffin wax. Samples were cut to obtain 4 μm thick sections. Tissue sections were prepared and stained either with hematoxylin and eosin (H/E) or used for immunohistochemistry (IHC) and examined by optical microscopy.

2.4.4. ITX immunohistochemistry

Deparaffinized tissue sections were treated with 1% hydrogen peroxide in methanol to block endogenous peroxidases, followed by heat-induced antigen retrieval in 0.01 M citrate acid buffer (pH 6). After that, sections of intestines were overlaid sequentially with an egg yolk polyclonal anti-ITX antibody (1/100, vol/vol), and peroxidase labeled rabbit anti-egg yolk (1/100, vol/vol; Sigma Aldrich Co) for 1 h each. Antibodies were diluted in PBS. Control sections were treated using the same buffer but omitting the primary antibody. Finally, preparations were revealed with diaminobenzidine and hydrogen peroxide solution (DAB cod K3468; DAKO) and observed by optical microscopy.

2.5. Effects of ITX on intraluminal fluid accumulation

2.5.1. Enteropooling

Mucosal transport of fluid was determined using the enteropooling assay that evaluates the net accumulation of fluid in the lumen of the small intestine [19]. After 18 h of fasting and 2 h of water deprivation, mice were treated as follows. Groups of 6 mice were dosed i. g. with 0.2 ml of 200 U/ml of ITX in 1.5% PBS $NaHCO_3$ or buffer solution without ITX. Mice in both groups were sacrificed 4 or 20 h later. The small intestine of all mice was clamped at the pyloric sphincter and immediately before the ileocaecal junction, and carefully removed from the abdomen. The small intestine length (L) was measured and then weighed (W1), dried of fluid and reweighed (W2). The difference between W1 and W2 divided by the length [(W1-W2)/L] represents the “enteropooling” in milligrams of fluid per centimeter of intestine, which is an indication of intestinal fluid accumulation [18].