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Clostridium perfringens epsilon toxin inhibits the gastrointestinal transit in mice

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ABSTRACT

Epsilon toxin produced by *Clostridium perfringens* type B and D is a potent toxin that is responsible for a highly fatal enterotoxemia in sheep and goats. *In vitro*, epsilon toxin produces contraction of the rat ileum as the result of an indirect action, presumably mediated through the autonomic nervous system. To examine the impact of epsilon toxin in the intestinal transit, gastric emptying (GE) and gastrointestinal transit (GIT) were evaluated after intravenous and oral administration of epsilon toxin in mice. Orally administered epsilon-toxin produced a delay on the GIT. Inhibition of the small intestinal transit was observed as early as 1 h after the toxin was administered orally but the effects were not observed after 1 week. Epsilon toxin also produced an inhibition in GE and a delay on the GIT when relatively high toxin concentrations were given intravenously. These results indicate that epsilon toxin administered orally or intravenously to mice transitorily inhibits the GIT. The delay in the GIT induced by epsilon toxin could be relevant in the pathogenesis of *C. perfringens* type B and D enterotoxemia.

1. Introduction

Epsilon-toxin produced by *Clostridium perfringens* type B and D is a potent toxin responsible for a highly fatal enterotoxemia in sheep and goats (Barker et al., 1993). Overgrowth of *C. perfringens* B or D in the intestine of susceptible animals, leads to the accumulation of large amounts of epsilon toxin that is absorbed through the intestinal mucosa and spreads in all the organs by the blood circulation (McClane et al., 2006). The toxin causes edema by damage to endothelial cells (Adamson et al., 2005), crosses the blood–brain barrier, it accumulates in the brain (Nagahama and Sakurai, 1991, 1992) and produce neurotoxicity (Finnie, 2003).

In goats and sheep, epsilon-toxin produced functional and histologic changes in the colon, and fluid imbalance without evident morphological alteration in ileum (Uzal et al., 1999; Fernandez Miyakawa and Uzal, 2003; Fernandez Miyakawa et al., 2003). Although intestinal disorders such as diarrhea are described in naturally occurring enterotoxemia of goats and occasionally in sheep, the disease in both species may occur rapidly without any evidence of intestinal changes (Barker et al., 1993; Finnie, 2003; Uzal, 2004). These cases are usually characterized by sudden death due to the systemic effects of epsilon toxin, almost certainly associated to a rapid absorption of epsilon toxin in the intestine.

In mice and sheep, epsilon toxin enhanced the absorption of immunoglobulins from the intestine (Batty and Bullen, 1956), suggesting an increased intestinal permeability for at least some macromolecules including epsilon toxin itself. Since the intestinal permeability to a given macromolecule can be influenced by the length of time during which the intestinal mucosa is exposed to that macromolecule, a delayed intestinal transit could increase the absorption of epsilon toxin. Sakurai et al. (1989) reported that, epsilon toxin caused contraction of the isolated rat ileum *in vitro* and that the toxin-elicited contraction was the result of an indirect action mediated through the nervous system. However, it is unclear if epsilon toxin alters the intestinal transit by itself. We have recently demonstrated that epsilon toxin can be absorbed from the intestine of mice into the systemic circulation (Fernandez-Miyakawa et al., 2007; Losada-Eaton et al., 2008). Therefore, the aim of this report was to evaluate the effects of epsilon toxin in the gastrointestinal transit of mice.

2. Materials and methods

2.1. Animals

Conventionally reared, 20–25 g Balb-c mice of either sex were used. The experiments were conducted between 09:00 AM and 1:00 PM. All procedures involving animals were reviewed by the Animal Care and Use Committee at Instituto Nacional de Tecnología Agropecuaria.

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2.2. Toxin

Epsilon-prototoxin was prepared from an overnight culture of *C. perfringens* type D (strain NCTC 8346) in Trypticase-yeast-glucose medium, under anaerobic conditions at 37 °C. The overnight cultures were centrifuged at 10,000 rpm for 30 min at 4 °C, and the supernatant containing epsilon-toxin was saved for toxin purification. The toxin was then precipitated by ammonium sulfate. Two columns were prepared with DEAE and CM Sepharose (Pharmacia, Sweden), respectively, equilibrated in 10 mM Tris, pH 7.5. The toxin was applied to the DEAE column, and the effluent was monitored at 220 nm. The initially eluted peak was saved and applied to the CM column. Again the effluent was monitored at 220 nm, and the first peak was collected, dialyzed against phosphate buffer solution (PBS), and freeze-dried. Prior to its use in these experiments, the prototoxin was activated by incubation at 37 °C during 30 min with 0.1% trypsin (Sigma).

2.3. Measurements of gastric emptying (GE) and gastrointestinal transit (GIT)

The animals were deprived of food for 18 h prior to the GE and GIT measurements, but allowed water ad libitum until 2 h before the experiments. For GE and GIT measurements, a modification of the technique described by Reynell and Spray (1956) was used. In one set of experiments, groups of 6–8 mice were dosed intragastrically (IG) with 0.5 ml of a solution of 1.5, 6, 12 or 24 μg per ml of epsilon toxin in PBS-1.5% $NaHCO_3$ by stomach gavage. In another experiment, six mice were inoculated IG with 12 μg of epsilon toxin. Additional groups of 6–8 mice were inoculated with 0.5 ml of 0.06 μg of epsilon toxin per ml of 1% peptone water intravenously (IV). Control groups were introduced in each experiment and consisted of 6–8 mice administered IG with 0.5 ml of PBS-1.5% $NaHCO_3$ or 0.5 ml of peptone water injected IV. The mice received 0.3 ml of a 0.5 mg/ml phenol red (phenolsulfonphthalein; Sigma–Aldrich) in 5% glucose 2, 3 or 4 h after toxin treatment in those IG groups and 1 h after treatment in the IV groups.

In all experiments, the animals were killed by cervical dislocation 30 min after the phenol red dosing. The cardial and pyloric sphincters as well as the terminal ileum immediately proximal to the ileocecal valve were quickly clamped and the stomach and small intestine were removed from the carcasses. The whole length of these organs was extended along a meter stick on a tabletop and cut into four consecutive segments: stomach, and proximal (40%), mid (30%) and distal (30%) small intestine. Each segment was placed in 3 ml of 0.1 N NaOH and agitated. The suspension was allowed to settle for 1 h at room temperature and 1 ml of the supernatant was added to 0.1 ml of 20% trichloroacetic acid (wt/vol) and then centrifuged at 3000*g* at 4 °C for 20 min. The supernatant was mixed with 3 ml of 0.5 N NaOH, and the absorbance of the sample was read at 560 nm (Spectronic 20D). The fractional (%) dye recovery value in each gut segment was expressed according to the following equation: recovery segment/amount of phenol red recovered in this segment × total amount of phenol red recovered from all four segments × 100. The recovering of phenol red in each of the segments is related to the movement of the dye inside the gastrointestinal tract. All the experiments were repeated at least two times to confirm the results but only representative experiments are shown.

A second technique was also used to measure the GIT. A charcoal meal (0.2 ml per mouse) containing a solution of 1.5% arabic gum and 5% charcoal as a marker was given intragastrically to conscious mice that were treated 2 h or 1 week earlier with 48 μg of epsilon toxin in PBS-1.5% $NaHCO_3$ IG. Thirty minutes later, the mice were euthanized by cervical dislocation. The abdominal cavity was opened, and the gastrointestinal tract was removed. The traveled distance of the marker was measured and expressed as a percentage of the total length of the small intestine from the pylorus to caecum and this percentage was used as a measurement of GIT.

2.4. Post-mortem examinations

In order to evaluate the morphologic effects of epsilon toxin in the small intestine, other mice inoculated IG with 12 μg of epsilon toxin were analyzed 2 h after inoculation. Post-mortem examination was performed immediately after death and the whole gastrointestinal tract, was immediately fixed by immersion in 10% formalin, and processed routinely for hematoxylin and eosin staining (HE).

2.5. Statistical analysis

Values are expressed as means ± standard error of mean (SEM). For statistical analysis, Student's *t*-test or one-way analysis of variance test was used. *P* values less than 0.05 were considered significant.

3. Results

3.1. Effect of orally administered epsilon toxin on GE and GIT

The results of recovery of phenol red from the stomach or small intestinal segments after the oral administration of different doses of epsilon toxin are shown in Fig. 1. The lower epsilon toxin doses used in this experiment did not significantly affect the distribution of recovered phenol red in the stomach and the intestine. Dye recovery in the stomach increased by 10% in relation to the control mice (buffer alone) when the highest epsilon toxin concentrations (12 and 24 μg) were used ($P < 0.05$), indicating that the GE was reduced.

In the mid-small intestine, dye recovery decreased after treatment with 24 μg of epsilon toxin. In the distal segment, the proportion of dye recovered decreased significantly in treatments with 12 and 24 μg of epsilon toxin ($P < 0.05$), indicating an inhibition of the gastrointestinal transit (Fig. 1). In contrast, 6 μg of toxin increased the amount of dye recovery.

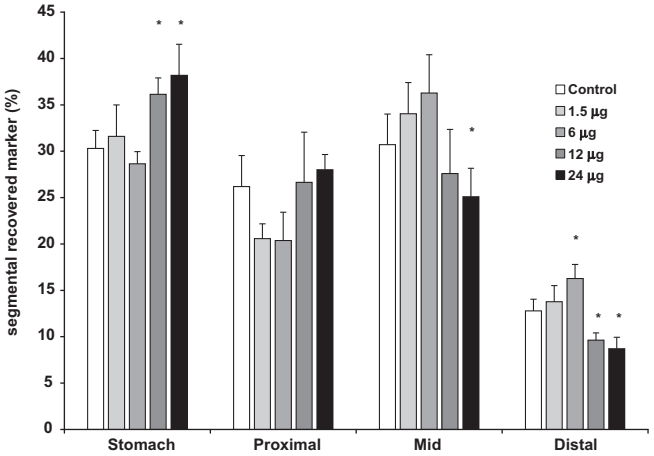

Fig. 1. Effects of different concentrations of epsilon toxin on fractional dye recovery (%) in stomach and proximal, medial and distal small intestine segments in awake fasted mice. Two hours after vehicle (PBS-1.5% $NaHCO_3$) or epsilon toxin administration (1.5, 6, 12 and 24 μg), the animals were gavage-fed with a test meal of phenol red and killed by cervical dislocation 30 min later. Fractional dye recovery values of treated and control animals (each group consisted of 6–8 mice) were determined by spectrophotometry. Bars represent average of fractional recovery (%) values in each segment and vertical lines indicate standard error of mean. *$P < 0.05$ significantly different from each control group.

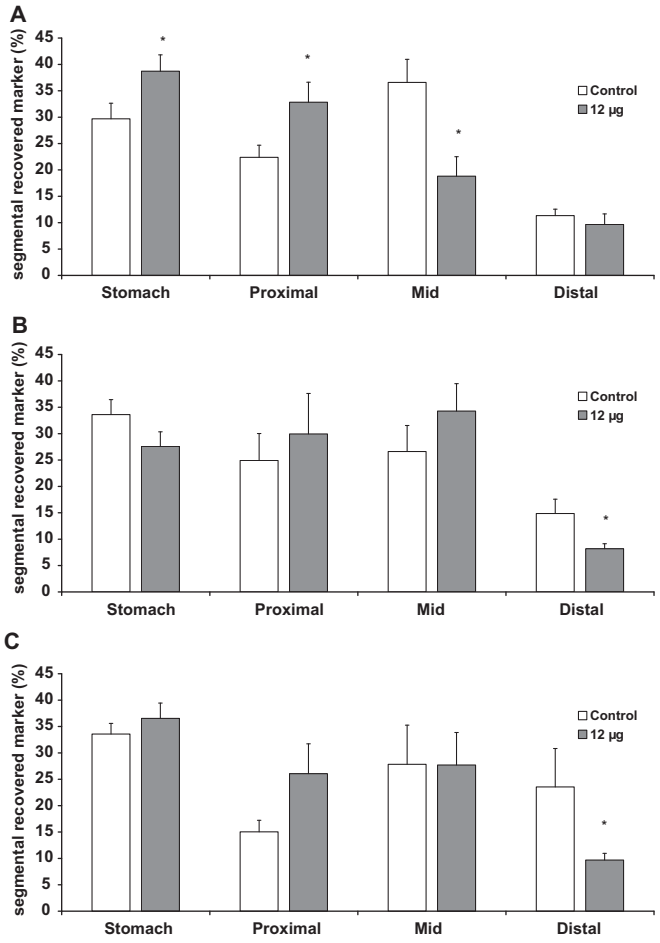

Fig. 2. Effects of epsilon toxin on fractional dye recovery (%) in stomach and proximal, medial and distal small intestine segments (A) 2, (B) 3 and (C) 4 h after vehicle (PBS-1.5% $NaHCO_3$) or epsilon toxin administration (12 µg), the animals were gavage-fed with a test meal of phenol red and killed by cervical dislocation 30 min later. Fractional dye recovery values of treated and control animals (each group consisted of six mice) were determined by spectrophotometry. Bars represent average of fractional recovery (%) values in each segment and vertical lines indicate standard error of mean. $^*P < 0.05$ significantly different from each control group.

Epsilon toxin (12 µg) or control solution were orally inoculated in mice and dye recovery was analyzed 2, 3 and 4 h later (Fig. 2). Dye recovered from the stomach was affected by epsilon toxin 2 h after inoculation but not at 3 or 4 h. In the proximal and mid-small intestine, dye recovery decreased 2 h after toxin administration and 3 and 4 h later in the distal segment ($P < 0.05$), indicating an inhibition of the gastrointestinal transit.

3.2. Effect of intravenously administered epsilon toxin on GE and GIT

Epsilon toxin (0.06 µg) administered IV did not modify the GE 1 h after inoculation. However, this toxin concentration decreased significantly the dye recovery in the mid and distal segments of the small intestine, demonstrating an inhibition of the intestinal transit (Fig. 3). The toxin increased the dye accumulation in the proximal small intestine ($P < 0.05$), possibly as cause of the inhibition of transit in the distal part of the intestine.

3.3. Reversible effect of epsilon toxin on GIT

The charcoal meal method was used to confirm the observation that epsilon toxin inhibits the intestinal transit and to determine if

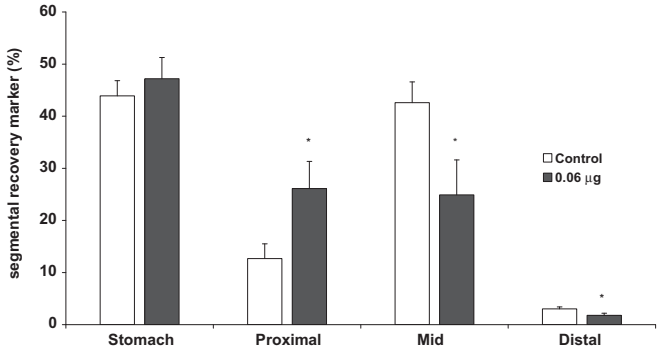

Fig. 3. Effects of systemic epsilon toxin treatment on fractional dye recovery (%) in stomach and proximal, medial and distal small intestine segments in awake fasted mice. Two hours after vehicle (PBS-1.5% $NaHCO_3$) or epsilon toxin administration (0.06 µg), the animals were gavage-fed with a test meal of phenol red and killed by cervical dislocation 30 min later. Fractional dye recovery values of treated and control animals (each group consisted of 6–8 mice) were determined by spectrophotometry. Bars represent average of fractional recovery (%) values in each segment and vertical lines indicate standard error of mean. $^*P < 0.05$ significantly different from each control group.

Table 1
Effect of epsilon toxin on gastrointestinal transit in mice measured by the charcoal meal method.

Period of pre-treatment	*n*	Group	Distance traveled by charcoal marker as % of total length of small intestine (mean ± SEM)	% of inhibition
2 h	8	Control	70.3 ± 6.2	–
		Epsilon	36.9 ± 10.6*	47.5
1 week	6	Control	68.3 ± 1.7	–
		Epsilon	68.2 ± 5.0	0.14

* Significant as compared to control, $P < 0.05$.

the effects are reversible (Table 1). Intragastric administration of 48 µg of epsilon toxin per mouse inhibited the gastrointestinal transit of charcoal 2 h later but that inhibition was not observed when a subset of the mice were evaluated 1 week later.

4. Discussion

The present report shows that *C. perfringens* epsilon toxin is able to induce a delay in GIT of a liquid meal in mice in a dose dependent mode. The delayed GIT was not permanent since animals recovered the normal motility when the transit was evaluated 1 week after toxin administration. The effects of epsilon toxin in the GIT were observed when the toxin was introduced orally and also when the toxin was injected systemically.

GE and GIT assessment, based on fractional dye retention, has been extensively used in different studies (Dundore et al., 1992; Gondim et al., 1999, 2001; De Rosalmeida et al., 2003; Suchitra et al., 2003; Izbéki et al., 2001). This technique allows the evaluation of gut motility in awake rodents, avoiding the anesthesia effects on the cardiovascular and autonomic functions. The gastrointestinal functions are under the tight control of various neurotransmitters, hormones, inflammatory mediators, and intraluminal contents. Therefore, the response of the intestine to epsilon toxin was evaluated in awake mice, mimicking more closely the natural scenario of toxin action during disease.

From a preliminary set of experiments, inhibition of GIT was observed as soon as 1 h after IG administration of 12 µg epsilon toxin. However, 2 h was the time selected to test the effects of different epsilon toxin concentrations since inhibition of GE and GIT

was more marked after this time. Since GIT is highly influenced by both GE and intestinal transit, then the observed GIT delay could be the result of changes in GE. However, GE was not inhibited 4 h after toxin administration or 1 h after intravenous injection, suggesting that reduced gastrointestinal transit could be produced by intestinal transit delay. The effects of epsilon toxin over the intestinal transit could be produced, at least in part, after the intestinal absorption of the toxin.

Relatively high intestinal concentrations of epsilon toxin delayed the GIT and lower concentrations of this toxin did not produce a delay but a slight increase of the intestinal transit. Although the GIT rise could be related to an enterotoxic action of the toxin, this acceleration of the GIT could be an effect per se. For instance alcohol has been reported to stimulate, inhibit or exert no effect on the gastrointestinal motility depending on the dose and the mode of administration (Pirola and Davis, 1970; Robles et al., 1974; Moor et al., 1981; Keshavarazian et al., 1986; Palasciano et al., 1995). Histological changes were not observed in the intestine of mice treated with epsilon toxin, which suggests that the changes in GIT observed in these experiments were not associated with morphological damage to the stomach or small intestine.

The effects of epsilon toxin administered orally were relatively fast (1–2 h) suggesting that the toxin could trigger a rapid reaction from the mucosa. However, the action of epsilon toxin on the GIT was observed when this toxin was administered IG and IV. Then it is also possible that epsilon toxin is absorbed very quickly from the lumen of the intestine and that the systemic effects are responsible for the inhibition of the GIT. It is known that epsilon toxin injected systemically affects primarily the central nervous system (CNS) (Finnie, 2003). In rodents, low doses of toxin produce damage to neurons in the hippocampus and cerebral cortex, while high doses also injure neurons in other brain regions such as thalamus, cerebellum, striatum and corpus callosum (Finnie, 2003). The toxin damage to CNS neurons could impair the intestinal motor function in the small intestine since the CNS structures mostly affected by infections are mainly in the brainstem from where they can influence extrinsic motor nerve output (Spiller, 2004).

The CNS is considered a primary target of epsilon toxin, but this toxin also can affect peripheral pathways (Sakurai et al., 1989; Nagahama et al., 1993). Sakurai et al. (1989) reported that epsilon toxin caused contraction of the isolated ileum of rats *in vitro* and that the toxin-elicited contraction was the result of an indirect action mediated through the nervous system. Several enteric bacterial pathogens and/or their toxins have been shown to alter intestinal myoelectric patterns. These include cholera toxin (Mathias et al., 1976), *Escherichia coli* heat-stable toxin (Mathias et al., 1982), wild-type and recombinant *Vibrio cholerae* strains (Mathias et al., 1976; Lind et al., 1991), and various classes of *E. coli* (Sjogren et al., 1989; Burns et al., 1978). Although it is likely that epsilon toxin activity in the gastrointestinal motility is mediated by a neuronal effect, the clarification of this subject requires further work.

The consequences of gastrointestinal motility inhibition caused by epsilon toxin might be crucial in the development of enterotoxemia. If the intestinal concentration of epsilon toxin is high enough to trigger a delay of the intestinal motility, this could predispose bacteria overgrowth and toxin production together with increased chances of epsilon toxin reaching the mucosal surface. Delayed intestinal motility induced by epsilon toxin could be a determinant factor for the sudden death observed in acute cases of enterotoxemia produced by *C. perfringens* type B and D in ruminants.

In summary, the results of the present report indicate that GE and GIT were inhibited by epsilon toxin. Additional studies are necessary to determine the mechanism involved and the significance of this phenomenon in the development of the disease.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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