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Neutrophil migration induced by staphylococcal enterotoxin type A in mice: a pharmacological analysis

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Abstract

Staphylococcal enterotoxin type A induced marked neutrophil migration into the mouse peritoneal cavity and was dependent on the number of resident macrophages. This migratory response was dose- (16–64 μg of staphylococcal enterotoxin type A/cavity) and time-dependent, peaking at 12 h and disappearing after 72 h. Dexamethasone (0.5 mg/kg) inhibited the neutrophil migration induced by staphylococcal enterotoxin type A (32 μg; 42% inhibition). A similar response was observed with the platelet-activating factor–acether receptor antagonist, BN 52021 (ginkgolide B, 3-(1,1-dimethylethyl)-hexahydro-1,4-7*b*-trihydroxy-8-methyl-9*H*-1,7α (epoxymethano-1*H*,6α*H*-cyclopenta (*c*) furo (2,3-*b*) furo (3′, 2′: 3,4) cyclopenta (1,2-*d*) furan-5, 9, 12 (4*H*)-trione); 10 mg/kg; 57% inhibition), the histamine H_2 receptor antagonist, cimetidine (2 mg/kg; 31% inhibition), the lipoxygenase inhibitor, BWA4C (*N*-(3-phenoxycinnamyl) acetohydroxamic acid); 10 mg/kg; 73% inhibition), and capsaicin (*trans*-8-methyl-*N*-vanilyl-6-nonamide), a sensory C-fiber neuropeptide depletor. In contrast, indomethacin (5 mg/kg) had no effect on staphylococcal enterotoxin type A-induced chemotaxis. We conclude that the peritonitis induced by staphylococcal enterotoxin type A in mice is macrophage-dependent. The mechanism whereby staphylococcal enterotoxin type A stimulates macrophages to induce neutrophil recruitment remains to be elucidated.

Keywords: Staphylococcal enterotoxin type A; Staphylococcal enterotoxin type B; Neutrophil migration; Macrophage; Skin air-pouch; Neurogenic inflammation; Peritoneal cavity; Interleukin-8

1. Introduction

The enterotoxins produced by *Staphylococcus aureus* are the most common cause of food poisoning in humans (Iandolo, 1989). These toxins comprise a group of closely related proteins (25 to 30 kDa) that are classified into several immunological types designated A to E (Iandolo, 1989). A unique feature of staphylococcal enterotoxins is their ability to provoke emesis and diarrhea in humans and other primates (Bergdoll, 1989). This illness has been characterized as acute because the classic signs of intoxication may occur within 2 h. Although such signs may last 8–12 h, in humans and experimental animals, they usually disappear after 24–72 h (Jett et al., 1990). Neutrophils appear to be the major cell type involved in the host's defense against *S. aureus* (Hill et al., 1976), and the accumulation of neutrophils is a prominent feature of staphylococcal enterotoxins-induced gastroenteritis (Zehavi-Willner et al., 1984).

Although much is known about the structural organization of the various types of staphylococcal enterotoxins, the mechanism by which they act remains unknown, mainly because staphylococcal enterotoxins induce symptoms only in primates (Freer and Arbuthnott, 1986; Bobak and Guerrant, 1992).

Based on similarities between the in vivo pharmacological profile of the mediators responsible for the paw edema and the neutrophil migration into the peritoneal cavity caused by staphylococcal enterotoxin type B in mice (Desouza, 1993; Desouza and Ribeiro-DaSilva, 1996; Desouza et al., 1996) on the one hand, and the vomiting, the main symptom of food poisoning and of experimental enterotoxemia with staphylococcal enterotoxins in monkeys (Scheuber et al., 1985, 1987a,b; Denzlinger et al., 1986; Alber et al., 1989) on the other, we have proposed that the phlogistic reaction in mice may serve as a valuable model for investigating the pathophysiological mechanism of enterotoxemias.

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Staphylococcal enterotoxin type B induces a long-lasting neutrophil migration into the mouse peritoneal cavity (Desouza and Ribeiro-DaSilva, 1996). This migratory effect is dependent on the number of resident macrophages. Dexamethasone inhibits the neutrophil migration induced by staphylococcal enterotoxin type B. A similar response is observed when the mice are pretreated with the BN52021, a platelet-activating factor–acether receptor antagonist, cimetidine, a histamine H_2 receptor antagonist, or BWA4C, an inhibitor of arachidonic acid lipoxygenation. In contrast, neither indomethacin nor diphenhydramine reduces the chemotactic activity of staphylococcal enterotoxin type B.

Staphylococcal enterotoxins are exoproteins with superantigen properties. In addition to their role in the pathophysiology of food poisoning, these toxins also have a profound effect on the immune system. In vitro, the staphylococcal enterotoxins are strong T-cell activators and in mice, staphylococcal enterotoxin type A has a specificity for Vβ1, 3, 10, 11 and 17 T-cell receptors (Bobak and Guerrant, 1992). Staphylococcal enterotoxin type B and staphylococcal enterotoxin type A have the same general structure but are different entities as shown by their antigenic unrelatedness (Bergdoll, 1973). Moreover, staphylococcal enterotoxin type A is unique in that it is the first zinc-binding staphylococcal enterotoxin whose structure has been determined (Schad et al., 1995).

In this work, through the use of inhibitors, we have investigated the pharmacological mediators involved in the neutrophil migration induced by staphylococcal enterotoxin type A into the mouse peritoneal cavity and compared these with those previously reported for staphylococcal enterotoxin type B (Desouza and Ribeiro-DaSilva, 1996). To determine whether the toxin-induced chemotaxis was cell-dependent, the migratory effect of the toxin was also evaluated using the 6-day old skin air-pouch technique which provides an artificial cell-free environment.

2. Materials and methods

2.1. Animals

These studies were approved by the Animals Committee of Brazilian College of Experimental Animals in accordance with the procedures laid down by the Universities Federation for Animal Welfare. Male Swiss mice (25–30 g) were housed in temperature-controlled rooms and received water and food ad libitum until used.

2.2. Work conditions

All the procedures described below were carried out under aseptic conditions in a laminar flow cabinet. The material used was autoclaved at 127°C for 1 h and all solutions were prepared with autoclaved, deionized water.

2.3. Staphylococcal enterotoxin type A

The toxin was dissolved in sterile distilled water and stored at −20°C at a concentration of 1 mg/ml.

2.4. Contamination with bacterial endotoxin

To check for possible contamination of the staphylococcal enterotoxin type A solutions with bacterial endotoxin, samples containing staphylococcal enterotoxin type A (32 μg) or lipopolysaccharide from *Escherichia coli* (200–800 ng) were incubated for 10 min with polymyxin B (3.5 μg/ml) and tested for their ability to induce neutrophil migration.

2.5. Neutrophil migration

2.5.1. Peritoneal cavities

Staphylococcal enterotoxin type A (16–64 μg) was injected intraperitoneally (i.p.), in 0.1 ml of sterile phosphate-buffered saline. Control animals received 0.1 ml of sterile phosphate-buffered saline alone. Neutrophil migration was assessed 4, 12, 24 and 72 h after injection of the toxin.

2.5.2. Skin air-pouches

Six-day old air-pouches were produced in the dorsal skin of mice as described previously (Edwards et al., 1981). The back of the mice were shaved and 5 ml of sterile air were injected subcutaneously. Three days later, 2.5 ml of sterile air was again injected to maintain pouch patency. Six days after the initial injection, the pouches were injected with staphylococcal enterotoxin type A (16–64 μg) or dextran (300 μg, in 0.1 ml of sterile phosphate-buffered saline). Control animals received 0.1 ml of sterile phosphate-buffered saline alone. Neutrophil migration was assessed 12 h after the injection of toxin or dextran.

2.6. Peritoneal cell harvesting and counting

The cells in the peritoneal cavity or air pouches were harvested by lavage of the cavities with 3 ml of sterile phosphate-buffered saline–heparin (5 IU/ml)–bovine serum albumin (0.1%) solution. Total and differential cell counts were performed as described elsewhere (Souza et al., 1988). The results are reported as the number of cells per cavity.

2.7. Changes in the number of peritoneal macrophages

2.7.1. Pretreatment with thioglycolate

To increase the number of peritoneal macrophages, groups of five mice were injected with thioglycolate (2 ml of sterile 3% w/v solution, i.p.). Three days later, the animals were injected with staphylococcal enterotoxin type

Table 1
Dose-dependence of staphylococcal enterotoxin type A-induced neutrophil migration into the mouse peritoneal cavity

SEA (μg)	Neutrophils/cavity ($\times 10^5$)	Fold-increase
0	3.0 ± 0.3	–
16	9.0 ± 1.5^a	3
32	19.0 ± 3.0^a	6
64	21.0 ± 3.0^a	7

Staphylococcal enterotoxin type A (SEA) was injected i.p. in 0.1 ml of sterile phosphate-buffered saline; the control animals received 0.1 ml of sterile phosphate-buffered saline alone. The neutrophil counts were obtained 12 h after staphylococcal enterotoxin type A administration. The data represent the mean ± S.E.M. of five mice. $^aP \leq 0.05$, compared with the control group (Student's unpaired *t*-test).

A (16 μg/0.1 ml sterile phosphate-buffered saline, i.p.). Neutrophil migration was evaluated after 12 h.

2.7.2. Peritoneal lavage

The number of peritoneal macrophages was reduced as described by Souza et al. (1988). Mice were anesthetized with ethyl ether and three hypodermic needles were inserted into the abdominal cavity. Sterile saline (10 ml) was injected through the needle placed near the sternum. The abdominal cavity was then gently massaged for 1 min and the peritoneal fluid collected via the two needles inserted into the inguinal region. This operation was repeated three times and 95% of the injected saline was recovered. If blood was detected visually in the lavage fluid, the animal was discarded. Control (sham) mice were manipulated as described above but no fluid was injected or withdrawn. Thirty minutes after this procedure, the peritoneal macrophage population was estimated in half of the mice of each group by injecting 3 ml of sterile phosphate-buffered saline–heparin–bovine serum albumin solution, as described above. The other animals of the group received staphylococcal enterotoxin type A (32 μg/0.1 ml sterile phosphate-buffered saline, i.p.) and the neutrophil migration was estimated 12 h later.

2.8. Pharmacological investigation with different drugs

To test the effectiveness of the dose- and time-schedules employed, lipopolysaccharide from *E. coli* was used as a positive control. Except for capsaicin (*trans*-8-methyl-*N*-vanilyl-6-nonamide), which was applied topically as a 1% solution (as described by Alber et al., 1989), all drugs were administered subcutaneously (s.c.) 1 h before the i.p. injection of staphylococcal enterotoxin type A (32 μg/0.1 ml sterile phosphate-buffered saline) or lipopolysaccharide from *E. coli* (200 ng/0.1 ml sterile phosphate-buffered saline). The following drugs were used at the indicated doses: dexamethasone (0.5 mg/kg; Barja-Fidalgo et al., 1992), indomethacin (5 mg/kg; Henriques et al., 1987), BWA4C (*N*-(3-phenoxycinnamyl) acetohydroxamic acid), 10 mg/kg; Tateson et al., 1988), cimetidine (2 mg/kg; Scheuber et al., 1985) and BN52021 (ginkgolide B, 3-(1, 1-dimethylethyl)-hexahydro-1,4-7*b*-trihydroxy-8-methyl-9-*H*1,7α(epoxymethano-1*H*,6α *H*-cyclopenta (*c*) furo (2,3-*b*) furo (3′, 2′: 3,4) cyclopenta (1,2-*d*) furan-5, 9, 12 (4*H*)-trione); 50 mg/kg; Barja-Fidalgo et al., 1992).

2.9. Drugs and chemicals

BN52021 was generously provided by Dr. Edson Antunes (Department of Pharmacology, State University of Campinas). BWA4C was a gift from Prof. Célia Regina Carlini (Department of Biochemistry, Federal University of Rio Grande do Sul). Staphylococcal enterotoxin type A, indomethacin, cimetidine, capsaicin, dexamethasone and polymyxin B were purchased from Sigma (St. Louis, MO, USA). The others chemicals were of the highest grade available.

2.10. Statistical analysis

The data are reported as the mean ± S.E.M. for five animals. The results from the pretreatment experiments were compared using analysis of variance followed by the Kruskal–Wallis test ($P \leq 0.05$). The other data were compared by Student's unpaired *t*-test ($P \leq 0.05$).

3. Results

Tables 1 and 2 show the migratory response to increasing concentrations of staphylococcal enterotoxin type A (16–64 μg/cavity) and the time course of the neutrophil migration induced by 32 μg of staphylococcal enterotoxin type A/cavity, respectively. The neutrophil recruitment induced by staphylococcal enterotoxin type A was dose- and time-dependent and lasted more than 24 h. This migratory effect peaked at 12 h and disappeared by 72 h.

The incubation of staphylococcal enterotoxin type A (32 μg/cavity) with polymyxin B (3.5 μg/ml) did not interfere with the toxin's chemotactic activity, whereas the

Table 2
Time-course of the staphylococcal enterotoxin type A-induced neutrophil migration into the mouse peritoneal cavity

Time (h)	Neutrophils/cavity ($\times 10^5$)	
	Control	Treated
4	3.0 ± 1.0	16.0 ± 1.0^a
12	3.0 ± 0.5	19.0 ± 3.0^a
24	3.0 ± 1.0	10.0 ± 1.0^a
72	2.0 ± 0.5	2.0 ± 0.5^a

The dose of staphylococcal enterotoxin type A was 32 μg/cavity. The neutrophil counts were obtained at the indicated times after staphylococcal enterotoxin type A administration. The data represent the mean ± S.E.M. of five mice. $^aP \leq 0.05$, compared with the control group (Student's unpaired *t*-test).

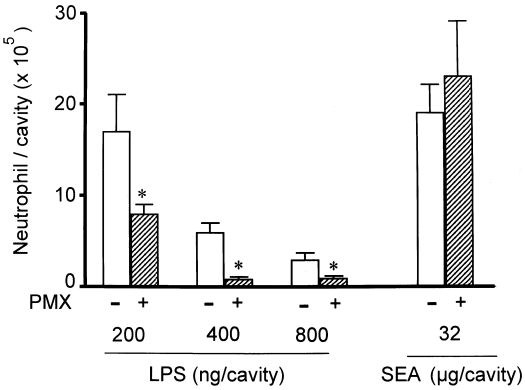

Fig. 1. The effect of polymyxin B on staphylococcal enterotoxin type A and lipopolysaccharide from *E. coli*-induced neutrophil migration into the mouse peritoneal cavity. Polymyxin B (PMX; 30 μg/cavity) was injected i.p. with staphylococcal enterotoxin type A (SEA-32 μg/cavity) or lipopolysaccharide from *E. coli* (LPS-200–800 ng/cavity) and the neutrophils counted 12 h later. The data are the mean ± S.E.M. for five mice. * $P \leq 0.05$, compared with the response in the absence of PMX (Kruskal–Wallis test).

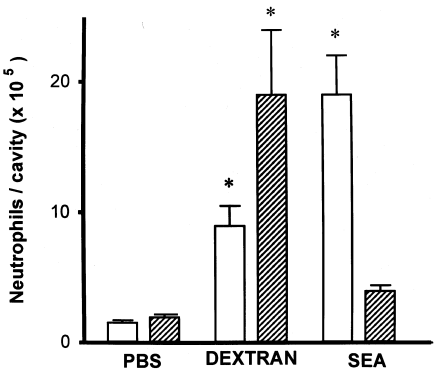

Fig. 3. Neutrophil migration induced by staphylococcal enterotoxin type A in 6-day old air-pouches. Staphylococcal enterotoxin type A (SEA-32 μg/cavity) or dextran (300 μg/cavity) in 0.1 ml of sterile phosphate-buffered saline was injected i.p., (rectangle) or into 6-day old air-pouches (rectangle with diagonal lines) while the control animals received 0.1 ml of sterile phosphate-buffered saline alone. The number of neutrophil was obtained 12 h after SEA or dextran administration. The data are the mean ± S.E.M. for five mice. * $P \leq 0.05$, compared with the corresponding PBS group (Student's unpaired *t*-test).

incubation of lipopolysaccharide from *E. coli* (200–800 ng) with the same dose of polymyxin reduced the corresponding neutrophil migration into the peritoneal cavity (Fig. 1).

Fig. 2A shows a significant increase (approximately fivefold) in the peritoneal macrophage population of mice pretreated with thioglycolate and a marked reduction (83%) in the number of these cells after peritoneal lavage. In thioglycolate-treated animals, the neutrophil migration observed 12 h after an i.p. injection of staphylococcal enterotoxin type A (32 μg/cavity) was about three times greater than in normal mice injected with the same dose of staphylococcal enterotoxin type A (Fig. 2B). On the other hand, 4 h after peritoneal lavage, the neutrophil migration induced by staphylococcal enterotoxin type A (32 μg/cavity) was significantly reduced compared with the sham-manipulated mice (Fig. 2C).

Dextran (positive control, 300 μg/cavity) caused neutrophil migration into the peritoneal cavity and 6-day old air-pouches. In contrast, the chemotactic activity of staphylococcal enterotoxin type A (32 μg/cavity) was observed only in the peritoneal cavity; staphylococcal enterotoxin type A showed no pro-migratory activity in 6-day air pouches (Fig. 3).

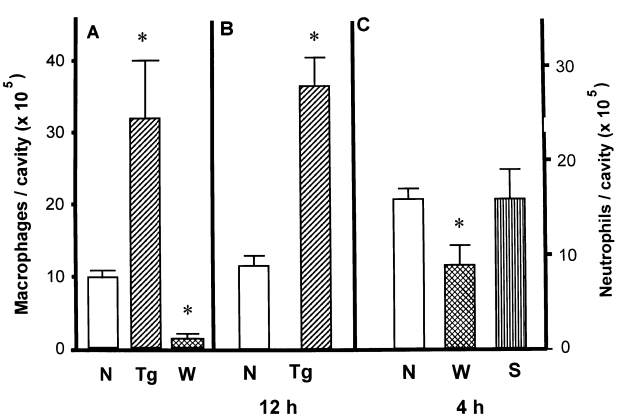

Fig. 2. The effect of increasing (Tg) or decreasing (W) the peritoneal macrophage population on the neutrophil migration induced by staphylococcal enterotoxin type A. Panel A shows the macrophage population in nontreated (N), thioglycolate-pretreated (Tg), and washed (W) peritoneal cavities. Panel B shows the neutrophil migration induced by staphylococcal enterotoxin type A (SEA-16 μg/cavity) in normal (N) and Tg mice after 12 h and Panel C shows the neutrophil migration induced by SEA (32 μg/cavity) in N, saline-washed (W) and sham (S) groups. The data are the mean ± S.E.M. for five mice. * $P \leq 0.05$, compared with the corresponding N group (Kruskal–Wallis test).

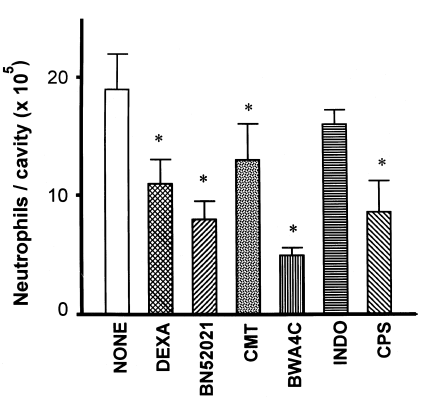

Fig. 4. The effects of anti-inflammatory drugs on the staphylococcal enterotoxin type A-induced neutrophil migration into the mouse peritoneal cavity. Drugs were given s.c. 1 h before staphylococcal enterotoxin type A (32 μg/cavity) and the number of neutrophils was determined 12 h after staphylococcal enterotoxin type A administration. None, no pretreatment; DEXA, dexamethasone (0.5 mg/kg), CMT, cimetidine (2 mg/kg), INDO, indomethacin (5 mg/kg) and CPS, capsaicin. The data are the mean ± S.E.M. for five mice. * $P \leq 0.05$, compared with the group without pretreatment (Kruskal–Wallis test).

Pretreatment with dexamethasone (0.5 mg/kg) inhibited by 42% the neutrophil migration induced by 32 μg of staphylococcal enterotoxin type A. A similar response was observed with the platelet-activating factor–acether receptor antagonist, BN 52021 (10 mg/kg; 57% inhibition), the histamine H_2 receptor antagonist, cimetidine (2 mg/kg; 31% inhibition), the lipoxygenase inhibitor, BWA4C (10 mg/kg; 73% inhibition) and capsaicin, a sensory C-fiber neuropeptide depletor. In contrast, indomethacin (5 mg/kg) had no effect on staphylococcal enterotoxin type A-induced chemotaxis (Fig. 4). All of the above drugs, except capsaicin, inhibited the neutrophil migration induced by lipopolysaccharide from *E. coli* (positive control, data not shown).

4. Discussion

Staphylococcal enterotoxin type A caused a long-lasting, dose- and time-dependent neutrophil migration into the mouse peritoneal cavity (Tables 1 and 2). This migratory effect was dependent on the number of resident macrophage and was inhibited when the animals were previously treated with various anti-inflammatory drugs.

The dose- and time-dependent neutrophil migration observed following the injection of staphylococcal enterotoxin type A into mouse peritoneal cavities contrasts with the inability of the toxin to induce neutrophil migration into subcutaneous air pouches (Fig. 3), a test which is sensitive to direct chemotactic substance (Ribeiro et al., 1991). This observation suggests that the chemotactic activity of staphylococcal enterotoxin type A is indirect and mediated by resident cells.

Interleukin-8 is a major neutrophil chemotaxin produced by human alveolar macrophages stimulated with staphylococcal enterotoxin type A (Miller et al., 1996a,b). This cytokine causes neutrophil migration by an indirect mechanism, possibly via the release of another cytokine from the resident cells (Ribeiro et al., 1991). It is therefore possible that, as in other models of neutrophil recruitment to the peritoneum (Ribeiro et al., 1991; Zeillemaker et al., 1995; Betjes et al., 1996), Interleukin-8 may mediate the staphylococcal enterotoxin type A-induced peritonitis described above.

The staphylococcal enterotoxin type A-induced neutrophil chemotaxis was blocked by pretreating the mice with BN52021, cimetidine or BWA4C but not by indomethacin (Fig. 4). Therefore, platelet-activating factor–acether, the histamine H_2 receptor and lipoxygenase products seem to be involved in neutrophil recruitment. The broad spectrum of drugs which inhibit this response may reflect the fact that staphylococcal enterotoxin type A stimulates various cell types including T-cells and mast cells (Micusan and Thibodeau, 1993). In particular, mast cells are able to release all of the mentioned above mediators. Thus, two or more cell types are probably involved in staphylococcal enterotoxin type A-stimulated neutrophil migration.

Staphylococcal enterotoxin type A is a potent inducer of several endogenous mediators including interleukin-1, tumor necrosis factor-α and interferon-γ (Bergdoll and Chesney, 1991). Interleukin-1 and tumor necrosis factor-α (Faccioli et al., 1990), as well as interferon-γ (Ribeiro et al., 1990) stimulate macrophages to release a neutrophil chemotactic factor in vitro. The neutrophil migration induced by this factor in vivo is independent of the number of resident macrophages and is observed even in dexamethasone-pretreated animals (Souza et al., 1988; Faccioli et al., 1990). In our experiments, the neutrophil migration induced by staphylococcal enterotoxin type A was dependent on the number of resident macrophages (Fig. 2) and was partially inhibited by dexamethasone (Fig. 4).

Lipopolysaccharide from *E. coli* can both stimulate and inhibit neutrophil migration (Rosenbaum et al., 1983). To exclude the possibility of contamination with lipopolysaccharide from *E. coli*, solutions of staphylococcal enterotoxin type A were incubated with polymyxin B (Cunha et al., 1989; Barja-Fidalgo et al., 1992). As shown in Fig. 1, the neutrophil migration responses to staphylococcal enterotoxin type A were not affected by polymyxin B whereas those to lipopolysaccharide from *E. coli* (200 ng) was.

Exudate is the principal component of the phlogistic response produced by staphylococcal enterotoxin type A and staphylococcal enterotoxin type B in the mouse hindpaw (Desouza et al., 1996; Desouza and Ribeiro-DaSilva, 1997). Although neutrophils are one of the components of staphylococcal enterotoxin type B-induced exudate (Desouza and Ribeiro-DaSilva, 1996), this is apparently not the case with staphylococcal enterotoxin type A since there was no correlation between the time-course of staphylococcal enterotoxin type A-induced mouse paw edema and the neutrophil migration. Thus, the staphylococcal enterotoxin type A-induced paw edema peaks 2 h after the toxin administration and is followed by a sharp decrease at 4 h (Desouza and Ribeiro-DaSilva, 1997), whereas the neutrophil migration peaked between 4 h and 12 h (Table 2) and disappeared only at 72 h.

Resident macrophages are involved in the neutrophil migration produced by staphylococcal enterotoxin type A (Fig. 2) and a similar response has been described for staphylococcal enterotoxin type B (Desouza and Ribeiro-DaSilva, 1996). However, staphylococcal enterotoxin type A was about four times more potent than staphylococcal enterotoxin type B in promoting neutrophil migration. Thus, while the i.p. injection of 16 μg of staphylococcal enterotoxin type A tripled the neutrophil population after 12 h (Table 1), a response of similar magnitude was observed only with an staphylococcal enterotoxin type B dose of ≥ 62.5 μg (Desouza and Ribeiro-DaSilva, 1996). Accordingly, we recently suggested the existence of a divergent mechanism in staphylococcal enterotoxin type

A- and staphylococcal enterotoxin type B-induced mouse paw edema (Desouza et al., 1996; Desouza and Ribeiro-DaSilva, 1997).

Substance P is the main peptide mediator of neurogenic inflammation and capsaicin depletes sensory neurons of their neuropeptides (Foreman, 1987). The mouse hindpaw edema induced by staphylococcal enterotoxin type A is a clinical manifestation of neurogenic inflammation (Desouza and Ribeiro-DaSilva, 1997). This conclusion was reinforced by the observation that capsaicin blocked the chemotactic effect of staphylococcal enterotoxin type A (Fig. 4). Thus, substance P may be involved in staphylococcal enterotoxin type A-induced neutrophil migration, although this peptide is not present in macrophages but occurs in sensory neurons (Foreman, 1987). Substance P-induced tissue swelling and granulocyte infiltration in mice are associated with mast cell degranulation and the neutrophil infiltration seen following the intradermal injection of substance P into mouse skin is mast-cell dependent (Yano et al., 1989). Thus, mast cells may be important intermediates between the release of neuropeptides and the local alterations in vascular tone, permeability and granulocyte infiltration following the administration of staphylococcal enterotoxin type A.

5. Conclusion

Macrophages are involved in the peritonitis induced by staphylococcal enterotoxin type A in mice. The mechanism whereby staphylococcal enterotoxin type A stimulates macrophages to induce neutrophil recruitment remains to be elucidated.

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