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Clostridium difficile Toxin A Induces the Release of Neutrophil Chemotactic Factors From Rat Peritoneal Macrophages: Role of Interleukin-1β, Tumor Necrosis Factor Alpha, and Leukotrienes

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Clostridium difficile **produces a potent enterotoxin and cytotoxin, toxins A and B, respectively, which appear to be responsible for pseudomenbranous colitis and antibiotic-associated diarrhea. In the present study we explored the neutrophil migration evoked by toxin A in the peritoneal cavities and subcutaneous air pouches of rats and examined the role of macrophages and their inflammatory mediators in this process. Toxin A causes a significant dose-dependent neutrophil influx into the peritoneal cavity, with a maximal response at 0.1 μg/ml and at 4 h. The depletion of macrophages by peritoneal washing prevents the toxin A-induced neutrophil migration into the peritoneal cavity. In contrast, an increase in macrophages induced by peritoneal injection of thioglycolate amplifies this toxin effect on neutrophil migration. Furthermore, the injection of supernatants from toxin A-stimulated macrophages into the rat peritoneal cavity causes significant neutrophil migration. Pretreatment of rats with BWA4C, nordihydroguaiaretic acid, mepacrine, or dexamethasone inhibits the neutrophil migration evoked by toxin A in the peritoneal cavities. However, pretreatment with the cyclooxygenase inhibitor indomethacin or the platelet-activating factor antagonist BN52021 fails to alter toxin A-induced neutrophil migration. Toxin A was also injected into air pouches of normal rats or rats pretreated with anti-interleukin-1β (anti-IL-1β) or anti-tumor necrosis factor alpha (anti-TNF-α) antibodies. Anti-TNF-α or anti-IL-1β antibodies significantly reduce the neutrophil migration induced by toxin A. These data suggest that neutrophil migration evoked by toxin A is in part dependent on macrophage-derived cytokines, such as TNF-α and IL-1β, and leukotrienes. These mediators may help to explain the intense inflammatory colitis caused by *C. difficile* toxin A in an experimental animal model of this disease.**

The anaerobic bacterium *Clostridium difficile* is one of the most frequently recognized bacterial causes of diarrheal disease in hospitalized adults in industrial countries (12). Antibiotic-associated colitis and pseudomembranous colitis are often associated with cytotoxigenic *C. difficile* (19, 24, 30).

C. difficile produces two antigenically and biologically distinct toxins that appear to be involved in the pathogenesis of the diseases. Toxin A is an exotoxin that possesses potent enterotoxic and weak cytotoxic properties (2, 39). Toxin B is a more potent cytotoxin than toxin A, but it has no enterotoxic activity in animal models studied to date (2, 22, 23). The genes for *C. difficile* toxins A and B have been cloned (3, 8); the gene for toxin A encodes a protein of 2,710 amino acids (308 kDa), and that for toxin B encodes a protein of 2,366 amino acids (269 kDa).

Colitis as a result of infection with toxigenic *C. difficile* is characterized by large numbers of polymorphonuclear leukocytes infiltrating the mucosal layers of the intestine (12). Lima and coworkers (21) showed in 1989 that as early as 2 h after inoculation with 0.1 μg of *C. difficile* toxin A in isolated rabbit intestinal loops, there was a diffuse lymphocytic and neutrophilic infiltrate in the lamina propria and the surface epithelium, with destruction of the mucosa. One of the distinctive features of toxin A action in the intestine is its ability to elicit an acute inflammatory response with activation of macrophages and mobilization of neutrophils (30). The signaling mechanisms involved in this inflammatory response are quite complex and involve the release of potent proinflammatory mediators from various cells that contributes to the increase epithelial permeability, damage, and concomitant intense fluid secretion (12, 26, 29, 40). We hypothesize that *C. difficile* toxin A might potentiate its damage and intestinal secretory effects in vivo via interaction with some cells of the immune system, like macrophages, which can then be activated to release inflammatory mediators. The effects of the toxin A on the cells of the immune system have been poorly investigated, and the mechanism for this toxin-induced release of mediators is unknown (12, 30).

Several laboratories have shown that neutrophil migration in vivo, induced by exogenous stimuli such as *Escherichia coli* lipopolysaccharide (LPS), bacteria, and zymosan (1, 25, 36) and by endogenous inflammatory mediators such as human purified interleukin-1 (IL-1), recombinant tumor necrosis factor alpha (TNF-α), or recombinant gamma interferon (5, 10, 32), was dependent on the release of cytokines or others me-

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diators from macrophages. The present study attempted to evaluate whether *C. difficile* toxin A causes neutrophil migration into the peritoneal cavities and subcutaneous air pouches of rats and whether this effect, as has been described for other exogenous stimuli, is dependent on the cytokines and leukotrienes released from resident cells.

MATERIALS AND METHODS

Animals. Male and female Wistar rats weighing 180 to 200 g received water and food ad libitum until use.

Preparation of *C. difficile* toxin A. Highly purified and characterized *C. difficile* toxin A was provided by David Lyerly, TechLab, Blacksburg, Va. Briefly, *C. difficile* (VPI 10463) was grown anaerobically in dialysis tubing suspended in brain heart infusion broth as described previously (37). Toxin A was purified by ammonium sulfate precipitation, ion-exchange chromatography on DEAE–Sepharose CL-60, and precipitation at pH 5.6. Toxin A was homogeneous as shown by crossed immunoelectrophoresis and polyacrylamide gel electrophoresis.

Neutrophil migration in rat peritoneal cavities and air pouches. Toxin A (0.1 μg/ml) was diluted in 1 ml of sterile phosphate-buffered saline (PBS) and injected intraperitoneally (i.p.) in normal rats or in rats treated 30 min earlier with indomethacin (2 mg/kg, subcutaneously [s.c.]), BWA4C (10 mg/kg, s.c.), nordihydroguaiaretic acid (NDGA) (60 mg/kg, s.c.), mepacrine (20 mg/kg, s.c.), dexamethasone (0.5 mg/kg, s.c.), or BN52021 (15 mg/kg, s.c.). The control animals received 1.0 ml of PBS i.p. Four hours later, the animals were sacrificed and the peritoneal cavities were harvested by injection of 10 ml of PBS containing 5 U of heparin per ml and 3% bovine serum albumin. Total and differential cell counts were performed as described elsewhere (35). The results were reported as the number of cells per milliliter of fluid.

Six-day-old rat skin air pouches were produced as described previously (9, 34). The backs of the rats were shaved, and 20 ml of sterile air was injected s.c. Three days later, 10 ml of sterile air was again injected to maintain pouch potency. Six days after the initial injection, the pouches were used. Toxin A (0.1 μg/ml) was diluted in PBS and injected into 6-day-old air pouches of normal rats or in rats pretreated 30 min earlier with dexamethasone (0.5 mg/kg, s.c.), anti-IL-1β serum (1:20, 1:40, and 1:80), or anti-TNF-α (1:40, 1:80, and 1:160) in the pouches. The control animals received 1.0 ml of PBS or preimmune serum in the pouches. Six hours after injection into the air pouches, the rats were sacrificed and the air pouches were harvested by injection of 5 ml of PBS containing 5 U of heparin per ml and 3% bovine serum albumin. Total and differential cell counts were performed as previously described (35). The results were reported as the number of cells per milliliter of exudate.

Harvest and depletion of rat peritoneal macrophages and mast cells. Thioglycolate (Tg) (3%, wt/vol; 10 ml) was injected i.p. in a group of rats, and after 4 days, peritoneal macrophages were collected from half of those rats and the number of cells was compared with that obtained from nontreated (control) rats. Toxin A (0.1 μg/ml) or *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) (10^{-8} M) was then injected i.p. into the remaining rats (control and Tg treated). Four hours later, the neutrophil migration was evaluated as described above.

The method employed to deplete peritoneal macrophages has been described previously (36). Briefly, male Wistar rats were anesthetized with ethyl ether, and three hypodermic needles were inserted into the abdominal cavity. Thirty milliliters of sterile saline was injected through the needle placed near the sternum. The abdominal cavity was then gently massaged for 1 min, and the peritoneal fluid was collected through two needles inserted into the inguinal region. This operation was repeated three times. More than 85% of the peritoneal macrophage population was harvested in the lavage fluid, and 95% of the injected saline was recovered. Control rats had the same procedures and manipulations, but no fluid was injected or withdrawn. Thirty minutes later, the peritoneal macrophage population in half of those rats was estimated by injecting 10 ml of PBS containing 5 U of heparin per ml and 3% bovine serum albumin as described above. The other half of each group received, i.p., toxin A (0.1 μg/ml) or fMLP (10^{-8} M). The neutrophil migration was evaluated 4 h later as described previously (35).

Animals were pretreated during 4 days with 48/80 compound in a dose of 0.6 mg/kg, i.p., two times a day on the first 3 days and at twice this dose on the fourth day (7). The peritoneal mast cell population in half of those rats was then estimated, comparing the numbers of these cells in rats pretreated or not (control). The other half of each group of rats received toxin A (0.1 μg/ml) or IL-8 (100 ng/ml), i.p. The neutrophil migration was evaluated 4 h later, as described previously (35).

Isolation and culture of rat macrophages. Rat peritoneal macrophages were harvested with 10 ml of RPMI containing 5 U of heparin per ml 4 days after i.p. injection of 10 ml of Tg (3%, wt/vol), as previously described (33). Macrophages were then cultured in plastic tissue culture dishes, as previously described (33). After 1 h of incubation, nonadherent cells were removed by washing three times with RPMI medium. The adherent population, consisting of 95% macrophages, was incubated for 1 h at 37°C in fresh medium (control), medium with toxin A (0.1 and 1.0 μg/ml), or medium with LPS (0.1 μg/ml). After that, the supernatants were removed, and after three further medium changes, the cells were incubated for 2 h in 4 ml of RPMI medium without stimuli. The supernatants were then harvested, ultrafiltered in YM-5, and immediately used to study peritoneal neutrophil migration as described above (35).

Chemotactic agents and drugs. Purified LPS and Tg were obtained from Difco Laboratories, Detroit, Mich.; fMLP, mepacrine, 48/80 compound, and NDGA were purchased from Sigma Chemical Company, St. Louis, Mo.; dexamethasone and indomethacin were purchased from Merck Sharp & Dohme, Sao Paulo, Brazil; BN 52021, BWA4C, and BW755C were obtained from Institute Pasteur, Paris, France; sheep anti-rat IL-1β was a gift from J. Gauldie (McMaster University, Hamilton, Ontario, Canada); and sheep anti-murine TNF-α serum and control preimmune serum were kindly provided by R. Thorpe and T. Meager (Division of Immunology, National Institute for Biological Standards and Control, London, England).

Statistical analysis. The statistical significance of differences in the mean numbers of cells in fluids of various groups was assessed by analysis of variance (ANOVA) (Fisher or Scheffé test). The data are presented as means ± standard errors of the means (SEM), and P values equal to or less than 0.05 were considered statistically significant.

RESULTS

Toxin A injected i.p. causes a dose-dependent neutrophil migration into rat peritoneal cavities at 4 h, with a maximal response at 0.1 μg/ml (2.4×10^6 neutrophils/ml [toxin A] versus 0.1×10^6 neutrophils/ml [PBS]; $P < 0.001$; $n = 6$) (Fig. 1A). The time course of toxin A (0.1 μg/ml)-induced neutrophil or mononuclear cell migration in rat peritoneal cavities is shown in Fig. 1B. Toxin A was studied at 2 to 72 h postinoculation. After 2 h, there is significant neutrophil migration (1.7×10^6 neutrophils/ml [toxin A] versus 0.01×10^6 neutrophils/ml [PBS]; $P < 0.001$; $n = 5$), which is greatest at 4 h (3.3×10^6 neutrophils/ml; $P < 0.001$; $n = 5$). This effect is transient and disappears by 8 h after inoculation. In contrast, toxin A does not alter the number of mononuclear cells in rat peritoneal cavities during the times studied over 2 to 72 h (Fig. 1B).

As shown in Fig. 2A, rats treated 4 days earlier with an i.p. injection of Tg showed a significant increase in the number of macrophages (5.6×10^6 macrophages/ml [Tg] versus 1.54×10^6 macrophages/ml [control]; $P < 0.001$; $n = 11$ and 17, respectively). Toxin A (0.1 μg/ml), but not fMLP (10^{-8} M), further increases the neutrophil migration in rats previously treated with Tg (3.5×10^6 neutrophils/ml [toxin A] versus 6.3×10^6 neutrophils/ml [toxin A plus Tg]; $P < 0.001$; $n = 6$; and 4.6×10^6 neutrophils/ml [fMLP] versus 4.5×10^6 neutrophils/ml [fMLP plus Tg]; $n = 6$) (Fig. 2B). On the other hand, when the resident peritoneal cells are depleted (0.3×10^6 mononuclear cells/ml [washed] versus 1.5×10^6 mononuclear cells/ml [control]; $P < 0.01$; $n = 10$ and 16, respectively) by previous peritoneal lavage (Fig. 3A), toxin A-induced neutrophil migration is also decreased (2.3×10^6 neutrophils/ml [toxin A] versus 1.1×10^6 neutrophils/ml [toxin A, washed]; $P < 0.01$; $n = 6$), (Fig. 3B). In contrast, the previous peritoneal lavage does not alter the neutrophil migration induced by fMLP (10^{-8} M) (2.1×10^6 neutrophils/ml [fMLP] versus 2.4×10^6 neutrophils/ml [fMLP, washed]; $n = 6$) (Fig. 3B).

To further evaluate if toxin A-induced neutrophil migration is dependent on mast cells, we treated a group of rats with 48/80 compound. Figure 4A shows that rats treated i.p. with 48/80 compound have a significantly decreased number of mast cells (0.75×10^3 mast cells/ml [48/80] versus 18.4×10^3 mast cells/ml [control]; $P < 0.001$; $n = 6$). However, the migration of neutrophils induced by toxin A (0.1 μg/ml) into the peritoneal cavities is not changed when the mast cell population is reduced by the pretreatment of rats with 48/80 compound (1.8×10^6 neutrophils/ml [toxin A] versus 1.6×10^6 neutrophils/ml [toxin A plus 48/80]; $n = 6$) (Fig. 4B). In contrast, neutrophil migration stimulated by IL-8 (100 ng/ml) appears to be dependent on the number of mast cells present in the abdominal

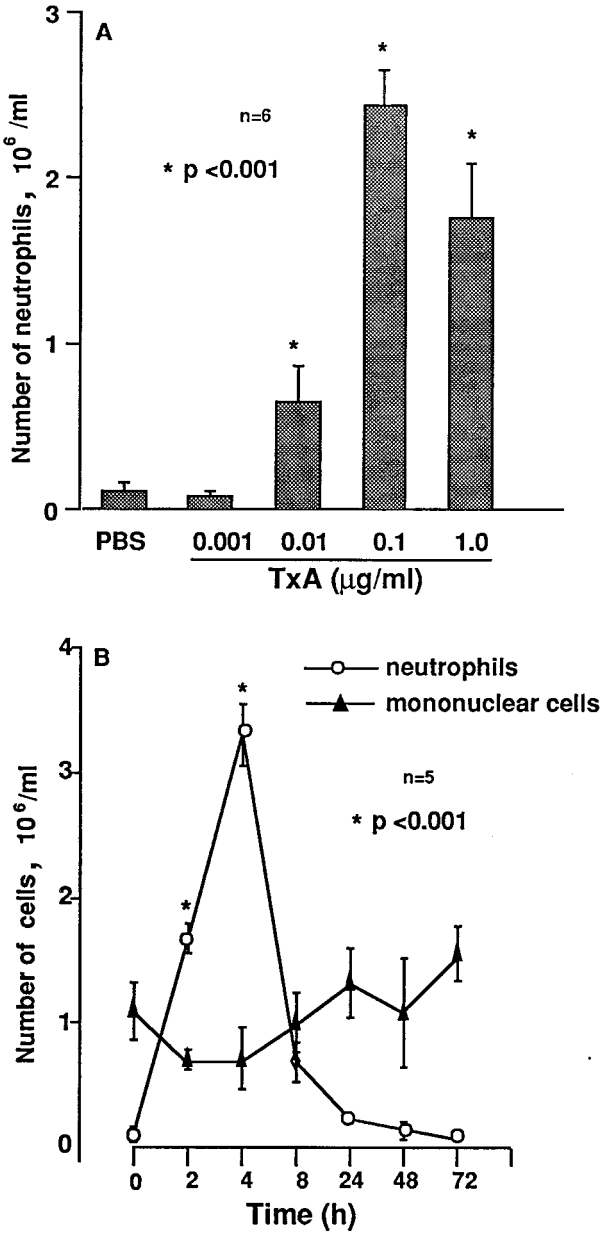

FIG. 1. (A) Dose-response of toxin A (TxA)-induced neutrophil migration into rat peritoneal cavities. (B) Time course of toxin A-induced neutrophil and mononuclear cell migration in the same animal model. *n*, number of animals for each dose and time. The results are reported as means ± SEM. *, $P < 0.001$ when compared with PBS or time zero (ANOVA test).

cavities (3.0×10^6 neutrophils/ml [IL-8] versus 1.7×10^6 neutrophils/ml [IL-8 plus 48/80]; $P < 0.01$; $n = 6$) (Fig. 4B).

To evaluate if the purified toxin A preparation was contaminated with LPS, we pretreated toxin A (0.1 μg/ml) with polymyxin (PMX) (100 μg/ml), a well-known LPS blocker, at 37°C for 30 min. In contrast to LPS at 0.1 μg/ml (3.0×10^6 neutrophils/ml [LPS] versus 0.3×10^6 neutrophils/ml [LPS plus PMX]; $P < 0.001$; $n = 6$), PMX-treated toxin A does not change the neutrophil migration induced by toxin A (2.9×10^6 neutrophils/ml [toxin A] versus 2.4×10^6 neutrophils/ml [toxin A plus PMX]; $P > 0.05$; $n = 6$) in rat peritoneal cavities.

We also examined if cultured supernatants from rat peritoneal macrophages stimulated by toxin A (1.0 μg/ml) induce neutrophil migration into rat peritoneal cavities. Injection of supernatants from macrophages treated with toxin A (1.0 μg/ml) significantly increased neutrophil migration (1.1×10^6 neutrophils/ml [supernatant-toxin A] versus $0.3\ 1 \times 10^6$ neutrophils/ml [supernatant-RPMI]; $P < 0.01$; $n = 5$). This effect is similar to that caused by supernatants from LPS (0.1 μg/ml)-stimulated macrophages (1.6×10^6 neutrophils/ml).

In order to examine if the phospholipase A_2 product of arachidonic acid and its metabolites produced via the cyclooxygenase or lipoxygenase pathway might be involved in toxin A-induced neutrophil migration, we studied the effects of indomethacin, BWA4C, NDGA, mepacrine, and dexamethasone. The doses and protocol used for each pharmacologic blocker were based on previous reports (5, 6, 10, 32). As shown in Table 1, BWA4C, NDGA, mepacrine, and dexamethasone significantly reduce the neutrophil migration into peritoneal cavities induced by toxin A. However, pretreatment with indomethacin or BN52021 fails to reduce significantly the toxin A effect.

To determine if IL-1β or TNF-α is involved in toxin A-induced neutrophil migration, we used the rat s.c. air pouch model. The pretreatment of rat s.c. air pouches, 30 min earlier, with serial dilutions of serum anti-IL-1β (1:20 [0.7×10^6 neutrophils/ml], 1:40 [1.1×10^6 neutrophils/ml], and 1:80 [1.4×10^6 neutrophils/ml] [versus 2.2×10^6 neutrophils/ml for toxin A; [$P < 0.05$; $n = 5$]) and anti-TNF-α (1:40 [1.1×10^6 neu-

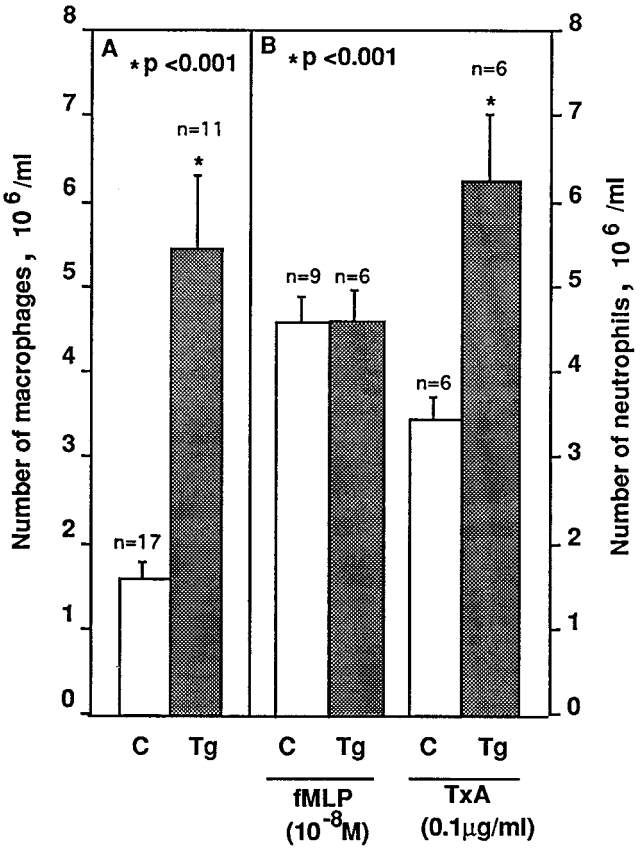

FIG. 2. (A) Increase in macrophage migration into the peritoneum in rats treated i.p. with 3% Tg over 4 days. (B) Toxin A (TxA) (0.1 μg/ml), but not fMLP (10^{-8} M), further increases the neutrophil migration into rat peritoneal cavities previously primed with 3% Tg. C, control rats injected with PBS; *n*, number of animals studied in each experimental group. The data are reported as means ± SEM. *, $P < 0.001$ for Tg versus control and for Tg plus TxA versus control plus TxA (ANOVA test).

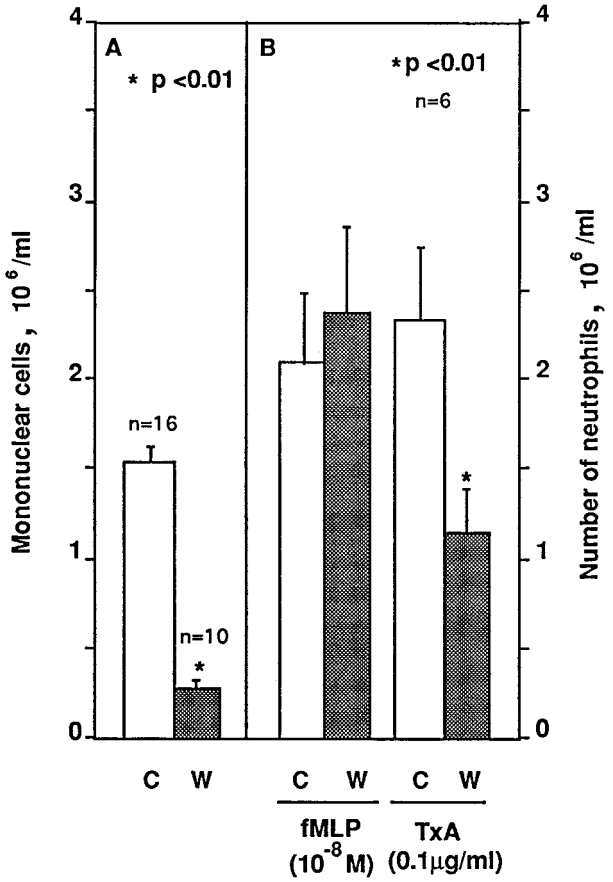

FIG. 3. Resident peritoneal cells were removed by lavage of the peritoneal cavity. (A) Macrophage population in control (C) and washed (W) rat peritoneal cavities. (B) Neutrophil migration induced by i.p. injections of fMLP (10^{-8} M) and toxin A) (TxA) (0.1 μg/ml) in C and W peritoneal cavities. *n*, number of animals in each treated group. The data are reported as means ± SEM. *, $P < 0.01$ for washed versus control and for washed plus TxA versus control plus TxA (ANOVA test).

trophils/ml], 1:80 [1.7×10^6 neutrophils/ml], and 1:160 [3.9×10^6 neutrophils/ml] [versus 4.3×10^6 neutrophils/ml for toxin A; $P < 0.01$ for the 1:40 and 1:80 dilutions; $n = 5$) significantly reduced the toxin A-induced neutrophil migration (Fig. 5 and 6).

DISCUSSION

C. difficile is a major recognized cause of antibiotic-associated inflammatory diarrhea and pseudomembranous colitis, through its toxins A and B. However, its pathogenesis remains poorly understood (11). We studied the effects of toxin A on neutrophil migration in vivo and found that toxin A-induced neutrophil migration is largely an indirect effect and is dependent on resident macrophages. The contribution of macrophages to the effect of toxin A is supported by the observations that neutrophil migration induced by toxin A is potentiated by the increased peritoneal macrophage population induced by Tg pretreatment. Conversely, lavage of peritoneal cavities, which reduces resident cells (36), decreases neutrophil migration induced by toxin A. Furthermore, incubation of macrophages with toxin A causes a release into the supernatant of a factor(s) which stimulated neutrophil migration when injected into rat peritoneal cavities. Additional indirect evidence favoring this hypothesis is the observed reduction of toxin A-induced neutrophil migration in dexamethasone-pretreated rats. This dexamethasone inhibitory effect can be explained by the blockade of the release of chemotactic factors, stimulated by inflammatory stimuli (5, 10).

In addition, mast cells can no longer be regarded simply as cells that initiate acute allergic reactions through the release of rapidly metabolized mediators, such as histamine and products of arachidonic acid oxidation. These cells clearly can also orchestrate the infiltration of leukocytes into sites of mast cell activation, an effect that was at least partially cytokine dependent (16, 33). Macrophages, neutrophils, and mast cells can be stimulated to synthesize TNF-α. However, only mast cells stored TNF-α in their granules. Activated mast cells, therefore, released TNF-α immediately from preformed stores and later released newly synthesized TNF (17). This suggests that TNF-α derived from mast cells might participate in the early phase of inflammation (43). Several reports showed indirect evidence of a role for mast cells in toxin A-induced rat intestinal inflammation (4, 27, 28, 31). However, two experiments suggest that mast cells do not appear to be involved in toxin A-induced neutrophil migration into the peritoneal cavity. First, treatment of rats with 48/80, a compound that induces degranulation and decreases the mast cell population (7), does not alter the migration of neutrophils induced by toxin A in the peritoneal cavity. Although these results come from a different model, they provide support for the indirect evidence of a role for mast cells in intestinal inflammation induced by toxin A, as shown by other investigators (4, 27, 28, 31). Second, toxin A induces a significant neutrophil migration into 6-day-old air pouches, a cavity where the cell population is essentially macrophages (9, 20). These data further suggest that toxin A causes neutrophil migration by an indirect mechanism, probably via the release of neutrophil chemotactic factors from resident macrophages.

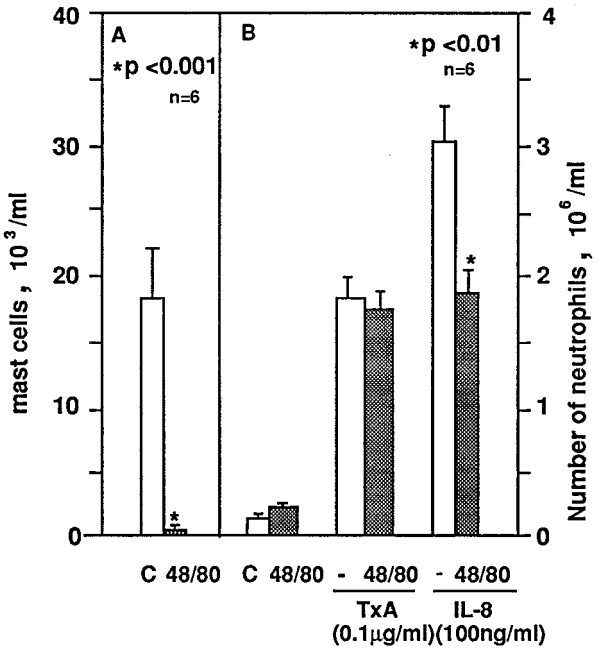

FIG. 4. (A) Number of mast cells present in PBS (control [C]) and 48/80-pretreated rat peritoneal cavities. (B) Neutrophil migration induced by toxin A (TxA) (0.1 μg/ml) and IL-8 (100 ng/ml) in the groups treated or not treated with 48/80 compound. *n*, number of animals in each group. The data are reported as means ± SEM. *, $P < 0.001$ for control versus 48/80 compound (A) and $P < 0.01$ for IL-8 versus IL-8 plus 48/80 compound (B) (ANOVA test).

TABLE 1. Effects of potential pharmacologic blockers on toxin A-induced neutrophil migration in rat peritoneal cavities

Treatment	n^a	Neutrophils (10^6)/ml (mean ± SEM)	Inhibition (%)	P	Pharmacology
PBS (1.0 ml, i.p.)	6	0.2 ± 0.12			
Toxin A (0.1 μg/ml, i.p.)	6	2.8 ± 0.30			Toxin A from *C. difficile*
Toxin A + NDGA (60 mg/kg, s.c.)	6	0.8 ± 0.24	71.4	<0.001	Dual cyclo- and lipoxygenase inhibitor
Toxin A + dexamethasone (0.5 mg/kg, s.c.)	6	0.5 ± 0.18	82.1	<0.001	Cytokine synthesis and phospholipase A_2 inhibitor
Toxin A + indomethacin (2 mg/kg, s.c.)	6	3.1 ± 0.29	NS[b]	NS	Cyclooxygenase inhibitor
Toxin A + mepacrine (20 mg/kg, s.c.)	5	1.8 ± 0.19	35.7	<0.01	Phospholipase A_2 inhibitor
Toxin A + BWA4C (10 mg/kg, s.c.)	6	1.4 ± 0.31	50.0	<0.01	Lipoxygenase inhibitor
Toxin A + BN52021 (15 mg/kg, s.c.)	5	2.3 ± 0.60	NS	NS	PAF antagonist

[a] *n*, number of animals in each treatment group.
[b] NS, not significant ($P > 0.05$).

Since toxin A-induced neutrophil migration appears to be indirect and mediated by macrophages, we sought to pharmacologically explore the possible mediators involved in this event, using phospholipase A_2 inhibitors, cyclooxygenase and lipoxygenase inhibitors, a platelet-activating factor (PAF) antagonist, and anti-TNF-α and anti-IL-1β antibodies. We find that two phospholipase A_2 inhibitors, dexamethasone and mepacrine, block the neutrophil migration into the peritoneal cavity induced by toxin A. When we examined the involvement of cyclooxygenase and lipoxygenase pathways, we found significant reductions in neutrophil migration with the selective lipoxygenase inhibitor BWA4C and with the dual cyclo- and lipoxygenase inhibitor NDGA but not with the selective cyclooxygenase inhibitor indomethacin or with the PAF antagonist BN52021. These findings suggest that the products of the lipoxygenase pathway, notably leukotriene B_4, which is a potent chemotactic agent for leukocytes (15), may be more relevant to toxin A-induced neutrophil migration than are the other products of arachidonic acid. On the other hand, the cyclooxygenase pathway does not appear to be involved in toxin A-induced neutrophil migration. However, Walker and coworkers (41) demonstrated that the inhibition of leukocyte migration by indomethacin is independent of its effects on cyclooxygenase activity.

Several reports indicated that prostaglandin synthesis could be involved in other steps of the inflammatory secretion induced by *C. difficile* toxin A or other microorganisms. Fang and coworkers (11) reported the inhibition of rabbit intestinal hemorrhagic fluid secretion by the cyclooxygenase inhibitor indomethacin, and they suggested that prostaglandin synthesis was

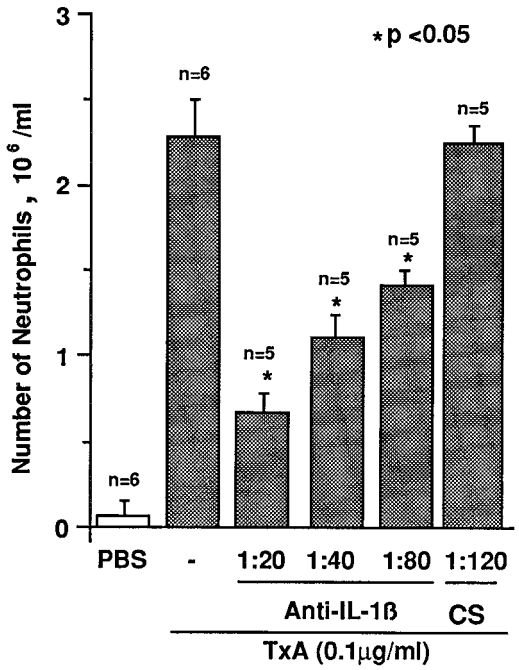

FIG. 5. Neutrophil migration induced by toxin A (TxA) (0.1 μg/ml) into air pouches pretreated, 30 min earlier, with serial dilutions of anti-IL-1β (1:20, 1:40, 1:80) in pouches. CS, control serum; *n*, number of animals in each group. The results are reported as means ± SEM. *, $P < 0.05$ for anti-IL-1 (1:20 or 1:40) plus TxA versus TxA and for anti-IL-1 (1:80) plus TxA versus TxA (ANOVA test).

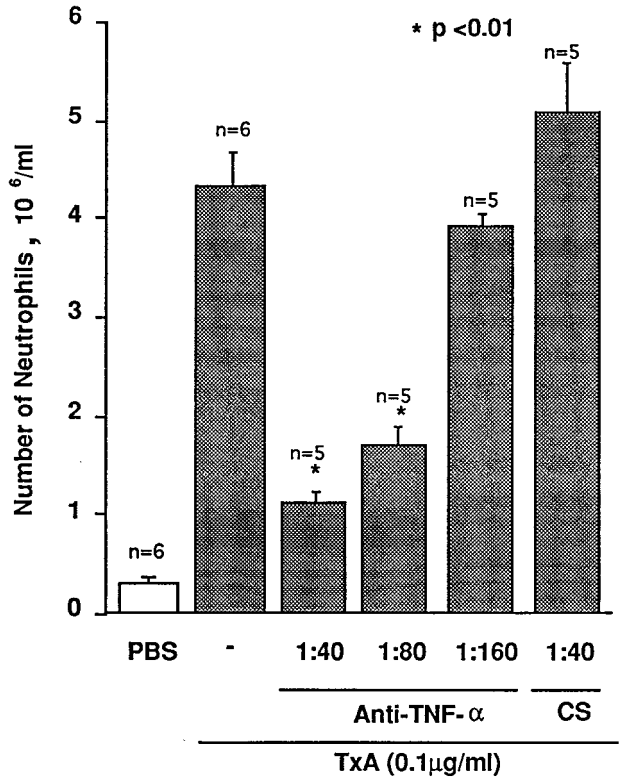

FIG. 6. Neutrophil migration induced by toxin A (TxA) (0.1 μg/ml) into air pouches pretreated, 30 min earlier, with anti-TNF-α serum (1:40, 1:80, and 1:160). CS, control serum; *n*, number of animals in each group. The results are reported as means ± SEM. *, $P < 0.01$ for anti-TNF-α (1:40 or 1:80) plus TxA versus TxA (ANOVA test).

involved in the inflammatory secretion seen with toxin A. Moreover, rabbit intestinal fluid secretion mediated by *Salmonella typhimurium*, *Shigella flexneri*, and *Vibrio clolerae* was also inhibited by pretreatment with indomethacin (18), suggesting that eicosanoids were involved in the secretory response.

The PAF antagonist BN52021 did not alter the neutrophil migration induced by toxin A. Nevertheless, additional evidence suggested that PAF was not able to induce neutrophil migration in various animals models of inflammation (6). However, it could be involved in others events of intestinal inflammation and secretion associated with toxin A from *C. difficile*. Recently, Fonteles and coworkers (14) demonstrated that the PAF antagonists BN52021, WEB 2170, and SR27417 inhibit the hemorrhagic inflammatory fluid secretion in rabbit ileum loops.

Anti-TNF-α antibody inhibits toxin A-induced neutrophil migration by 74.4% in cutaneous air pouches. Similarly, when anti-IL-1β is injected into 6-day-old air pouches, toxin A-induced neutrophil accumulation is inhibited by 70.1%. As with LPS (42), these data provide evidence that TNF-α and IL-1β likely play an important role in neutrophil accumulation at sites of toxin A action and suggest that these cytokines indirectly mediate toxin A-induced neutrophil migration. Despite the potent effects of LPS on neutrophils, the initial effector cells of endotoxin-induced organ injury are probably the tissue macrophages. These cells produce many inflammatory mediators and respond to endotoxin by quickly producing large amounts of TNF and IL-1 (38). These observations are similar to the report of toxin A-induced neutrophil migration in vivo. Therefore, the data indicate that TNF-α and IL-1β may play an important role in neutrophil migration induced by toxin A. It has also been reported that toxin A could induce IL-1 in mouse peritoneal macrophages (26). In addition, Flegel et al. (13) showed that toxins A and B from *C. difficile* activated human monocytes as measured by the release of IL-1, TNF-α, or IL-6.

We conclude that the neutrophil migration in rat peritoneal cavities and air pouches evoked by toxin A is mediated by cytokines such as TNF-α and IL-1β and by leukotrienes, released by resident macrophages. These findings suggest that such inflammatory mediators may be important contributors to the inflammatory colitis caused by *C. difficile* toxin A.

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