Inhibitory effect upon neutrophil migration to the inflammatory focus was previously detected in the cell-free incubation fluid of lipopolysaccharide (LPS)-stimulated macrophage monolayers. In the present study we showed that the neutrophil recruitment inhibitory activity from this supernatant was mainly detected in a fraction (P2) obtained by gel filtration chromatography on Sephacryl S-300. P2 fraction was able to inhibit 'in vivo' neutrophil emigration induced by different inflammatory stimuli, but it did not affect 'in vitro' neutrophil chemotaxis induced by FMLP. When injected intravenously, P2 inhibited oedema induced by carrageenin or immunological stimuli but not the oedema induced by dextran, thus affecting cell-dependent inflammatory responses. It was observed that P2 also induced neutrophil migration when injected locally in peritoneal cavities. This activity was significantly reduced by pretreatment of the animals with dexamethasone. Cytokines, such as IL-8 and TNF-α that are known to exhibit inhibitory effect upon neutrophil migration, were not detected in P2 fraction by highly sensitive assays. Overall the results suggest the existence of a novel cytokine exhibiting 'in vivo' neutrophil inhibitory activity, referred as NRIF.

Key words: Cytokines, Macrophage-derived neutrophil recruitment inhibitory factor, Neutrophil migration

Neutrophil recruitment inhibitory factor: a possible candidate for a novel cytokine


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Introduction

Cytokines, such as IL-6, IL-8 and TNF-α, besides their pro-inflammatory effects also exhibit anti-inflammatory activities, such as inhibition of neutrophil adhesion to activated endothelial cells and 'in vivo' inhibition of neutrophil migration into inflammatory focus. These cytokines are released by various types of activated cells, such as those of phagocyte mononuclear system, polymorphonuclear leucocytes, endothelial cells and T-lymphocytes.

In our laboratory we have examined the activity of supernatants from macrophage monolayers stimulated with LPS. It was found that this crude supernatant is able to induce neutrophil migration when injected into peritoneal cavities of dexamethasone-pretreated rats. This activity was referred as macrophage-derived neutrophil chemotactic factor (MNCF). In contrast, neutrophil migration to peritoneal cavities induced by cytokines such as TNF-α and β, IL-1β and IL-8, gamma interferon and IL-8 are inhibited by dexamethasone pretreatment. In addition, the same supernatant when intravenously (i.v.) injected was shown to inhibit in a dose dependent manner neutrophil migration to an inflamed site. The factor responsible for this activity was named neutrophil recruitment inhibitory factor (NRIF).

Therefore the objective of this study was to characterize some biological properties of NRIF. For this purpose, we have submitted crude macrophage supernatant to gel filtration chromatography obtaining a fraction (P2) that retained the original inhibitory activity. We also observed that the i.v. administration of P2 was able to block cell dependent oedema. As IL-8 and TNF, cytokines for which neutrophil recruitment inhibitory activity has been described, were not detected in P2 fraction, that in addition was eluted far from the elution volume expected for these cytokines, we suggest that NRIF may constitute a novel cytokine.

Material and Methods

Animals: Adult male Wistar rats weighing 150–180 g were housed in a temperature-controlled room and received water and food ad libitum until use.

Immunization procedure: Animals were actively immunized with 200 μg ovalbumin (OVA) emulsified in 50% complete Freund adjuvant (CFA) (DIFCO, Detroit, MI, USA), by subcutaneous (s.c.) injection. Controls received s.c. injections of CFA. Rats were used in oedema paw assays on 28th day after immunization.

Preparation of macrophage supernatant: The method for obtaining NRIF by stimulating macrophage mono-
layers has been described elsewhere. Briefly, the peritoneal cavities of rats were stimulated by injection of thioglycollate medium (DIFCO) (10 ml of 3% thioglycollate medium/rat). Four days later, the peritoneal cells were harvested with RPMI 1640 (Flow Laboratories, McLean, VG, USA) and allowed to adhere in plastic tissue culture dishes for 1 h at 37°C, in an atmosphere of air with 5% of CO2. The monolayers were then washed three times with phosphate buffered saline (PBS, pH 7.4) and incubated for 30 min at 37°C with 10 μg ml⁻¹ of lipopolysaccharide of E. coli (LPS, 0111:B4, DIFCO). The supernatants were discarded and the cells (95% macrophages) washed another three more times with PBS, followed by a final 1 h incubation with 5 ml of RPMI medium per dish, at 37°C. The cell-free incubation fluids were subsequently ultradiafiltered through an YM 10 membrane (Amicon Corp, Lexington, MA, USA). The final volume (corresponding to 5% of the original volume) was filtered on 0.22 μm membrane (Millipore, Bedford, MA, USA).

Chromatographic procedure: One millilitre of macrophage supernatants prepared as described above, containing the material released by 2.5 x 10⁸ adherent cells, were dialysed against 0.5 M NaCl solution buffered with 0.02 M phosphate pH 7.4, and applied on Sephacryl S-300 column (2.6 x 70 cm; Pharmacia, Uppsala, Sweden) at a flow rate of 8 ml h⁻¹. The S-300 column was previously calibrated with a molecular weight standards kit (Pierce Chemical Co., Rockford, IL, USA) containing: ferritin (540 KDa), catalase (240 KDa), aldolase (158 KDa), BSA (67 KDa), OVA (43 KDa), chemotrypsinogen (25 KDa) and cytochrome c (12.4 KDa). Blue dextran 20 000 (Pharmacia) was used to determine the void volume of the column. Absorbance at 280 nm was determined for each fraction collected (2 ml fractions). After desalting on a PD10 column (Pharmacia), the fractions were pooled (Pool 1–Pool 10; three fractions/pool), starting with the chromatographic fractions of macrophage supernatant, now referred as fractions P1 to P10, prior to testing for chemotactic response to N-formyl-methionyl-L-leucyl-L-phenylalanine (FMLP, Sigma) in microchemotaxis assay. The chromatographic fractions were diluted in RPMI-BSA in order to give solutions containing the equivalent to the product released by 10⁷ adherent macrophages/ml and were present throughout the assay.

Chemotaxis assay. Chemotaxis was performed in 48-well chemotaxis chambers (Neuroprobe Inc., Cabin John, MD, USA). Polyvinylpyrroldone free polycarbonate filters (0.5 μm) (Millipore) were placed between the upper and lower chambers. Twenty-five μl samples of either chemotactic or RPMI-BSA were added to the wells of the lower chamber. FMLP was used at concentration of 5 x 10⁻⁸ M in RPMI-BSA. Fifty μl samples of pretreated neutrophils were added to the wells of the upper chamber. The chemotaxis chambers were incubated for 1 h at 37°C in 5% CO₂. The cells which have migrated to the lower side of the filter were fixed and stained with Diff-Quick Stain Kit (American Scientific Products, McGraw Park, IL). Neutrophils were counted by using a 100 x objective in at least five random fields. Experiments were performed in triplicate for each variable, and the means determined. The results were expressed as number of neutrophils per field. In each experiment, untreated neutrophils migrating toward FMLP were used as positive reference.

In vivo neutrophil migration assay:
Inhibition assay. Inhibition assays were performed as described elsewhere. Briefly, S-300 chromatographic fractions (P1–P10 fractions) or different amounts (3 pg–3 μg) of mouse recombinant TNF-α (mTNF-α), which was a gift from Dr. M. A. Palladino Jr. of the Cell Biology Department, Genentech Inc., South San Francisco, CA, USA, were i.v. injected into penial venous sinus of the rats. Thirty min later, the animals received an intraperitoneal (i.p.) injection of carrageenin (300 μg/3 ml) (Marine Colloids, Inc., USA), FMLP solution (10⁻⁷ M/3 ml) or LPS (5 ng/3 ml). Controls received i.v. injections of PBS. Four hours after i.p. injections, the rats were sacrificed in a CO₂ chamber, their peritoneal cavities were washed with 10 ml of PBS containing 5 IU ml⁻¹ of heparin and total and differential cell counts in the lavage fluid were performed as described elsewhere. In subsequent biological assays, 0.2 ml (equivalent to the product released by 5 x 10⁷ cells) of the fraction S-300 chromatographic fractions presented ‘in vitro’ inhibitory activity, neutrophil suspensions (10⁶ cells/ml) were incubated for 30 min at 37°C, in a humidified incubator with 5% CO₂, either in the absence or in the presence of the pooled chromatographic fractions of macrophage supernatant, now referred as fractions P1 to P10, prior to testing for chemotactic response to N-formyl-methionyl-L-leucyl-L-phenylalanine (FMLP, Sigma) in microchemotaxis assay. The chromatographic fractions were diluted in RPMI-BSA in order to give solutions containing the equivalent to the product released by 10⁷ adherent macrophages/ml and were present throughout the assay.
Neutrophil recruitment inhibitory factor

retaining maximal inhibitory activity was i.v. injected per animal, except when indicated in the Results section. The results are expressed as means ± SEM of the number of neutrophils per ml of peritoneal wash.

Detection of MNCF activity. The S-300 chromatographic fractions were also assayed for the presence of MNCF activity as described elsewhere. Briefly, the fractions were injected into peritoneal cavities (3 ml/cavity, equivalent to the material released by 5 × 10^6 macrophages) of dexamethasone-pretreated rats (0.5 mg kg^-1, s.c., 1 h before). Controls received sterile saline both s.c. and i.p. injected. After 4 h, neutrophil migration was measured as indicated for inhibition assay.

**Results**

**Neutrophil migration activities of S-300 chromatographic fractions:** The “in vivo” inhibitory activity displayed by crude NRIF was detected in fraction P2 of S-300 chromatography of macrophage supernatants (Fig. 1, Panel A). The elution volume of P2 corresponded to an apparent molecular weight of 500 KDa.

When tested for TNF activity, crude supernatant was demonstrated to contain significant amounts (500 ng/0.2 ml), but only small amounts of such cytokine (1 pg ml^-1) were detected in P2 fraction. Antibodies raised against mouse recombinant TNF-α abolished the cytotoxicity of the mrTNF-α and strongly reduced the activity of the crude supernatant (50–60% of inhibition). We have tested increasing doses of mrTNF-α (0.3 pg–3 μg) upon carrageenin-induced neutrophil migration. Only TNF-α doses 10^6 times greater than that found in P2 were able to significantly inhibit neutrophil migration to rat peritoneal cavities (data not shown).

In panel B of Fig. 1, it is shown that P2 fraction of S-300 chromatography did not inhibit “in vitro” chemotaxis induced by FMLP, while it was found significant inhibition in fractions 5 to 10.

When chromatographic fractions were tested for MNCF, the highest activity was eluted in the void volume (P1) and in a fraction corresponding to 30–60 KDa (P7) (data not shown). In addition, we have separately studied the effect of P2 injected into peritoneal cavities. Figure 2 shows that P2 caused neutrophil migration (left block of bars) that was significantly reduced by pretreatment of the animals with dexamethasone (right block of bars).

**Effect of P2 fraction upon oedema:** Figure 4 shows that P2 fraction inhibited carrageenan, P2 fraction also inhibited neutrophil recruitment induced by either LPS or FMLP into rat peritoneal cavities (Fig. 3). Besides its inhibitory effect upon neutrophil migration induced by carrageenan, P2 fraction also inhibited neutrophil recruitment induced by either LPS or FMLP into rat peritoneal cavities (Fig. 3).

**Discussion**

Previous work from our laboratory have indicated the presence of a neutrophil recruitment inhibitory activity in thioglycollate-elicited and
FIG. 1. Effect of S-300 chromatographic fractions on neutrophil migration and chemotaxis. Panel A shows neutrophil migrations induced by i.p. injection of carrageenin (300 μg/rat) in animals pretreated 15 min before with i.v. injections of PBS (C, control), S-300 chromatographic fractions (P1–P10) or crude supernatant (NRIF), as indicated in Material and Methods. Traced line (---O---) represents the absorbance of each fraction (280 nm), starting with fraction 20. The results are reported as means ± SEM of six animals per experimental group. *p < 0.01 and **p < 0.05 as compared to control group. Panel B shows the effect of the pretreatment of human neutrophils with S-300 fractions on chemotaxis induced by FMLP (5 × 10⁻⁸ M). The results are expressed as means ± SEM of cells counted in 15 microscopic fields. *p < 0.01 and **p < 0.05 as compared to control group. Student’s t-test.

FIG. 2. Detection of MNCF activity in P2 fraction. The bars represent neutrophil migration into peritoneal cavities induced by either saline (C, control) or P2 fraction, in either saline or dexamethasone (0.5 mg kg⁻¹, s.c., 1 h before) pretreated animals. The results are reported as means ± SEM of the number of animals indicated up each bar. *p < 0.01 as compared to control. Student’s t-test.

FIG. 3. Inhibitory effect of P2 fraction upon neutrophil migration induced by different inflammatory stimuli. Bars represent neutrophil migrations induced by i.p. injections of carrageenin (Cg, 300 μg), LPS (5 ng) or FMLP (130 ng), in animals pretreated with i.v. injection of P2 fraction. The results are reported as means ± SEM of the number of animals indicated up each bar. *p < 0.05 as compared to control group. Student’s t-test.
LPS-stimulated macrophage supernatants. This activity was referred to as neutrophil recruitment inhibitory factor (NRIF), since it was specific for polymorphonuclear leukocytes and in contrast with other substances, such as LPS, it did not inhibit mononuclear cell migration to rat peritoneal cavities. This result indicates that NRIF is not contaminated with LPS. Further evidence that NRIF activity is not due to LPS contamination is based upon the fact that polymyxin B did not affect NRIF activity but abolished LPS neutrophil recruitment inhibitory effect. Furthermore, resident macrophage monolayers release the same inhibitory activity without LPS stimulation. In the present study we showed that NRIF activity was present in a fraction, referred as P2, obtained by gel filtration chromatography of the crude macrophage supernatant on Sephacryl S-300. This fraction was eluted in a volume corresponding to high molecular weight proteins (240–550 KDa) as deduced by standard calibration curve.

P2 fraction was effective against other inflammatory stimuli than carrageenin, such as LPS or FMLP, the latter being a direct chemotactic attractant in 'in vivo' inhibitory assays. In contrast, P2 fraction did not inhibit neutrophil chemotaxis induced by FMLP in microchemotaxis assay. These results taken together, suggest that NRIF did not affect the ability of neutrophils to respond to chemotactic stimuli, thus supporting the suggestion that NRIF may be acting by blocking neutrophil-endothelial adhesion mechanisms. However, additional experiments with more purified material are required to clarify its precise mechanism of action.

Similar to NRIF, i.v. administration of TNF-α has been shown to inhibit neutrophil accumulation at the site of inflammatory stimulus injection. Our results showed that crude macrophage supernatants contain significant TNF amounts (blocked by rabbit anti-mrTNF antibodies) that may contribute to inhibitory activity displayed by this material. However, P2 fraction presented only traces of TNF, corresponding to a quantity 10 times slower than that necessary to present neutrophil recruitment inhibitory activity. Circumstantial evidence that NRIF inhibitory activity is not due to TNF is supported by the high apparent molecular weight of the P2 fraction.

IL-8 is another cytokine presently shown to inhibit neutrophil accumulation at sites of acute inflammation when administered intravenously. The possibility that NRIF activity may be contaminated by IL-8 is minimized by its high molecular weight elution volume and by the evidence that no traces of IL-8 was found in a sample of P2 fraction (ELISA, using a monoclonal antibody raised against human recombinant IL-8, sensitivity of detection smaller than 50 pg, see acknowledgements). It should be pointed out, however, that the absence of detection could be due to the low sensitivity of the test to rat IL-8. However, in an on going experiment in our laboratory, it was found that antibodies raised against human IL-8 are able to inhibit either carrageenin as well as hIL-8-induced hyperalgesia in the rat.

Previous results obtained in our laboratory showed the presence of IL-1 in crude supernatant of LPS-stimulated macrophages. However, as...
demonstrated by the authors, IL-1 does not block neutrophil migration to peritoneal cavities even if it is injected in doses higher than those detected in such supernatants.

Another inflammatory parameter, rat paw oedema induced by either carrageenin or antigen challenge, was significantly reduced by P2 fraction administration. This effect was not observed with dextran-induced oedema. Carrageenin and dextrans have been shown to induce increased vascular permeability by different mechanisms. While dextran induces fluid accumulation due to mast cell degranulation with little protein and few neutrophils, carrageenin induces a protein-rich exudate containing large number of neutrophils. Since NRIF did not present inhibitory activity upon dextran-induced oedema, we suggest that the factor only acts in polymorphonuclear-leucocyte dependent inflammatory responses. Thus, it is plausible that P2 is blocking immunologically-induced oedema by a similar mechanism. If this hypothesis is correct, we can envisage its usefulness in the control of inflammatory diseases such as bronchial asthma, where infiltration of inflammatory cells has been related to bronchial hyperresponsiveness.

Further evidence that NRIF activity is not due to IL-1 or TNF presence has been demonstrated by the results that i.v. injected rh-IL-1 (α and β) and TNF-α inhibit both carrageenin and dextran-induced oedema.

Several cytokines, including TNF-α and β, IL-1α and β, and γ interferon are reported to induce neutrophil migration. In contrast to MNCF, dexamethasone inhibited the activity of those known cytokines. We have observed that in vivo chemotactic activity displayed by P2 was significantly blocked by pretreatment of the animals with dexamethasone, thus indicating that there is a material which indirectly stimulates neutrophil emigration. This activity could result from the presence of traces of several cytokines or due to NRIF by itself. The residual emigration induced by P2 in rats pretreated with dexamethasone may result from the presence of a direct acting cytokine, such as MNCF since this activity was eluted in two peaks, one of them corresponding to the void volume fraction, eluted just before P2. Further studies are necessary to clarify this point.

In the light of the present results it is possible to assume NRIF as a candidate for a novel cytokine, capable of inhibiting neutrophil migration to an inflammatory site when present in the circulation, as might occur in endotoxemic shock.

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