British Journal of Pharmacology (1998) 124, 1369–1374
http://www.stockton-press.co.uk/bjp

The intravenous administration of tumor necrosis factor alpha, interleukin 8 and macrophage-derived neutrophil chemotactic factor inhibits neutrophil migration by stimulating nitric oxide production

[2]B.M. Tavares-Murta, [1,3]F.Q. Cunha & [1]S.H. Ferreira

[1]Department of Pharmacology, Faculty of Medicine of Ribeirão Preto, USP, 14049-900 Ribeirão Preto, SP, Brazil

1 The i.v. administration of tumor necrosis factor-α (TNF-α), interleukin-8 (IL-8) and the recently described macrophage-derived neutrophil chemotactic factor (MNCF) inhibits the recruitment of neutrophils to the inflammatory site.

2 Pretreatment of mice with the NO synthase antagonist, N^G-monomethyl-L-arginine (L-NMMA, 15–60 mg kg^{-1}), but not the inactive enantiomer D-NMMA (30 mg kg^{-1}), prevented in a dose-dependent manner the TNF-α, IL-8 and MNCF-mediated inhibition of neutrophil migration into thioglycollate-challenged peritoneal cavities.

3 Treatment of the neutrophils with TNFα (10^{-7} M), IL-8 (10^{-7} M) or MNCF blocked their migration towards FMLP in the chemotaxis assay. The pretreatment of the neutrophils with L-NMMA (50–200 μM) prevented in a dose-dependent manner the inhibition of FMLP-induced chemotaxis by IL-8, but did not alter the inhibition caused by TNF-α or MNCF. Different concentrations of the NO donors, *S*-nitroso-*N*-acetylpenicillamine (SNAP) or 3-morpholino-sydnonimine (SIN-1), did not alter this chemotaxis.

4 Preincubating the neutrophils with L-NMMA (200 μM) significantly increased the TNF-α (10^{-7} M) and MNCF-mediated neutrophil adhesion to unstimulated endothelial cells, but had no effect on IL-8 (10^{-7} M)-mediated adhesion.

5 Although NO donors did not directly affect the mechanisms of neutrophil motility, NO is involved in the *in vitro* inhibitory action of IL-8 on chemotaxis. The TNF-α and MNCF-mediated inhibition of neutrophil migration seems to be indirect, by affecting the mechanisms of adhesion. It was concluded that TNF-α-, IL-8- and MNCF-mediated inhibition of neutrophil migration is associated with the stimulation of NO production.

Keywords: Cytokine; chemotaxis; cell adhesion; neutrophils; tumor necrosis factor alpha; interleukin 8; neutrophil migration; neutrophil migration inhibition; nitric oxide; L-NMMA

Introduction

The demonstration that the nitric oxide (NO) synthase inhibitors N^G-monomethyl-L-arginine (L-NMMA) and N^G-nitro-L-arginine methyl ester (L-NAME) increased leukocyte adherence suggested that NO is an important endogenous modulator of adhesion in these cells (Kubes *et al*., 1991). There are other studies which reinforce this suggestion. For example, in a feline model of myocardial ischemia and reperfusion, the decreased basal release of NO after myocardial injury preceded an enhanced polymorphonuclear leukocyte (PMN) adherence to the coronary endothelium (Ma *et al*., 1993). Furthermore, *in vivo* superfusion of the rat cremaster muscle with L-NAME also increased leukocyte adhesion to the venular endothelium (Akimitsu *et al*., 1995).

Several studies have demonstrated that Gram-negative bacteremia or circulating endotoxin decreases the migration of neutrophils into inflammatory sites (Smith *et al*., 1977; Van Dijk *et al*., 1980; Rocha & Ferreira, 1986), a phenomenon which may play an important role in the evolution of sepsis. The cytokines TNF-α, IL-8 and MNCF are among the possible mediators involved in this process. MNCF is an acidic chemotactic protein (pI < 4.0) with a MW of 54 kDa, that is obtained from LPS-stimulated macrophages. The protein's lectin-like property of being able to recognize D-galactose binding sites is important for its *in vitro* and *in vivo* neutrophil chemotactic activity (Dias-Baruffi *et al*., 1995a). MNCF induces neutrophil migration even in dexamethasone-pretreated animals, thus differentiating it from other known chemotactic mediators (Faccioli *et al*., 1990; Ribeiro *et al*., 1991; Dias-Baruffi *et al*., 1995a). Despite the fact that IL-8 and MNCF are chemotactic for neutrophils both *in vitro* and *in vivo* (Ribeiro *et al*., 1991; Dias-Baruffi *et al*., 1995a), whilst TNF-α is only chemotactic *in vivo* (Faccioli *et al*., 1990), the i.v. administration of either TNF-α or IL-8 (Otsuka *et al*., 1990; Hechtman *et al*., 1991; Cunha & Tamashiro, 1992), or of MNCF (Tavares-Murta *et al*., 1996) dose-dependently inhibits the neutrophil migration induced by several inflammatory stimuli in different animal species. Preincubating neutrophils with TNF-α or IL-8 also dose-dependently inhibits the *in vitro* chemotactic response to stimuli such as FMLP (Cunha & Tamashiro, 1992). The presence of circulating TNF-α and/or IL-8 during sepsis has been also extensively demonstrated (Van Zee *et al*., 1991; Gardlund *et al*., 1995; Watanabe *et al*., 1995).

Considering that NO blocks neutrophil adhesion, we have investigated whether the inhibitory activity of TNF-α, IL-8 and MNCF on neutrophil migration could be mediated by the release of NO.

[2] Present address: Department of Biological Sciences, Faculty of Medicine of Triângulo Mineiro, Praça Monoel Terra, 330, 38015-050 Uberaba, MG, Brazil.
[3] Author for correspondence.

Methods

Animals

Male Wistar rats (180 g) were used as the source of peritoneal macrophages for MNCF production and Balb/c mice for the *in vivo* inhibition of neutrophil migration. The animals were housed in temperature-controlled rooms and received water and food *ad libitum*.

Production of MNCF by LPS-stimulated macrophages

Preparation of macrophage supernatants containing MNCF The method for obtaining MNCF from LPS-stimulated macrophage supernatants has been described (Cunha & Ferreira, 1986). Briefly, rat macrophages were harvested in heparinized (5 IU ml^{-1}) RPMI medium from peritoneal cavities elicited 4 days earlier with thioglycollate (3%, 10 ml, w/v) and incubated in tissue culture dishes for 1 h at 37°C, in atmosphere of air containing 5% CO_2. The adherent monolayers (95% viable macrophages) were washed three times with phosphate-buffered saline (PBS, pH 7.4) and incubated with LPS (5 μg ml^{-1} of RPMI) for 30 min (37°C). The cells were washed three times with PBS followed by a final incubation with 5 ml of LPS-free medium for 90 min at 37°C. The cell-free media were collected, centrifuged (20,000 g, 5 min, 25°C) and ultrafiltered through a YM-10 membrane against sterile, deionized water (4°C). The samples were concentrated to 5 ml and chromatographed on an agarose/D-galactose column.

Purification of MNCF by affinity chromatography The LPS-stimulated macrophage supernatants (5 ml, derived from 3×10^8 cells) were chromatographed at 4°C on an agarose/D-galactose column (Pierce Chemical Co., Rockford, IL, USA), as described (Dias-Baruffi *et al*., 1995b). The material not retained by the column was eluted with sterile, deionized water, while the retarded substances were eluted with 0.4 M D-galactose. The ligand fraction (D-gal+) containing MNCF was ultrafiltered through a YM-10 membrane against sterile, deionized water at 4°C. SDS–PAGE of this preparation revealed four bands with one of them corresponding to an apparent MW of 54 kDa. Further chromatography of this fraction on a Superdex 75 column showed that the chemotactic and the inhibitory activities were recovered in the same fraction, which eluted in the volume corresponding to a MW of 54 kDa (Tavares-Murta *et al*., 1996). SDS–PAGE of this sample revealed a single band, also corresponding to 54 kDa. Chromatofocusing and isoelectrofocusing procedures showed that MNCF is an acidic protein with a pI < 4 (Dias-Baruffi *et al*., 1995b). When i.v. injected, purified MNCF inhibited the migration of neutrophils into rat peritoneal cavities stimulated with carrageenin. Since the inhibitory activity observed in the D-gal+ fraction was similar to that obtained with purified MNCF (Tavares-Murta *et al*., 1996), we have used the D-gal+ fraction in all experiments, in the present paper, and have equated it to MNCF.

The effect of L-NMMA or D-NMMA on the inhibition of in vivo *neutrophil migration by TNF-α, IL-8 and MNCF*

Murine recombinant TNF-α (mrTNF-α, 0.2 μg in 0.2 ml), human recombinant IL-8 (hrIL-8, 0.5 μg in 0.2 ml) or the macrophage-derived neutrophil chemotactic factor (MNCF, released by 6×10^6 macrophages in 0.2 ml) were injected i.v. into mice via the retro-orbital plexus. Control animals received i.v. injections of PBS. Thirty minutes later, the animals received an i.p. injection of thioglycollate 3% (w/v, 0.5 ml) to induce neutrophil migration. This inflammatory stimulus was used because it is more efficient in causing neutrophil migration in Balb/c mice (unpublished observation).

In order to test the effect of L-NMMA (15, 30 or 60 mg kg^{-1}) or D-NMMA (30 mg kg^{-1}) on the inhibitory activity of the cytokines, the animals were systemically pretreated with these substances by i.p. route, 30 min before the i.v. injections. Six hours after the thioglycollate administration, the mice were sacrificed by cervical dislocation and their peritoneal cavities were washed with 4 ml of PBS containing heparin (5 IU ml^{-1}) and the total and differential cell counts were determined, as described (Souza & Ferreira, 1985). The results are expressed as the number of neutrophils per ml of peritoneal wash, and are representative of two or three different experiments.

In vitro *neutrophil chemotaxis*

Preparation of neutrophils Viable and purified human neutrophils were obtained from the heparinized venous blood of a healthy subject using mono-poly-resolving medium fractionation, according to the manufacturer's instructions. The isolated neutrophils were washed three times with RPMI medium (180 g, 10 min) and then resuspended in RPMI containing 0.01% bovine serum albumin (RPMI-BSA).

Chemotaxis assay Chemotaxis was studied in 48-well chambers (Neuroprobe Inc., Cabin John, MD) separated by 5 μm pore size polyvinylpyrrolidone-free polycarbonate membranes. Twenty eight microliters of FMLP (10^{-7} M) diluted in RPMI-BSA were placed in the bottom chamber and 50 μl of the PMN suspension (10^6 cells ml^{-1}) were added to the top chamber. The chambers were then incubated for 1 h at 37°C with 5% CO_2, after which they were removed, fixed and stained with a Diff-Quick stain kit. The number of neutrophils, which had migrated to the lower side of the filter were counted (100 × objective) in five random fields. The results are representative of three different experiments performed in triplicate for each sample, and are expressed as the number of neutrophils per field. Neutrophils treated with RPMI which migrated towards FMLP were the positive control while neutrophils migrating towards RPMI served as the negative control.

The effect of L-NMMA on the inhibition of neutrophil chemotaxis caused by TNF-α, IL-8 and MNCF

In order to determine the *in vitro* inhibitory activity of the cytokines, neutrophils (10^6 cells ml^{-1}) were incubated for 30 min at 37°C and 5% CO_2 either in the absence (RPMI) or in the presence of hrTNF-α (10^{-7} M), hrIL-8 (10^{-7} M) or MNCF, prior to testing for the chemotactic response to FMLP as described above. MNCF was diluted in RPMI-BSA to give solutions containing the equivalent to the amount of product released by different numbers of macrophages (from 0.003 to 30×10^6 cells).

To test the effect of L-NMMA on the inhibitory activity of the cytokines, neutrophils were incubated with this substance (50, 100 or 200 μM) at 37°C with 5% CO_2 for 10 min before the addition of the cytokines.

The effect of NO donors on the FMLP-induced neutrophil chemotaxis

Neutrophils were incubated with different concentrations (30–1000 μM) of S-nitroso-N-acetyl-D,L-penicillamine (SNAP) and

3-morpholino sydnonimine (SIN-1) for 30 min, immediately before the chemotaxis assay.

In vitro *neutrophil-endothelium adhesion assay*

Endothelial monolayers A pig endothelial cell line (obtained in the Department of Nephrology, Middlesex Hospital, London University, England) was cultured in medium 199 containing 10% fetal calf serum and penicillin (100 U ml^{-1}) in 25-cm^2 flasks at 37°C in a CO_2 incubator. When required, the cells were trypsinized and plated onto 96-flat well plates. Confluent monolayers of cells were used for these experiments.

Pretreatment of neutrophils Neutrophils from healthy donors were purified as described above and incubated (10^6 cells ml^{-1}) with hrTNF-α (10^{-7} M), hrIL-8 (10^{-7} M), or MNCF (released by 6×10^6 cells) for 30 min (37°C, 5% CO_2). Control cells were treated with medium. To test the effect of L-NMMA on adhesion, neutrophils were preincubated with this substance (200 μM) for 30 min at 37°C before the addition of the cytokines.

Neutrophil adherence assay Endothelial cells (2×10^6 cells ml^{-1}) were incubated for 1 h (37°C, 5% CO_2) with medium 199. Subsequently, the neutrophils (200 μl, 10^6 cells ml^{-1}) were added in triplicate and allowed to adhere to the endothelial cells for 1 h (37°C, 5% CO_2). After this period, the wells were washed three times with previously warmed Hank's solution to remove the non-adherent neutrophils. Adherence of neutrophils on the endothelial cells was estimated by measuring the level of myeloperoxidase (MPO) in each well. The results are expressed as the percentage (%) increase in adhesion compared to the control wells (neutrophils treated with the cytokines alone).

Measurement of myeloperoxidase activity in adherent neutrophils

The MPO content of adherent neutrophils in each well was measured as a modification of the method described elsewhere (Bradley *et al*., 1982). Briefly, 50 μl of hexa-methyl-ammonium bromide solution (0.5%, w/v) diluted in PBS (pH 6.0) were added to each well, and the plates incubated for 30 min at room temperature. After, 100 μl of TMB (3,3′,5,5′,tetramethyl benzidine dihydrochloride) solution were added to the wells and incubated at room temperature for 15 min. The reaction was then stopped with 50 μl of 2% H_2SO_4. The MPO content was quantitated spectrophotometrically (570 nm) using a multiwell plate reader (Multiskan MCC/340 MKII, Flow Laboratories).

Materials

RPMI 1640 culture medium, medium 199, D-galactose, BSA fraction V, antibiotics, fetal calf serum, Hank's solution, reagents for MPO assay, L-NMMA and D-NMMA, endotoxin (*E. coli* 0111:B4 lipopolysaccharide), FMLP (N-formyl-methionyl-L-leucyl-L-phenylalanine) were all obtained from Sigma Chemical Co. (St. Louis, MO, USA). Mono-poly-resolving medium (Flow Laboratories, Irvine, Scotland), Diff-Quick stain kit (American Scientific Products, McGraw Park, IL), thioglycollate medium (Difco), heparine (liquemine, Roche), YM-10 membrane (Amicon Division, W.R. Grace & Co., Beverly, MA, U.S.A.), SNAP and SIN-1 (Galenik, Frankfurt, Germany). mrTNF-α (lot 88/532, biological activity 200,000 IU/1 μg), hrTNF-α (lot 87/650, biological activity 40,000 IU/1 μg) and hrIL-8 (lot 89/520, biological activity 1000 IU/1 μg) were a gift from Dr. Steve Poole of the National Institute for Biological Standards and Control (NIBSB), Herts, U.K.

Statistical analysis

The results are reported as the means ± s.e.mean and are representative of two or three different experiments. The means between different treatments were compared by ANOVA. If significance was determined, individual comparisons were subsequently tested with the Bonferroni's *t*-test for unpaired values. The test of difference between two proportions was used for analysis of increase in adhesion. Statistical significance was set at $P < 0.05$.

Results

Effect of L-NMMA or D-NMMA on the in vivo *inhibition of neutrophil migration caused by TNF-α, IL-8 and MNCF*

Figure 1 shows the inhibitory effect of the i.v. administration of mrTNF-α (0.2 μg), hrIL-8 (0.5 μg) or MNCF (obtained from 6×10^6 macrophages) on the neutrophil migration induced by thioglycollate (3% w/v, 0.5 ml) in mouse peritoneal cavities, as compared to PBS (closed bars). Pretreating the animals 30 min before with L-NMMA (15–60 mg kg^{-1}, i.p., open bars) dose–dependently prevented the inhibitory effect of the cytokines. In contrast, the inactive NO synthase inhibitor, D-NMMA (30 mg kg^{-1}), was ineffective against the TNF-α- and MNCF-mediated inhibition (cross-hatched bars). The administration of L-NMMA or D-NMMA alone (first group of bars) did not interfere with the neutrophil migration induced by the i.p. injection of thioglycollate.

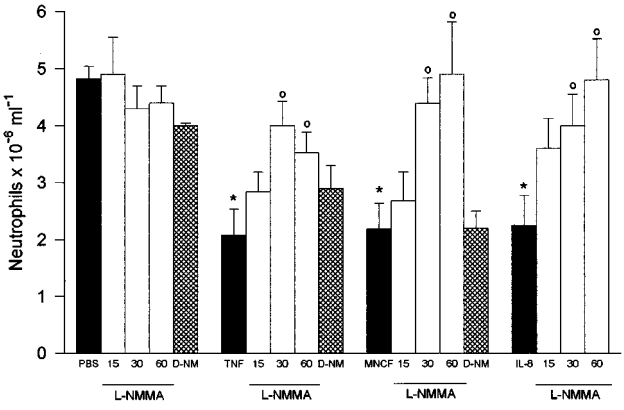

Figure 1 L-NMMA prevented the inhibitory activity of TNF-α, IL-8 and MNCF on neutrophil migration. The mice received i.v. injections (0.2 ml) of PBS, mrTNF-α (0.2 μg), hrIL-8 (0.5 μg) or MNCF (obtained from 6×10^6 macrophages) 30 min before the i.p. administration of thioglycollate (3%, 0.5 ml, closed bars). In some cases, the animals were pretreated i.p. with 15, 30 or 60 mg kg^{-1} of L-NMMA (open bars) or 30 mg kg^{-1} of D-NMMA (cross-hatched bars), 30 min before the i.v. injection of PBS or cytokines. The neutrophil migration was assessed 6 h after giving the inflammatory stimulus. The results are representative of two or three different experiments ($n = 5$ each). $^*P < 0.05$ compared to the PBS control, $^\circ P < 0.05$ compared to the response to cytokine alone (ANOVA + Bonferroni test).

The effect of L-NMMA on the in vitro *inhibition of neutrophil chemotaxis caused by TNF-α, IL-8 and MNCF*

In the FMLP-induced chemotaxis assay, the *in vitro* pretreatment of the neutrophils with L-NMMA (50–200 μM) for 10 min dose-dependently prevented the inhibitory effect of IL-8 (10^{-7} M) on neutrophil chemotaxis. In contrast, the same pretreatment did not interfere with the inhibition of chemotaxis caused by hrTNF-α (10^{-7} M) or MNCF (Figure 2). A dose-response curve for MNCF (material obtained from 0.003 to 30×10^6 macrophages per well) showed that this factor inhibited neutrophil chemotaxis at the three highest concentrations and that L-NMMA did not interfere with this effect at any of the concentrations tested (data not shown). Also, the *in vitro* pretreatment of the neutrophils with L-NMMA alone did not alter FMLP-induced chemotaxis (Figure 2).

The effect of NO donors on FMLP-induced neutrophil chemotaxis

Figure 3 shows that the NO donors SNAP or SIN-1 (30–1000 μM), when incubated for 30 min with the neutrophils, did not alter the chemotaxis induced by FMLP (10^{-7} M).

Effect of pretreatment of neutrophils with L-NMMA on the cell adhesion caused by TNF-α, IL-8 and MNCF

Table 1 shows that pretreating the neutrophils with L-NMMA (200 μM) 30 min before the addition of TNF-α (10^{-7} M) or MNCF (obtained from 6×10^6 macrophages), a significant increase in the percentage of adherent neutrophils (30.7% and 78%, respectively) was observed compared to the cells treated with the cytokines alone (data not shown). However, treating the neutrophils with L-NMMA plus IL-8 (10^{-7} M) did not increase the adherence to endothelial cells (0%). L-NMMA alone did not increase the adhesion of medium-treated neutrophils to unstimulated endothelial cells (first line).

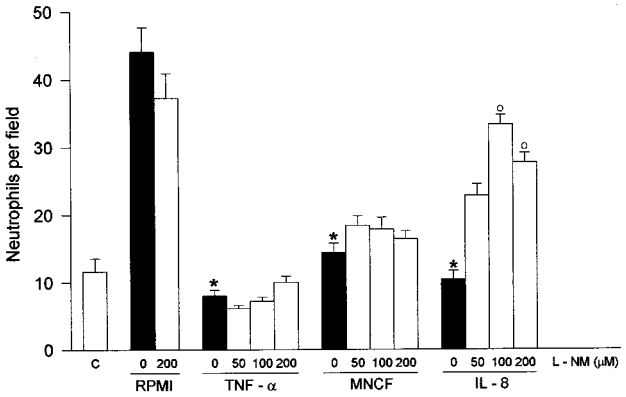

Figure 2 Divergent effects of L-NMMA on the inhibition of FMLP-induced neutrophil chemotaxis by TNF-α, MNCF and IL-8. The neutrophils were treated with RPMI, hrTNF-α (10^{-7} M), hrIL-8 (10^{-7} M) or MNCF (obtained from 3×10^6 macrophages) 30 min before the FMLP-induced chemotaxis assay (closed bars). The cells were pretreated with RPMI (0) or L-NMMA (50, 100 and 200 μM, open bars) 10 min before the addition of the desired cytokine or RPMI alone. Chemotaxis was induced by FMLP (10^{-7} M). C represents the negative control. The experiments were performed 3-fold, each time in triplicate. The results are expressed as the number of neutrophils per field. $^*P<0.05$ compared to FMLP alone in RPMI, $^\circ P<0.05$ compared to IL-8 alone (ANOVA + Bonferroni test).

Discussion

The present study demonstrates that pretreating mice with the NO synthase inhibitor, L-NMMA, dose-dependently prevented the inhibitory effect of TNF-α, IL-8 and MNCF on the neutrophil migration induced by thioglycollate injected i.p. This response may reflect the inhibition of NO synthase, since the D-enantiomer, D-NMMA, which does not inhibit NO synthase, had no effect on the inhibitory action of TNF-α or MNCF. In this context, there is *in vitro* (Ma *et al*., 1993) and *in vivo* (Gauthier *et al*., 1995) evidence showing that NO inhibits neutrophil adhesion and that NO synthase inhibitors increase neutrophil-endothelium adhesion (Kubes *et al*., 1991; Niu *et al*., 1994; Akimitsu *et al*., 1995), as well as emigration of these cells to extravascular tissues (Kubes *et al*., 1991).

Despite the evidence that the inhibitory action of circulating TNF-α, IL-8 and MNCF on neutrophil migration contributes for the evolution of sepsis (Otsuka *et al*., 1990; Hechtman *et al*., 1991; Cunha & Tamashiro, 1992; Tavares-Murta *et al*.,

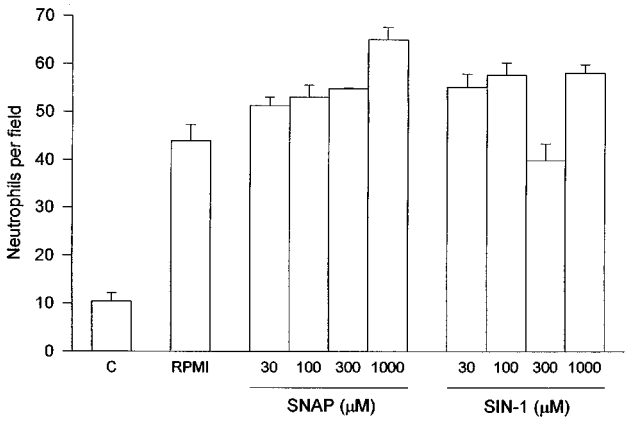

Figure 3 NO donors do not influence FMLP-induced neutrophil chemotaxis. The neutrophils were treated with RPMI or different concentrations of SNAP or SIN-1 (30–1000 μM) 30 min before testing the FMLP-induced chemotaxis (10^{-7} M). The chemotaxis in RPMI alone served as a positive control. C represents the negative control. The experiments were performed threefold, each time in triplicate. The results are expressed as the number of neutrophils per field.

Table 1 L-NMMA selectively enhanced the adhesion of neutrophils treated with TNF-α or MNCF, but not IL-8, to unstimulated endothelial cells

Treatment of neutrophils	*Increase in adhesion (%)*
medium 199	0
hrTNF-α	30.7*
MNCF	78.0*
hrIL-8	0

The neutrophils which have been preincubated with L-NMMA (200 μM) for 30 min before the addition of cytokines were then exposed to medium (control), hrTNF-α (10^{-7} M), hrIL-8 (10^{-7} M) or MNCF (obtained from 6×10^6 macrophages) 30 min before the adhesion assay. The PMNs (10^6 cells ml^{-1}) were added in triplicate and allowed to adhere to the plated endothelial cells for 1 h. Adherence was estimated by measuring the level of MPO in each well. The results are representative of three experiments, each in triplicate, and expressed as the percentage (%) in increase in adhesion compared to medium-treated (first line) or cytokine-treated wells alone (in the absence of L-NMMA). $^*P<0.05$ (Test difference between two proportions).

1996), the mechanisms involved in this process are still not clear. Our results suggest that NO is involved in the inhibition of neutrophil recruitment seen after the intravenous administration of these cytokines. NO synthase inhibitors have been reported to ameliorate the symptoms of septic shock through a beneficial effect on cardiovascular function (reviewed by Moncada & Higgs, 1995). In addition to these effects, our data show that NO synthase inhibitors may prevent the inhibition of leukocyte emigration to the inflammatory focus, thus restoring the host's defense against bacterial infection.

The adhesion to endothelial cells and the neutrophil motility in the direction of the chemotactic gradient are crucial steps in neutrophil emigration (Furie & Randolph, 1995). For this reason, the effect of L-NMMA was tested in both *in vitro* chemotaxis assay and in neutrophil-endothelium adhesion.

In the chemotaxis assay L-NMMA dose-dependently prevented the inhibitory action of IL-8, without affecting that of TNF-α or MNCF when FMLP was used as the chemotactic stimulus. On the other hand, in the neutrophil-endothelium adhesion assay, L-NMMA increased the adhesion of neutrophils incubated with TNF-α or MNCF, but not those incubated with IL-8. Thus, the *in vivo* inhibitory effect of TNF-α, IL-8 and MNCF on neutrophil migration cannot be explained by a single NO-mediated mechanism. Our results indicate that TNF-α and MNCF inhibit neutrophil chemotaxis and adhesion by a mechanism(s) different from that of IL-8.

That the *in vitro* inhibitory activity of IL-8 on chemotaxis seems to be mediated by an NO-dependent mechanism is not supported by the observation that NO donors (SNAP or SIN-1) *per se* did not inhibit neutrophil chemotaxis. A possible explanation for this may be that NO released outside the cell can not reach the intracellular structures responsible for neutrophil locomotion, because of the presence of cytosolic thiol compounds such as gluthatione (Buchmuller-Rouiller *et al*., 1995), which can trap NO. In support of the suggestion that IL-8 inhibits neutrophil motility by activating an NO-sensitive intracellular pathway, there is evidence showing that IL-8 interferes with the cytoskeletal integrity (Furie & Randolph, 1995). Also, human neutrophil actin has been reported to be a substrate for NO-dependent ADP ribosylation, being this event accompanied by inhibition of cytoskeletal assembly of these cells (Clancy *et al*., 1995).

Although TNF-α and MNCF block neutrophil locomotion by an NO-independent mechanism, NO seems to be involved in the *in vivo* inhibitory activity of these cytokines, since L-NMMA prevented the inhibition of neutrophil migration normally seen following the intravenous administration of TNF-α or MNCF. The fact that L-NMMA increased the adhesion of TNF-α or MNCF-treated neutrophils suggests that these substances inhibit *in vivo* neutrophil migration by interfering with an NO-dependent adhesion mechanism. There are several *in vivo* (Gauthier *et al*., 1995) and *in vitro* (Ma *et al*., 1993; Niu *et al*., 1994) studies showing that NO inhibits leukocyte adhesion, but the mechanism(s) involved are not yet clear. One possibility may be that the inhibition of NO synthesis leads to increased intracellular oxidative stress, which subsequently inhibits the neutrophil-endothelial cell interaction (Niu *et al*., 1994). Recently, NO was shown to inhibit IL-1-stimulated VCAM-1 expression in parallel with a reduction in the monocyte adhesion to endothelial cells (De Caterina *et al*., 1995). Our present data do not rule out the possibility that the *in vivo* inhibitory effect of TNF-α, IL-8 or MNCF on neutrophil migration is also mediated by endothelium-derived NO.

Overall, our results indicate that TNF-α, IL-8 and MNCF inhibit neutrophil recruitment to the inflammatory site by stimulating the release of NO, although the final pathways involved are different. Thus, IL-8 interferes principally with the mechanisms of neutrophil motility to impair the migration of these cells while TNF-α and MNCF affect the mechanisms of neutrophil adhesion to the endothelium, part of which may involve an indirect action via NO release.

The authors wish to thank N.I.S. Freiri and A.K. dos Santos for technical assistance and N.I.S. Freiri for performing the neutrophil adhesion assay. The authors also thank CNPq and FAPESP financial support.

References

AKIMITSU, T., GUTE, D.C. & KORTHUIS, R.J. (1995). Leukocyte adhesion induced by inhibition of nitric oxide in skeletal muscle. *J. Appl. Physiol.,* **78,** 1725–1732.

BRADLEY, P.P., PRIEBAT, D.A., CHRISTENSEN, R.D. & ROTHSTEIN, G. (1982). Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker. *J. Invest. Dermatol.,* **78,** 206–209.

BUCHMULLER-ROUILLER, Y., CORRADIN, S.B., SMITH, J., SCHNEIDER, P., RANSIJN, A., JONGENEEL, C.V. & MAUEL, J. (1995). Role of glutathione in macrophage activation: effect of cellular glutathione depletion on nitrite production and leishmanicidal activity. *Cell. Immunol.,* **164,** 73–80.

CLANCY, R., LESZCZYNSKA, J., AMIN, A., LEVARTOVSKY, D. & ABRAMSON, S.B. (1995). Nitric oxide stimulates ADP ribosylation of actin in association with the inhibition of actin polymerization in human neutrophils. *J. Leukoc. Biol.,* **58,** 196–202.

CUNHA, F.Q. & FERREIRA, S.H. (1986). The release of a neutrophil chemotactic factor from peritoneal macrophages by endotoxin: inhibition of glucocorticoids. *Eur. J. Pharmacol.,* **129,** 65–76.

CUNHA, F.Q. & TAMASHIRO, W.M.S.C. (1992). Tumour necrosis factor-alpha and interleukin-8 inhibit neutrophil migration *in vitro* and *in vivo*. *Med. Inflamm.,* **1,** 397–401.

DE CATERINA, R., LIBBY, P., PENG, H., THANNICKAL, V.J., RAJAVASHISTH, T.B., GIMBRONE JR, M.A., SHIN, W.S. & LIAO, J.K. (1995). Nitric oxide decreases cytokine-induced endothelial activation. *J. Clin. Invest.,* **96,** 60–68.

DIAS-BARUFFI, M., CUNHA, F.Q., FERREIRA, S.H. & ROQUE-BARREIRA, M.C. (1995a). Biological characterization of purified macrophage-derived neutrophil chemotactic factor. *Med. Inflamm.,* **4,** 263–269.

DIAS-BARUFFI, M., CUNHA, F.Q., FERREIRA, S.H. & ROQUE-BARREIRA, M.C. (1995b). Isolation and partial characterization of neutrophil chemotactic factor released from macrophages (MNCF). *Med. Inflamm.,* **4,** 257–262.

FACCIOLI, L.H., SOUZA, G.E.P., CUNHA, F.Q., POOLE, S. & FERREIRA S.H. (1990). Recombinant interleukin-1 and tumour necrosis factor induce neutrophil migration *in vivo* by indirect mechanisms. *Agents Actions,* **30,** 344–349.

FURIE, M.B. & RANDOLPH, G.J. (1995). Chemokines and tissue injury. *Am. J. Pathol.,* **146,** 1287–1301.

GARDLUND, B., SJOLIN, J., NILSSON, A., ROLL, M., WICKERTS, C. & WRETLIND B. (1995). Plasma levels of cytokines in primary septic shock in humans: correlation with disease severity. *J. Infect. Dis.,* **172,** 296–301.

GAUTHIER, T.W., DAVENPECK, K.L. & LEFER, A.M. (1995). Nitric oxide attenuates leukocyte-endothelial interaction via P-selectin in splanchnic ischemia-reperfusion. *Am. J. Physiol.,* **267,** G562–568.

HECHTMAN, D.H., CYBULSKY, M.I., FUCHS, H.J., BAKER, J.B. & GIMBRONE JR, M.A. (1991). Intravascular IL-8. Inhibitor of polymorphonuclear leukocyte accumulation at sites of acute inflammation. *J. Immunol.,* **147,** 883–892.

KUBES, P., SUZUKI, M. & GRANGER, D.N. (1991). Nitric oxide: an endogenous modulator of leukocyte ahesion. *Proc. Natl. Acad. Sci. U.S.A.,* **88,** 4651–4655.

MA, X., WEYRICH, A.S., LEFER, D.J. & LEFER, M. (1993). Diminished basal nitric oxide release after myocardial ischemia and reperfusion promotes neutrophil adherence to coronary endothelium. *Circ. Res.,* **72,** 403–412.

MONCADA, S. & HIGGS, E.A. (1995). Molecular mechanisms and therapeutic strategies related to nitric oxide. *FASEB J.,* **9,** 1319–1330.

NIU, X., SMITH, C.W. & KUBES, P. (1994). Intracellular oxidative stress induced by nitric oxide synthesis inhibition increases endothelial cell adhesion to neutrophils. *Circ. Res.,* **74,** 1133–1140.

OTSUKA, Y., NAGANO, K., NAGANO, K., HORI, K., OH-ISHI, J., HAYASHI, H., WATANABE, N. & NIITSU, Y. (1990). Inhibition of neutrophil migration by tumor necrosis factor. Ex vivo and in vivo studies in comparison with in vitro effect. *J. Immunol.,* **145,** 2639–2643.

RIBEIRO, R.A., FLORES, C.A., CUNHA, F.Q. & FERREIRA, S.H. (1991). IL-8 causes *in vivo* neutrophil migration by a cell-dependent mechanism. *Immunol.,* **73,** 472–477.

ROCHA, N.P. & FERREIRA, S.H. (1986). Restoration by levamisole of endotoxin-inhibited neutrophil migration, oedema and increased vascular permeability induced by carrageenin. *Eur. J. Pharmacol.,* **122,** 87–92.

SMITH, M.J.H., FORD-HUTCHINSON, A.W. & WALKER, J.R. (1977). Anti-inflammatory activity of bacterial endotoxin. *J. Pharm. Pharmacol.,* **29,** 702–703.

SOUZA, G.E.P. & FERREIRA, S.H. (1985). Blockade by anti-macrophage serum of the migration of PMN neutrophils into the inflamed peritoneal cavity. *Agents Actions,* **17,** 97–103.

TAVARES-MURTA, B.M., CUNHA, F.Q., DIAS-BARUFFI, M., ROQUE-BARREIRA, M.C. & FERREIRA, S.H. (1996). The macrophage-derived neutrophil chemotactic factor is involved in the neutrophil recruitment inhibitory activity present in the LPS-stimulated macrophage supernatants. *Med. Inflamm.,* **5,** 116–120.

VAN DIJK, W.C., VERBRUGH, H.A., VAN DER TOL, M.E., PETERS, R., PETERSON, P.K., QUIE, P.G. & VERHOEF, J. (1980). Interactions of phagocytic and bacterial cells in patients with bacteremia caused by Gram-negative rods. *J. Infect. Dis.,* **141,** 441–448.

VAN ZEE, K.J., DEFORGE, L.E., FISCHER, E., MARANO, M.A., KENNEY, J.S., REMICK, D.G., LOWRY, S.F. & MOLDAWER, L.L. (1991). IL-8 in septic shock, endotoxemia, and after IL-1 administration. *J. Immunol.,* **146,** 3478–3482.

WATANABE, S., MUKAIDA, N., IKEDA, N., AKIYAMA, M., HARADA, A., NAKANISHI, I., NARIUCHI, H., WATANABE, Y. & MATSUSHIMA, K. (1995). Prevention of endotoxin shock by an antibody against leukocyte integrin β2 through inhibiting production and action of TNF. *Int. Immunol.,* **7,** 1037–1046.

(Received November 12, 1998
Revised April 6, 1998
Accepted April 27 1998)