Effects of Cytokine-Induced Neutrophil Chemoattractants (CINCs) on Shape Change, Adhesiveness and Phagocytosis of Rat Neutrophils

Maher AL-MOKDAD,* Futoshi SHIBATA, and Hideo NAKAGAWA

Department of Physiological Chemistry, Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-01, Japan. Received March 27, 1997; accepted May 8, 1997

Rat cytokine-induced neutrophil chemoattractants (CINC)-1, -2 α , -2 β and -3 belong to α -chemokines and are known to be potent chemoattractants for rat neutrophils *in vivo* and *in vitro*. In this study, the abilities of CINCs to affect several functions of rat neutrophils are estimated. All CINCs at a concentration of 10^{-8} M induced the polarization of rat neutrophils. The adhesion of each CINC-treated neutrophil to fibrinogen significantly increased, reaching a maximal adhesion at 10^{-8} M of each CINC. The CINCs enhanced the phagocytosis of heat-killed yeast cells by neutrophils in a dose-dependent manner. In contrast, CINCs did not induce the production of reactive nitrogen intermediates. The results suggest that CINCs play a significant role in neutrophil infiltration and phagocytosis at inflammatory sites in rats.

Key words neutrophil function; cytokine-induced neutrophil chemoattractant; chemokine; neutrophil adhesion; phagocytosis; polarization

The hallmark of inflammation is the migration, accumulation, and activation of leukocytes at the site of injury.¹⁾ Leukocyte migration from the blood into tissue is a complex phenomenon including the endothelial adhesion and transendothelial migration of leukocytes. The accumulation of leukocytes at inflammatory sites is due in part to the effect of neutrophil-/monocyte-specific chemokines.²⁾ The chemokines are divided into two sub-families, α - and β -chemokines, as defined by the spacing of the first two cysteines in a highly conserved four-cysteine motif. Recently, lymphotactin, a member of a new class of chemokines with preferential effects on lymphocytes, has been characterized.³⁾ Cytokine-induced neutrophil chemoattractants (CINCs) and macrophage inflammatory protein (MIP)-2 are members of the α -chemokine family. We have purified four CINCs (CINC-1, -2 α , -2 β and CINC-3/rat MIP-2) from the conditioned medium of a rat granulation-tissue culture.⁴⁾ CINC-2 α and CINC-2 β differ only in the sequence of carboxy-terminal three amino acid residues.⁴⁾ None of the CINCs clearly correspond to any of human GRO- α , β and γ , though the CINCs are the rat counterparts of the GRO proteins on the basis of the similarities of amino acid sequences between CINCs and GRO proteins.⁴⁾ In previous studies using recombinant CINCs, we demonstrated that each CINC induced neutrophil chemotaxis *in vitro* and *in vivo*, increased intracellular Ca^{2+} concentration, and released cathepsin G from azurophil granules of rat neutrophils.⁵⁾ In addition, desensitization experiments suggested the presence of two putative receptors mediating the activities of CINCs for rat neutrophils.^{5,6)}

The present studies are a further investigation of the biological activities of recombinant CINCs on rat neutrophils. We report here that CINCs stimulate neutrophils to cause shape change (polarization), increased adhesiveness, and increased phagocytosis toward heat-killed yeast cells, though no effect was detected on the nitrite production.

MATERIALS AND METHODS

Purification of Recombinant CINCs Recombinant CINC-1, -2 α , -2 β and -3/rat MIP-2 were purified from *Escherichia coli* JM109 carrying expression vectors as described previously,^{5,7)} and were used throughout the present studies.

Isolation of Rat Neutrophils Neutrophils were collected from the rat peritoneal cavities 16 h after the intraperitoneal injection of Krebs–Ringer bicarbonate solution containing 1% (w/v) casein (120 ml/kg body weight). Neutrophils were washed two times with Gey's balanced salt solution (GBSS) and then suspended in RPMI-1640 medium at a concentration of 10^7 cells/ml.

Polarization Analysis The effect of CINCs on neutrophil shape change was investigated as reported by Lomas *et al.*⁸⁾ Neutrophils (1×10^7 cells/ml) were incubated with each CINC at a concentration of 10^{-8} M for 2 min prior to fixation with 10 volumes of 2% (v/v) formaldehyde. In another experiment, formyl-methionyl-leucyl-phenylalanine (fMLP), a positive control, was added to the neutrophil suspension at a final concentration of 10^{-8} M after the addition of the CINCs, and the suspension was left at room temperature for another hour before fixation. Polarization was measured by flow cytometry (Epics®-profile II analyzer, Coulter cooperation).

Adhesion Assay The assay was performed as described by Devi *et al.*¹⁾ In order to provide a substrate for neutrophil binding, 96-well flat-bottom plates were coated with 25 μ l of fibrinogen solution (2 mg/ml phosphate-buffered saline) (PBS) for 90 min at room temperature. Plates were washed three times with Hank's balanced salt solution (HBSS) containing 10 mM HEPES (pH 7.3) and 0.5% (w/v) bovine serum albumin (BSA). Rat neutrophils were treated with different concentrations of each CINC at 37 °C for 30 min, and the neutrophil suspension (5×10^5 cells/well) was added to the fibrinogen-coated well. After incubation at 37 °C for 30 min, plates were washed 5 times with the same buffer. Two hundred microliters of

* To whom correspondence should be addressed.

the buffer without BSA was added, followed by 10 μ l of the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (5 mg/ml). This mixture was incubated for 4 h at 37°C. The supernatant was very carefully removed and 100 μ l of acid isopropanol (2 mM HCl) was added to the cells in order to solubilize the formazan crystals. The absorbance of each well was then read at 595 nm using a microplate reader (model 450; Bio-Rad Laboratories).

Phagocytosis Assay Different concentrations of CINC_s in 0.3 ml of neutrophil suspension (10^7 cells/ml) were incubated with 0.6 ml of a heat-killed yeast cell suspension (1×10^6 cells/ml) at 37°C for 30 min. After centrifugation, the cell pellets were collected. Staining with Giemsa was performed after fixation with methanol, and the phagocytic index was determined. The phagocytic index was defined as the percent positive ingestion multiplied by the average number of ingested yeast cells per cell.

Nitrite Assay Neutrophils (5×10^5 cells/ml) were cultured in RPMI-1640 containing 10% fetal calf serum for 2 h at 37°C before use. The medium was replaced with 200 μ l of fresh medium and the cells were incubated with chemoattractants at concentrations of 10^{-9} M— 10^{-7} M for 20 h at 37°C. The nitrite concentration of the conditioned medium was measured as described by Ding *et al.*⁹⁾ The conditioned medium (100 μ l) was incubated with an equal volume of Greiss reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2.5% H_3PO_4) at room temperature for 10 min. The absorbance at 550 nm was determined using a microplate reader. Serial dilutions of 100 μ M of sodium nitrite were used to establish a standard curve.

RESULTS

Polarization At a concentration of 10^{-8} M all CINC_s induced the polarization of neutrophils above the negative control (Fig. 1). The effects of CINC_s on the shape change of neutrophils were comparable with those induced by the same concentration of fMLP, a positive control. The effect on shape change was more clear when neutrophils were treated with a combination of fMLP and each CINC.

Adhesion to Fibrinogen To estimate the contribution of CINC_s in the adhesiveness of neutrophils, the adhesion of CINC-treated neutrophils to fibrinogen was evaluated. In comparison with non-stimulated neutrophils, treatment with CINC_s significantly enhanced the binding of neutrophils to fibrinogen (Fig. 2). The adhesion was maximal when neutrophils were treated with each CINC at a concentration of 10^{-8} M, and CINC-1, CINC-2 α , CINC-2 β and CINC-3 increased the adhesion 5.9, 3.7, 3.6 and 4.7-fold, respectively. The adhesion induced by CINC-1 at a concentration of 10^{-8} M was comparable with that induced by the same concentration of fMLP.

Phagocytosis The effect of CINC_s on phagocytosis toward heat-killed yeast cells was investigated. All CINC_s caused concentration-dependent increases in the phagocytosis of heat-killed yeast cells by neutrophils (Table 1). In comparison with the control, the phagocytic activities of neutrophils treated with CINC-1, CINC-2 α , CINC-2 β and CINC-3 increased 2.2, 1.8, 2.1 and 2-fold, respectively.

Fig. 1. Polarization of Neutrophils Stimulated with CINC_s

Rat neutrophils (1×10^7 cells/ml) were incubated with each CINC at a concentration of 10^{-8} M for 2 min prior to fixation. A, negative control; B, fMLP; C, CINC-1; D, CINC-2 α ; E, CINC-2 β ; F, CINC-3. In the following experiments, at 2 min after the addition of the CINC_s, the fMLP was added to the neutrophil suspensions at a final concentration of 10^{-8} M, and the suspensions were left at room temperature for another hour before fixation. Polarization was measured by flow cytometry. G, CINC-1 with fMLP; H, CINC-2 α with fMLP; I, CINC-2 β with fMLP; J, CINC-3 with fMLP.

Production of Nitrite We examined the ability of CINC_s to stimulate the production of reactive nitrogen intermediates (RNI) by neutrophils as measured by the production of nitrite. Treatment of neutrophils with different concentrations of CINC_s resulted in no induction of RNI production from stimulated neutrophils (data are not shown). In contrast, the treatment of neutrophils with 10^{-8} M of fMLP resulted in a 1.6-fold higher level of nitrite production by non-stimulated neutrophils. The present findings indicate that CINC_s are incapable of generating

Fig. 2. Effect of CINC on the Neutrophil Adhesion to Fibrinogen

Rat neutrophils treated with CINC at 37°C for 30 min were added to fibrinogen-coated plates (5×10^5 cells/well). After incubation at 37°C for 30 min, the number of neutrophils which adhered to fibrinogen was determined. Each point represents the means $\pm$ S.E. of six determinations. Values are significantly different from the control: * $p < 0.01$.

Table 1. Effect of CINC on Phagocytosis of Heat-Killed Yeast Cells by Neutrophils

CINC, M	Phagocytosis ^{a)} (%)	Phagocytic index ^{b)}
CINC-1		
10 ⁻¹⁰ M	16.3 $\pm$ 4.5	27.7 $\pm$ 6.5
10 ⁻⁹ M	17.7 $\pm$ 2.0	25.0 $\pm$ 2.3
10 ⁻⁸ M	24.3 $\pm$ 4.8	30.0 $\pm$ 9.4
10 ⁻⁷ M	23.3 $\pm$ 3.5	34.0 $\pm$ 4.1*
CINC-2α		
10 ⁻¹⁰ M	18.7 $\pm$ 4.8	29.0 $\pm$ 8.0
10 ⁻⁹ M	22.0 $\pm$ 2.1	30.0 $\pm$ 1.0*
10 ⁻⁸ M	28.0 $\pm$ 6.2	30.0 $\pm$ 8.1
10 ⁻⁷ M	32.0 $\pm$ 3.5	45.0 $\pm$ 4.9*
CINC-2β		
10 ⁻¹⁰ M	21.0 $\pm$ 2.6	30.0 $\pm$ 7.0
10 ⁻⁹ M	17.0 $\pm$ 2.6	32.7 $\pm$ 7.0
10 ⁻⁸ M	26.0 $\pm$ 3.5	35.2 $\pm$ 2.5*
10 ⁻⁷ M	28.0 $\pm$ 3.1	38.3 $\pm$ 3.3*
CINC-3		
10 ⁻¹⁰ M	14.0 $\pm$ 3.5	17.5 $\pm$ 4.4
10 ⁻⁹ M	15.7 $\pm$ 3.0	20.0 $\pm$ 5.0
10 ⁻⁸ M	26.0 $\pm$ 4.5	30.0 $\pm$ 1.3*
10 ⁻⁷ M	32.0 $\pm$ 4.0	46.0 $\pm$ 4.9*
Control	14.0 $\pm$ 1.5	21.0 $\pm$ 2.5

a) Rat neutrophil suspensions (5×10^5 cells/ml) containing different concentrations of CINC were incubated with heat-killed yeast cells (1×10^6 cells/ml) in a total volume of 0.9 ml at 37°C for 30 min. After incubation, phagocytosis was microscopically assayed. The values represent the means $\pm$ S.E. of 3 determinations. b) Phagocytic index is the percentage of neutrophils with particles ingested, multiplied by the average number of particles ingested per cell. Values are significantly different from the control: * $p < 0.01$.

extracellular RNI detectable with the method utilized.

DISCUSSION

CINC belongs to the α -chemokine family, which includes IL-8, growth regulated/melanoma growth stimulatory activity (GRO/MGSA) and MIP-2. CINC are chemoattractants for neutrophils, as evidenced by both *in vitro* and *in vivo* studies.^{4,5} Although many studies described the biological activities of α -chemokines, little

is known about the activities of CINC on their target cells. The most studied α -chemokine, IL-8, acts mainly on neutrophils, and induces chemotaxis and adhesion to endothelial cells.^{10,11} In neutrophils, IL-8 binds to type A and type B receptors, induces Ca^{2+} -transients, and stimulates the release of elastase as well as vitamin B₁₂-binding protein.¹⁰⁻¹³ The present study demonstrated that CINC can also activate target cells. It is thought that the morphological polarization of neutrophils affects the ability to migrate through the vascular endothelium. The increasing volume of neutrophils, which is accompanied by neutrophil migration *in vitro*, suggested that the volume change may contribute to the migratory process itself.¹⁴ In this study, the exposure of neutrophils to each CINC induced a shape change of the neutrophils (Fig. 1). It had previously been reported that the effect of IL-8 on the shape change of human neutrophils reached a maximum in approximately 30 s, and reverted to prestimulation level within 1.5–2 min.¹⁵ In our experiments, the shape change of neutrophils occurred 2 min after exposure to CINC, indicating that shape change is a very early response to stimulation by chemotactic factors.

An essential process for the migration of neutrophils from the circulation into an inflammatory site is the enhanced adhesion of neutrophils to the vascular endothelium and matrix proteins. CINC are capable of inducing neutrophil adhesion to fibrinogen, as shown in Fig. 2. Our observations are in good agreement with the finding that IL-8 at a concentration of 5×10^{-8} M increased the binding of neutrophils to fibrinogen immobilized on plastic substrates by an average of 4.3-fold.¹⁶ Since the protocol of the adhesion assay includes rather vigorous washing steps, the neutrophils detected in our experiments may represent the neutrophils exerting very strong adhesive interactions. Despite the relatively low adhesion of neutrophils to fibrinogen, it may be physiologically relevant. It has been suggested that CD11b/CD18 functions as a receptor not only for C3bi, but also for fibrinogen, because the binding of neutrophils to fibrinogen-coated surfaces was blocked by monoclonal antibodies against complement receptor type 3 (CR3; CD11b/CD18), a molecule identified as a receptor for complement protein fragment C3bi.¹⁷ A significant increase in CD11b/CD18 receptor level on neutrophils was found when rat KC/CINC-1 was added to rat whole blood.¹⁸ The results and our data suggest that CINC modulate neutrophil adherence by affecting the expression of adhesion molecules, including CD11b/CD18.

Phagocytosis is an important function of neutrophils in the host defense against infectious organisms. The phagocytic response is a complex, multistep, and probably multibranch process.¹⁹ In the present study, the effect of CINC on the phagocytosis of heat-killed yeast cells by neutrophils was investigated. As shown in Table 1, CINC enhanced the phagocytosis of heat-killed yeast cells by neutrophils in a dose-dependent manner, suggesting that CINC may be involved in the elimination of microorganisms during acute inflammation. We previously demonstrated that CINC stimulated the release of cathepsin G from neutrophils in a dose-dependent

manner.⁵⁾ Similarly, in the present study we found that CINC also stimulate the release of β -glucuronidase and myeloperoxidase, constituents of azurophil granules (data are not shown). The results indicate that CINC induce the exocytosis of azurophil granules from rat neutrophils.

Reactive nitrogen intermediates (RNI) are derived from nitric oxide (NO) and include nitrite and nitrate. RNI are secretory products of activated macrophages,²⁰⁾ but are also secreted by numerous cell types, including neutrophils.²¹⁾ Others, however, have not detected the formation of RNI by neutrophils.²²⁾ In this study we investigated the ability of CINC to induce the production of nitric oxide/nitrite. No significant amount of nitrite was detected following the activation of neutrophils with CINC.

In summary, CINC caused a neutrophil shape change (polarization), adhesiveness, exocytosis and increased phagocytic activity toward heat-killed yeast cells. However, CINC failed to induce the release of reactive nitrogen intermediates. Our results suggest that CINC play an important role in the processes of rat inflammation.

REFERENCES

- 1) Devi S., Laning J., Luo Y., Dorf, M. E., *J. Immunol.*, **154**, 5376—5383 (1995).
- 2) Oppenheim J. J., Zachariae C. O., Mukaida N., Matsushima K., *Annu. Rev. Immunol.*, **9**, 617—648 (1991).
- 3) Kennedy J., Kelner G. S., Kleyensteuber S., Schall T. J., Weiss M. C., Yssel H., Scheider P. V., Cocks B. G., Bacon K. B., Zlotnik A., *J. Immunol.*, **155**, 203—209 (1995).
- 4) Nakagawa H., Komorita N., Shibata F., Ikesue A., Konishi K., Fujioka M., Kato H., *Biochem. J.*, **301**, 545—550 (1994).
- 5) Shibata F., Konishi K., Kato H., Komorita N., AL-mokdad M., Fujioka M., Nakagawa H., *Eur. J. Biochem.*, **231**, 306—311 (1995).
- 6) AL-mokdad M., Shibata F., Nakagawa H., *Biol. Pharm. Bull.*, **19**, 879—881 (1996).
- 7) Konishi K., Takata Y., Watanabe K., Date T., Yamamoto M., Murase A., Yoshida H., Suzuki T., Tsurufuji S., Fujioka M., *Cytokine*, **5**, 506—511 (1993).
- 8) Lomas D. A., Stone S. R., Llewellyn-Jones C., Keogan M. T., Wang Z. M., Rubin H., Carrell R. W., Stockley, R. A., *J. Biol. Chem.*, **270**, 23437—23443 (1995).
- 9) Ding A. H., Nathan C. F., Stuehr D. J., *J. Immunol.*, **141**, 2407—2412 (1988).
- 10) Baggiolini M., Walz A., Kunkel S. L., *J. Clin. Invest.*, **84**, 1045—1049 (1989).
- 11) Moser B., Clark-Lewis I., Zwahlen R., Baggiolini M., *J. Exp. Med.*, **171**, 1797—1802 (1990).
- 12) LaRosa G. J., Thomas K. M., Kaufmann M. E., Mark R., White M., Taylor L., Gray G., Witt D., Navarro J., *J. Biol. Chem.*, **267**, 25402—25406 (1992).
- 13) Norgauer J., Krutmann J., Dobos G. J., Traynor-Kaplan A. E., Oades Z. G., Schraufstatter I. U., *J. Invest. Dermatol.*, **102**, 310—314 (1994).
- 14) Rosengren S., Henson P. M., Worthen G. S., *Am. J. Physiol.*, **267**, 1623—1633 (1994).
- 15) Thelen M., Peveri P., Kernen P., Vinzeng T. V., Walz A., Baggiolini M., *Faseb. J.*, **2**, 2702—2706 (1988).
- 16) Detmers P. A., Lo S. K., Olsen-Egbert E., Walz A., Baggiolini M., Cohn Z. A., *J. Exp. Med.*, **171**, 1155—1162 (1990).
- 17) Wright S. D., Weitz J. I., Huang A. J., Levin S. M., Silverstien S. C., Loike J. D., *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 7734—7738 (1988).
- 18) Frevert C. W., Huang S., Danaee H., Paulauskis J. D., Kobzik L., *J. Immunol.*, **154**, 335—344 (1995).
- 19) Ishibashi Y., Yamashita T., *Infect. Immun.*, **38**, 825—833 (1982).
- 20) Stuehr D. J., Marletta M. A., *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 7738—7742 (1985).
- 21) Wright C. D., Mulsch A., Busse R., Osswald H., *Biochem. Biophys. Res. Commun.*, **160**, 813—819 (1989).
- 22) Rimele T. J., Sturm R. J., Adams L. M., Henry D. E., Heaslip R. J., Weichman B. M., Grimes D., *J. Pharmacol. Exp. Ther.*, **245**, 102—111 (1988).