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### Research Article

Toxin A, a 308,000-Mr enterotoxin from *Clostridium difficile*, mediates antibiotic-associated diarrhea and colitis in humans. Injection of toxin A into animal intestine triggers an acute inflammatory response characterized by activation of sensory neurons and immune cells of the intestinal lamina propria, including mast cells and macrophages, and migration of circulating neutrophils in the involved intestinal segment. In this study we show that mice genetically deficient in the neurokinin-1 receptor are protected from the secretory and inflammatory changes as well as from epithelial cell damage induced by toxin A. The protective effect of neurokinin-1R deletion correlates with diminished intestinal levels of the cytokine TNF- $\alpha$  and its mRNA and the leukocyte enzyme myeloperoxidase. These results demonstrate a major requirement for substance P receptors in the pathogenesis of acute inflammatory diarrhea.

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## Neurokinin-1 (NK-1) Receptor Is Required in *Clostridium difficile*-induced Enteritis

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### Abstract

Toxin A, a 308,000- $M_r$  enterotoxin from *Clostridium difficile*, mediates antibiotic-associated diarrhea and colitis in humans. Injection of toxin A into animal intestine triggers an acute inflammatory response characterized by activation of sensory neurons and immune cells of the intestinal lamina propria, including mast cells and macrophages, and migration of circulating neutrophils in the involved intestinal segment. In this study we show that mice genetically deficient in the neurokinin-1 receptor are protected from the secretory and inflammatory changes as well as from epithelial cell damage induced by toxin A. The protective effect of neurokinin-1R deletion correlates with diminished intestinal levels of the cytokine TNF- $\alpha$  and its mRNA and the leukocyte enzyme myeloperoxidase. These results demonstrate a major requirement for substance P receptors in the pathogenesis of acute inflammatory diarrhea. (*J. Clin. Invest.* 1998. 101:1547–1550.) Key words: substance P • neurokinin-1 receptor • intestinal inflammation • enterotoxins • *Clostridium difficile*

### Introduction

*Clostridium difficile* is the principal cause of antibiotic-associated colitis and represents a major intestinal pathogen in hospitals and nursing homes (1). This bacterium mediates diarrhea and inflammation by releasing two protein exotoxins, toxin A (308,000  $M_r$ ) and toxin B (275,000  $M_r$ ) (1). The cellular mechanism of these toxins involves binding to their receptors (2) and inactivation of the family of *Rho* proteins (3), leading to disaggregation of actin microfilaments and cell rounding (3). Experiments in intact animals have inferred that toxin A causes intestinal secretion and acute inflammation by stimulating a complex cascade which involves substance P (SP)<sup>1</sup> (4, 5),

enteric nerves (5), lamina propria macrophages (4), and leukocytes (4). The latter include, but are not necessarily limited to, mucosal mast cells and neutrophils (6). The potential importance of SP and its neurokinin-1 (NK-1) receptor in initiating or propagating this inflammatory cascade is underscored by in vivo studies showing that previous desensitization of sensory neurons by capsaicin (5, 7) or administration of specific SP receptor antagonists (5, 8) inhibits fluid secretion, intestinal inflammation, and mediator release in response to toxin A.

We have recently generated mice deficient in NK-1 (SP) receptor (NK-1R<sup>-/-</sup>) by targeted disruption of the SP receptor gene in embryonic stem cells (9). These mice showed reduced lung injury and neutrophil infiltration as compared to the wild-type mice in an experimental model of immune complex-mediated lung injury (9). However, levels of TNF- $\alpha$  in the lungs of NK-1R<sup>-/-</sup> mice after immune complex injury were similar to those found in wild-type animals (9), indicating a lack of association between SP and TNF- $\alpha$  in this model. The availability of NK-1R<sup>-/-</sup> mice allowed us to directly evaluate the importance of SP receptors in the regulation of inflammatory diarrhea and mucosal damage mediated by *C. difficile* toxin A and examine whether TNF- $\alpha$  synthesis and release during intestinal inflammation is modulated by SP.

### Methods

**Ussing chamber experiments.** Mouse ileal mucosa was stripped off the serosa and muscle layers using a dissecting microscope. This preparation contains the mucosa and submucosa layers, including submucosa nerves and immune cells (10). Mucosal preparations were mounted in Ussing chambers and incubated with buffer (10). After 30 min of baseline incubation, mucosa was exposed to serosal SP or forskolin at 10<sup>-6</sup> M. Short circuit current (Isc,  $\mu$ A/cm<sup>2</sup>) was continuously measured and recorded every minute. Drug-induced maximal Isc increases from baseline values are given as  $\Delta$ Isc.

**Preparation of ileal loops and measurement of fluid secretion and histologic damage.** Toxin A was purified to homogeneity from broth culture supernatants of *C. difficile* strain 10,463 (11). Fasted (16 h) mice matched for sex and age (12–14 wk) were anesthetized with intraperitoneal sodium pentobarbital (600  $\mu$ g per mouse) and kept at room temperature (37°C). Laparotomy was performed and two 5-cm-long closed distal ileal loops were formed (4, 8) and injected with either 50 mM Tris buffer (pH 7.4) containing 10  $\mu$ g of toxin A or buffer alone (control). At 4 h animals were killed by an intraperitoneal bolus of pentobarbital (40 mg/kg), the ileal loops were removed, their weights and lengths recorded, and fluid secretion was assessed by

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1. Abbreviations used in this paper: Isc, short circuit current; MPO, myeloperoxidase; NK-1, neurokinin-1; NK-1R<sup>-/-</sup>, mice deficient in NK-1 receptor; SP, substance P.

loop weight (mg) to length (cm) ratio (4, 8). Full-thickness sections of loops were fixed in formalin, paraffin embedded, and stained with hematoxylin and eosin and histologic severity of enteritis was graded in a blinded fashion by a gastrointestinal pathologist (Sigfus Nikulasson) (4, 8). Animal studies were approved by the institutional animal care and use committee.

**Measurement of myeloperoxidase activity.** Ileal loops of wild-type and NK-1R<sup>-/-</sup> mice were injected with either toxin A or buffer. At 4 h loops were homogenized in 1 ml of 50 mM KH<sub>2</sub>PO<sub>4</sub> buffer (pH 6) containing 0.5% hexadecyltrimethylammonium bromide and processed for myeloperoxidase measurements using a colorimetric assay (12) modified from the method of Bradley et al. (13).

**SP and TNF- $\alpha$  measurements.** Ileal loops of wild-type and NK-1R<sup>-/-</sup> mice were injected with either toxin A or buffer. Loops were homogenized in 1 ml of ice-cold 0.1 M HCl buffer for 20 s and processed for SP (4) and TNF- $\alpha$  (14) measurements, using an enzyme immunoassay (Peninsula Laboratories, Belmont, CA) or an ELISA kit (Biosource International, Camarillo, CA), respectively, according to the manufacturer's recommendations.

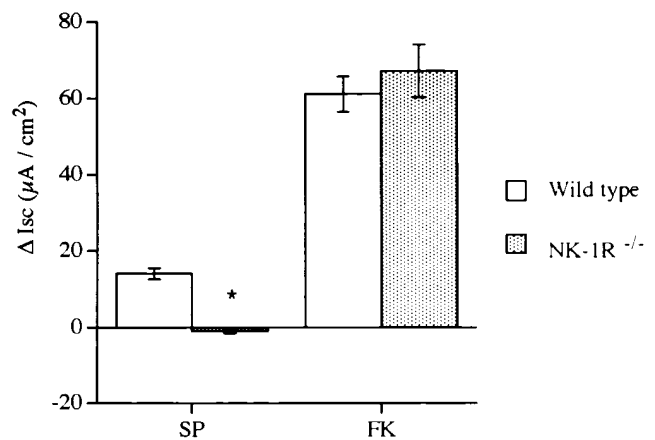
**Total RNA extraction and reverse-transcription PCR (RT-PCR) amplification for TNF- $\alpha$  mRNA.** Mouse ileal loops were formed and injected with buffer or toxin A as described above. Animals were killed at different time points, the loops removed, washed in ice-cold HBSS, and ileal tissues were collected as described above. Total RNA from these samples was isolated and cDNA was prepared by reverse transcribing 1  $\mu$ g of total RNA (4). RNA integrity was tested by 1% agarose formaldehyde gel electrophoresis. RT-PCR reactions for TNF- $\alpha$  and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) RNA mRNA were performed using a thermal cycler (Perkin-Elmer Corp., Norwalk, CT) (14).

**Statistical analyses.** Statistical analyses of all data were performed using SIGMASTAT (version 1.0; Jandel, San Rafael, CA). ANOVA was used for intergroup comparisons.

## Results

We first examined whether the intestine of NK-1R<sup>-/-</sup> mice responds *in vitro* to SP or to the cAMP-dependent secretagogue, forskolin (15). Our results showed that tissues from both genotypes responded similarly to administration of forskolin (Fig. 1). Serosal administration of SP to ileal mucosa caused an Isc increase in wild-type mice. Previous studies indicate that SP-mediated Isc increase in ileum of normal mice was inhibited by tetrodotoxin, indicating involvement of enteric nerves (16). However, administration of SP to ileal mucosa of NK-1R<sup>-/-</sup> mice did not increase Isc (Fig. 1). Although NK-1, NK-2, and NK-3 tachykinin receptor subtypes have been identified in the gastrointestinal tract (17), and some of the effects of SP on enteric neurons may not be mediated by NK-1 receptors (18, 19), our results are consistent with those of Wang et al. (16) indicating that NK-1 receptors mediate SP-induced secretion in mouse ileum.

To directly assess the role of SP in the toxin A model of intestinal inflammation, we injected ileal loops of anesthetized wild-type and NK-1R<sup>-/-</sup> mice with purified toxin A and measured ileal fluid secretion after 4 h. PMN infiltration was determined biochemically by measuring levels of the leukocyte enzyme MPO in ileal homogenates and histologically by counting the number of PMNs. Basal intestinal fluid secretion was similar in NK-1R<sup>-/-</sup> and wild-type mice (Table I). However, NK-1R<sup>-/-</sup> mice had significantly diminished fluid secretion in response to toxin A administration (Table I). Histologically, toxin A-exposed ileal tissues of these mice appeared to be no different from those exposed to buffer control (Fig. 2). Histologic quantification of the ileal sections indicated that NK-1R<sup>-/-</sup>



**Figure 1.** Effect of SP and forskolin (FK) on ileal Isc changes of wild-type and NK-1R<sup>-/-</sup> mice. Ussing chambered ileal mucosae of wild-type and NK-1R<sup>-/-</sup> mice were exposed to serosal SP or FK (10<sup>-6</sup> M). Bars represent mean  $\pm$  SEM changes in Isc ( $n = 5$  per group) obtained 5 and 7 min after SP or FK administration, respectively. \* $P < 0.05$  vs. wild-type.

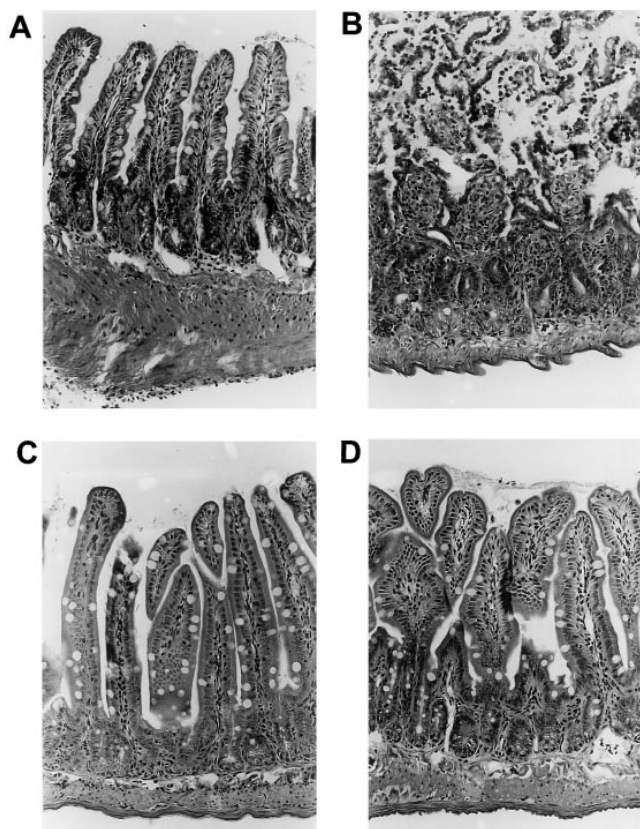
mice had dramatically attenuated epithelial damage, congestion and edema of the mucosa as well as PMN infiltration in response to toxin A (Table I). Furthermore, tissue MPO levels after toxin A administration were almost normalized in NK-1R<sup>-/-</sup> mice (Table II).

We then examined the levels of SP and TNF- $\alpha$  in ileal loops 1 and 4 h after toxin A administration, respectively. In both wild-type and NK-1R<sup>-/-</sup> mice basal tissue levels of SP and TNF- $\alpha$  obtained from buffer-exposed loops were statistically indistinguishable (Table II). Toxin A caused significant increases in SP levels in both genotypes, whereas TNF- $\alpha$  levels in NK-1R<sup>-/-</sup> mice were dramatically reduced in response to toxin A compared to the wild-type mice (Table II). While steady state levels of TNF- $\alpha$  mRNA were elevated in ileal tis-

**Table I. Toxin A-induced Fluid Secretion and Histologic Severity of Enteritis Are Reduced in NK-1R<sup>-/-</sup> Mice Compared to Wild-Type Mice**

	Intestinal secretion	Histologic severity of enteritis		
		Epithelial damage	Congestion and edema	Neutrophil infiltration
	mg/cm			
Wild-type, control	38 $\pm$ 4	0.5 $\pm$ 0.3	0.5 $\pm$ 0.2	0.25 $\pm$ 0.1
Wild-type, toxin A	211 $\pm$ 6 <sup>‡</sup>	2.2 $\pm$ 0.1 <sup>‡</sup>	3.0 $\pm$ 0.1 <sup>‡</sup>	2.8 $\pm$ 0.2 <sup>‡</sup>
NK-1R <sup>-/-</sup> , control	42 $\pm$ 4	0.2 $\pm$ 0.2	0.4 $\pm$ 0.2	0.4 $\pm$ 0.2
NK-1R <sup>-/-</sup> , toxin A	88 $\pm$ 11* <sup>§</sup>	0.4 $\pm$ 0.2 <sup>  </sup>	0.6 $\pm$ 0.1 <sup>  </sup>	0.4 $\pm$ 0.1 <sup>  </sup>

Ileal loops of fasted anesthetized mice were injected with toxin A or buffer and fluid secretion was assessed after 4 h as described in Methods (10 animals per group). Ileal tissues were fixed in formalin, paraffin embedded, and stained with hematoxylin and eosin (seven animals per group). The histologic severity of enteritis was graded by a score of 0–3 in a blinded fashion by a gastrointestinal pathologist. Values are means  $\pm$  SEM. \* $P < 0.05$  and <sup>‡</sup> $P < 0.01$  vs. the respective controls; <sup>§</sup> $P < 0.05$  and <sup>||</sup> $P < 0.01$  vs. wild-type, toxin A.



**Figure 2.** Histologic evaluation of toxin A-induced enteritis in wild-type and NK-1R<sup>-/-</sup> mice. (A) Control wild-type mouse ileum 4 h after injection of buffer; there is normal villus architecture. (B) Wild-type mouse ileum 4 h after injection of toxin A; there is severe necrosis and disruption of mucosal architecture and infiltration of lamina propria with inflammatory cells. (C) NK-1R<sup>-/-</sup> mouse ileum 4 h after injection of buffer showing normal mucosal architecture. (D) NK-1R<sup>-/-</sup> mouse ileum after 4-h exposure to toxin A showing lack of toxin-associated necroinflammatory changes. All sections were stained with hematoxylin and eosin and analyzed by the investigator in a blinded fashion. Original magnification was  $\times 160$ . Results are representative of 28 wild-type and NK-1R<sup>-/-</sup> mice.

sue of wild-type mice 4 h after toxin A administration, these were only slightly increased in NK-1R<sup>-/-</sup> mice in response to the toxin (Fig. 3). These results indicate a major requirement for SP receptors in TNF- $\alpha$  synthesis and release in mouse intestine during acute inflammation.

## Discussion

SP is an 11-amino acid peptide distributed throughout the intestine (20). Intestinal sources of SP include capsaicin-sensitive sensory neurons, enteric neurons, and enteroendocrine cells (21), as well as intestinal macrophages (4). Functional SP binding sites are present on enteric nerves (5), monocytes (22), macrophages (4, 23), and mast cells (24) and binding of SP to intestinal macrophages stimulates release of TNF- $\alpha$  (4). Although the involvement of SP in acute and chronic intestinal inflammation has been suggested previously (4, 5, 8, 25), our results provide direct evidence for the importance of SP-NK-1R

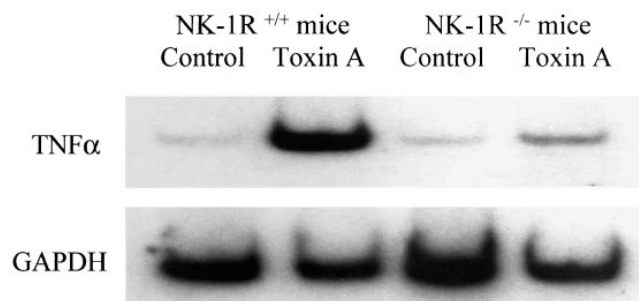
**Table II.** Effect of Toxin A Administration in SP, TNF- $\alpha$ , and MPO Ileal Tissue Levels

	SP	TNF- $\alpha$	MPO
	pmol/mg protein	pg/mg tissue	mU/mg tissue
Wild-type, control	4.4 $\pm$ 0.5	0.6 $\pm$ 0.1	5.3 $\pm$ 1
Wild-type, toxin A	9.7 $\pm$ 0.8 <sup>‡</sup>	7.6 $\pm$ 1.5 <sup>‡</sup>	124 $\pm$ 3.9 <sup>‡</sup>
NK-1R <sup>-/-</sup> , control	4.7 $\pm$ 0.6	1.0 $\pm$ 0.1	4.6 $\pm$ 1
NK-1R <sup>-/-</sup> , toxin A	9.0 $\pm$ 0.5 <sup>‡</sup>	1.6 $\pm$ 0.1 <sup>§</sup>	19.1 $\pm$ 3 <sup>‡  </sup>

After injection of toxin A or buffer (seven mice per group), ileal loops were homogenized and SP and TNF- $\alpha$  were determined 1 and 4 h after injection, respectively, as described in Methods. MPO activity was measured in tissue homogenates 4 h after toxin A or buffer administration by a colorimetric assay (see Methods). Values are means $\pm$ SEM. \* $P$  < 0.05, and <sup>‡</sup> $P$  < 0.01 vs. the respective controls; <sup>§</sup> $P$  < 0.05, and <sup>||</sup> $P$  < 0.01 vs. wild-type, toxin A.

interaction(s) in inflammatory diarrhea caused by *C. difficile* toxin A. Previous studies also point to an early involvement of SP in intestinal inflammation. Release of SP from dorsal root ganglia occurred before secretory and inflammatory changes in response to toxin A were evident (4), and administration of a NK-1R antagonist after ileal injection of the toxin failed to inhibit toxin A-associated responses (8). Thus, release of SP and its subsequent interaction with enteric nerves and immune and inflammatory cells of the intestinal lamina propria represent an important amplification system in the pathophysiology of *C. difficile* toxin A enteritis.

Although our results directly implicate SP as an early mediator of toxin A-mediated enteritis, the mechanism(s) by which signals from the intestinal epithelium in response to the toxin are communicated to sensory neurons to release SP remains to be elucidated. It would be interesting to determine if inflammatory mediators, released from intestinal epithelial cells in response to toxin A (26), are involved in this signaling process and whether this mechanism(s) involves *Rho* proteins. It would also be interesting to identify the mechanisms of upregulation of NK-1R in enteric nerves (5) and intestinal macrophages (4), particularly the cellular pathways that lead to cell



**Figure 3.** Reduced toxin A-mediated accumulation of TNF- $\alpha$  mRNA in mouse ileum of NK-1R<sup>-/-</sup> mice as compared to wild-type. Ileal loops were injected with buffer or toxin A and after 4 h RNA was prepared and reverse transcribed to obtain cDNA. TNF- $\alpha$  and GAPDH mRNA were measured by RT-PCR as described in Methods. Results are representative of 20 wild-type and NK-1R<sup>-/-</sup> mice.

activation and mediator release in response to SP. These results may also be relevant to the pathophysiology of human *C. difficile* colitis and inflammatory bowel disease since NK-1R sites are elevated in the colon of patients with *C. difficile*-induced pseudomembranous colitis (27), Crohn's disease, and ulcerative colitis (28).

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