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Possible roles of protein kinases in neutrophil chemotactic factor production by leucocytes in allergic inflammation in rats

Jun-ichi Tanabe, [1]Masako Watanabe, Sachiko Kondoh, *Suetsugu Mue & Kazuo Ohuchi

Department of Pathophysiological Biochemistry, Faculty of Pharmaceutical Sciences, Tohoku University, Aoba Aramaki, Aoba-ku, Sendai, Miyagi 980-77, Japan, and *College of Medical Sciences, Tohoku University, Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-77, Japan

1 In an air pouch-type allergic inflammation model in rats, leucocytes that had infiltrated into the pouch fluid collected 4 h after the antigen challenge produced proteinaceous chemotactic factors for neutrophils when they were incubated in the medium.

2 To clarify the mechanism of activation of the infiltrated leucocytes in producing these factors, the effects of protein kinase inhibitors on neutrophil chemotactic factor production were examined.

3 When the infiltrated leucocytes were incubated for 4 h in medium containing the non-selective protein kinase inhibitor K-252a (1–100 ng ml^{-1}, 2.14–214 nM), the tyrosine kinase inhibitor genistein (1–50 μg ml^{-1}, 3.7–185 μM), and the more selective protein kinase C inhibitor H-7 (5–100 μg ml^{-1}, 13.7–274 μM); neutrophil chemotactic activity in the conditioned medium was decreased in a concentration-dependent manner, but the adenosine 3':5'-cyclic monophosphate (cAMP)-dependent protein kinase inhibitor H-89 (1–1000 ng ml^{-1}, 2.24–2240 nM) showed no effect.

4 Isoelectric focusing of the conditioned medium revealed that the leucocytes produced two neutrophil chemotactic factors, leucocyte-derived neutrophil chemotactic factor (LDNCF) 1 and LDNCF-2. Treatment of the leucocytes with K-252a, genistein, and H-7, but not H-89, inhibited production of both LDNCF-1 and LDNCF-2.

5 These results suggest that activation of tyrosine kinase and protein kinase C, but not cAMP-dependent protein kinase, is responsible for the production of LDNCF-1 and LDNCF-2.

6 The steroidal anti-inflammatory drug dexamethasone and the protein synthesis inhibitor cycloheximide inhibited neutrophil chemotactic factor production in a concentration-dependent manner. Time-course experiments showed that the inhibitory effect by dexamethasone was apparent even 30 min after the incubation.

7 Mechanism for inhibiting the production of LDNCF-1 and LDNCF-2 by dexamethasone is also discussed.

Keywords: Allergic inflammation; lecocyte; neutrophil chemotactic factor; protein kinase C; tyrosine kinase; cAMP-dependent protein kinase; dexamethasone

Introduction

Cytokines are protein mediators that play important roles in inflammation and immune response (Elias *et al.*, 1990; Antin & Ferrara, 1992). For example, in rheumatoid arthritis, granulocyte–macrophage colony-stimulating factor (GM-CSF) (Xu *et al.*, 1989), interleukin (IL) 1, IL-6, tumour necrosis factor α (Thorbecke *et al.*, 1992) and IL-8 (Koch *et al.*, 1991) exist in the synovial fluid. These cytokines are produced by such inflammatory cells as mononuclear cells, lymphocytes and fibroblasts in the synovial fluid and tissues, and are responsible for the development of rheumatoid arthritis. Many studies suggest that cytokines produced by infiltrated leucocytes regulate the production of each cytokine, and thus control the function of inflammatory cells (Elias *et al.*, 1990; Feldman *et al.*, 1990; Haworth *et al.*, 1991). Therefore, to further define the inflammatory reaction, it is important to clarify the regulatory mechanism of cytokine production by leucocytes infiltrated into inflammatory sites.

During the course of experiments to identify the difference between allergic and non-allergic inflammation employing an air pouch-type inflammation model in rats (Ohuchi *et al.*, 1982; 1985; Tsurufuji *et al.*, 1982; Hirasawa *et al.*, 1986; 1987; Watanabe *et al.*,1987; 1990), focused especially on the qualitative difference in the function of infiltrated leucocytes, we found (Watanabe *et al.*, 1994) that the leucocytes infiltrated into the pouch fluid in the allergic inflammation model are much more active than those in the non-allergic inflammation model with respect to neutrophil chemotactic factor production. When the infiltrated leucocytes in the pouch fluid were collected 4 h after injection of the antigen solution and incubated, leucocytes from the immunized rats produced a larger amount of neutrophil chemotactic factors than did leucocytes from the non-immunized rats, thereby suggesting that the leucocytes infiltrated into the pouch fluid in the immunized rats are much more active than those in the non-immunized rats (Watanabe *et al.*, 1994). Our present investigation was intended to clarify pharmacologically the mechanism of activation of leucocytes for the production of neutrophil chemotactic factors in the allergic inflammation. We have reported that the leucocytes infiltrated into the pouch fluid produce two neutrophil chemotactic proteins, leucocyte-derived neutrophil chemotactic factor (LDNCF) 1 and LDNCF-2 (Watanabe *et al.*, 1994). It is possible that the production of the two chemotactic proteins by the infiltrated leucocytes is induced after activation of leucocytes at the inflammatory sites. Because cytokine production by inflammatory cells is regulated through the signal transduction pathway of protein tyrosine phosphorylation (Muñoz *et al.*, 1992) or serine/threonine phosphorylation (Zhang *et al.*, 1992), we investigated whether the production of LDNCF-1 and LDNCF-2 by leucocytes infiltrated into the inflammatory locus is also associated with protein phosphorylation. Using several types of protein kinase inhibitors,

[1] Author for correspondence.

we found that the inhibitors of protein kinase C and tyrosine kinase, but not cAMP-dependent protein kinase, inhibit the production of both LDNCF-1 and LDNCF-2. These results suggest that protein kinase C and tyrosine kinase in the infiltrated leucocytes are activated by certain inflammatory stimuli to produce LDNCF-1 and LDNCF-2.

Several studies have demonstrated that the anti-inflammatory drug dexamethasone inhibits production of cytokines or expression of their genes by inflammatory cells *in vitro* (Lew *et al.*, 1988; Villiger *et al.*, 1992; Cassatella *et al.*, 1993). The present investigation also demonstrates that production of LDNCF-1 and LDNCF-2 by infiltrated leucocytes is inhibited by dexamethasone treatment.

Methods

Induction of allergic inflammation

The immunization and induction of air pouch-type allergic inflammation in rats were carried out as described previously (Tsurufuji *et al.*, 1982), with a slight modification. Male rats of the Sprague–Dawley strain, specific pathogen free and weighing 120–140 g (Charles River Japan, Kanagawa, Japan) were used. An antigen, azobenzenearsonate-conjugated acetyl bovine serum albumin (ABA-AcBSA) was synthesized according to the procedure described by Tabachnick & Sobotka (1962). The rats were immunized by i.d. injection of ABA-AcBSA and Freund's complete adjuvant (Difco Laboratories, Detroit, MI, U.S.A.). Nine days after immunization, 8 ml of air was injected s.c. in the dorsum to form an ellipsoid-shape air pouch. Twenty-four hours after the injection of air, 2 mg of the antigen dissolved in 4 ml of a sterilized solution of 0.8% (w/v) sodium carboxymethylcellulose (CMC-Na, Cellogen F3H; Daiichi Kogyo Seiyaku, Niigata, Japan) in 0.9% (w/v) NaCl solution was injected into the air pouch to provoke allergic inflammation. The CMC-Na solution was supplemented with penicillin G potassium and dihydrostreptomycin sulphate (Meiji Seika, Tokyo, Japan), each at a 0.1 mg ml^{-1} concentration.

Culture of leucocytes collected from the pouch fluid

Four hours after the injection of the antigen solution into the air pouch of the immunized rats, rats were sacrificed by cutting the carotid artery under diethyl ether anaesthesia and the entire pouch fluid was collected. The pouch fluid was diluted 4-fold with RPMI-1640 medium (Nissui Seiyaku, Tokyo, Japan) and leucocytes infiltrated into the pouch fluid were precipitated by centrifugation at 350 *g* and 4°C for 5 min. The precipitate was washed three times with the medium and suspended in RPMI-1640 medium containing 0.25% (w/v) bovine serum albumin (BSA, essentially fatty acid-free; Sigma, St Louis, MO, U.S.A.) at a concentration of 1×10^7 cells ml^{-1}. The rest of the cells were differentially counted for each type of cells after May–Gruenwald–Giemsa staining. Four millilitres of the cell suspension (4×10^7 cells) was incubated at 37°C for various lengths of time in the presence or absence of drugs. After appropriate incubation times, the cell suspension was centrifuged at 1,500 *g* and 4°C for 5 min. The supernatant fraction was obtained, finally diluted to 20-fold with RPMI-1640 medium containing 0.25% (w/v) BSA, and used for the measurement of neutrophil chemotactic activity.

Measurement of neutrophil chemotactic activity

Neutrophil chemotactic activity in the diluted supernatant fraction of the conditioned medium was assayed using modified Boyden chambers as described previously (Watanabe *et al.*, 1994).

Drug treatment

The non-specific protein kinase inhibitor K-252a (Elliott *et al.*, 1990; Nye *et al.*, 1992) (Kyowa Medex, Tokyo, Japan), the tyrosine kinase inhibitor genistein (Tremblay *et al.*, 1992) (Wako), the more selective protein kinase C inhibitor H-7 [1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride] (Hidaka *et al.*, 1991) (Seikagaku Kogyo, Tokyo, Japan), the protein kinase A inhibitor H-89 (*N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinoline-sulphonamide) (Chijiwa *et al.*, 1990) (Seikagaku Kogyo Co.), the protein synthesis inhibitor cycloheximide (Sigma) and the synthetic glucocorticoid dexamethasone (Sigma) were dissolved in ethanol and an aliquot of the ethanol solution was added to RPMI-1640 medium containing 0.25% (w/v) BSA. The final ethanol concentration in the medium was adjusted to 0.1% (v/v). After treatment with drugs, viability of the leucocytes was examined by trypan blue exclusion test, and no cytotoxic effect was observed.

Isoelectric focusing to separate neutrophil chemotactic activity in the conditioned medium

Leucocytes infiltrated into the pouch fluid of immunized rats were collected 4 h after the antigen challenge, and were incubated for 4 h at 37°C in RPMI-1640 medium containing 0.25% (w/v) BSA at a concentration of 1×10^7 cells ml^{-1} with or without drugs. After incubation, the cell suspension was centrifuged at 1,500 *g* and 4°C for 5 min. The supernatant fraction (49 ml) was dialysed against 10 mM NaCl and mixed with 1 ml of Bio-Lyte ampholytes (pH range 3–10, Bio-Rad, Richmond, CA, U.S.A.). The mixture was loaded into the focusing chamber of the Rotofor cell (Bio-Rad), and isoelectric focusing carried out at 12 W constant power for 3 h at 4°C (Watanabe *et al.*, 1994). Twenty fractions were harvested and their pH values measured. Each fraction (2.5 ml) was dialysed against 1 M NaCl to remove ampholytes, and further dialysed against phosphate-buffered saline (pH 7.4) at 4°C. Chemotactic activity in each fraction was then determined after a 10-fold dilution with RPMI-1640 medium containing 0.25% (w/v) BSA.

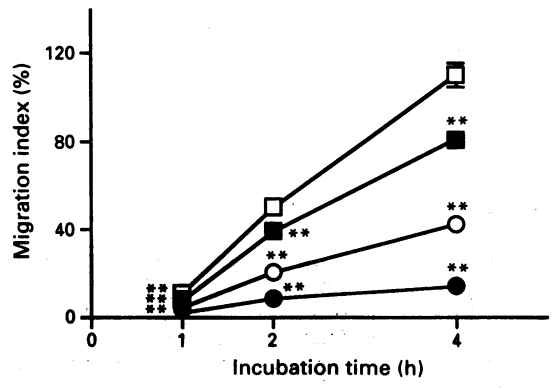

Figure 1 Effects of cycloheximide on chemotactic factor production by infiltrated leucocytes. Leucocytes (4×10^7 cells) in the pouch fluid collected 4 h after injection of the antigen solution into the air pouch of immunized rats were incubated for various periods at 37 °C in 4 ml of the medium containing cycloheximide at 0 μM (□), 0.018 μM (■), 0.18 μM (○) and 1.8 μM (●). A concentration of cycloheximide at 0.5 μg ml^{-1} corresponds to 1.8 μM. Neutrophil chemotactic activity in the conditioned medium was determined after 20-fold dilution. Values are the means from four samples in one experiment with s.e.mean shown by vertical bars. Where vertical bars are not depicted, s.e.mean is inside the symbol. Statistical significance: $^{**}P < 0.001$ vs the corresponding control. The results were confirmed by two separate experiments.

Statistical analysis

Results were analysed for statistical significance by Dunett's test for multiple comparisons and Student's *t*-test for unpaired observations.

Results

Production of neutrophil chemotactic factor by infiltrated leucocytes and its inhibition by cycloheximide

When leucocytes (4×10^7 cells) in the pouch fluid collected 4 h after injection of the antigen solution into the air pouch of immunized rats were incubated at 37°C in 4 ml of RPMI-1640 medium containing 0.25% (w/v) BSA, the chemotactic activity for neutrophils in the conditioned medium was increased time dependently (Figure 1). Differential cell count for the leucocytes collected at 4 h showed that 86.2% of the leucocytes were neutrophils, 9.9% were mononuclear cells and 3.9% were eosinophils (Table 1). Adding cycloheximide to the medium inhibited the production of neutrophil chemotactic factor in a concentration-dependent manner (Figure 1). Cycloheximide alone at these concentrations showed no direct effect on neutrophil chemotaxis towards 10 nM of platelet-activating factor (PAF) (Figure 2).

Effects of K-252a on chemotactic factor production by infiltrated leucocytes

To elucidate the significant roles of protein phosphorylation in neutrophil chemotactic factor production, the infiltrated leucocytes (4×10^7 cells) were incubated for 4 h in 4 ml of RPMI-1640 medium containing 0.25% (w/v) BSA and various concentrations of K-252a, a non-selective inhibitor of protein kinases. As shown in Figure 3, the neutrophil chemotactic activity in the conditioned medium was decreased in a concentration-dependent manner. A half-maximal effect was induced at a concentration of 17 ng ml^{-1} (36.4 nM). K-252a by itself, at these concentrations, showed no direct effect on neutrophil chemotaxis towards 10 nM PAF (data not shown).

Effects of specific inhibitor of protein kinases on chemotactic factor production by infiltrated leucocytes

To determine what types of protein kinases are associated with neutrophil chemotactic factor production by infiltrated leucocytes, the effects of the tyrosine kinase inhibitor genistein, the more selective protein kinase C inhibitor H-7 and the cAMP-dependent protein kinase inhibitor H-89 were examined. The infiltrated leucocytes (4×10^7 cells) were incubated for 4 h in 4 ml of the medium in the presence of various protein kinase inhibitors. Treatment with the tyrosine

Table 1 Differential cell count of leucocytes infiltrated into the pouch fluid at 4 h

Total cells (%)	*Neutrophils* (%)	*Mononuclear cells* (%)	*Eosinophils* (%)
100	86.2 ± 4.1	9.9 ± 0.4	3.9 ± 0.2

Leucocytes in the pouch fluid collected 4 h after challenge injection were collected and differential cell counts was performed after May–Gimsa–Grünwald staining. Values are percentages of total cells, and expressed as means ± s.e.mean from eight rats. The same results were obtained by three separate experiments.

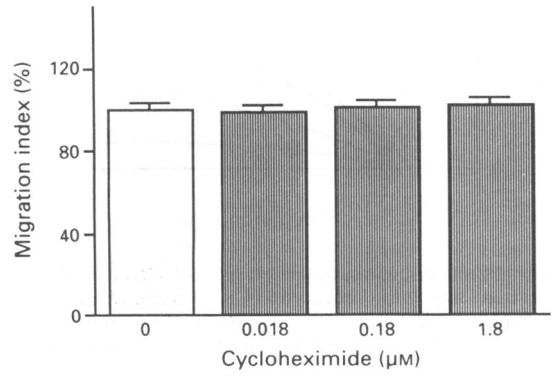

Figure 2 Effects of cycloheximide on PAF-induced neutrophil chemotaxis. Leucocytes (4×10^7 cells) in the pouch fluid were incubated at 37°C for 4 h in 4 ml of the medium containing 10 nM PAF and indicated concentrations of cycloheximide. A concentration of cycloheximide at 0.5 μg ml^{-1} corresponds to 1.8 μM. Values are the means from four samples with s.e.mean shown by vertical bars. The results were confirmed by three separate experiments.

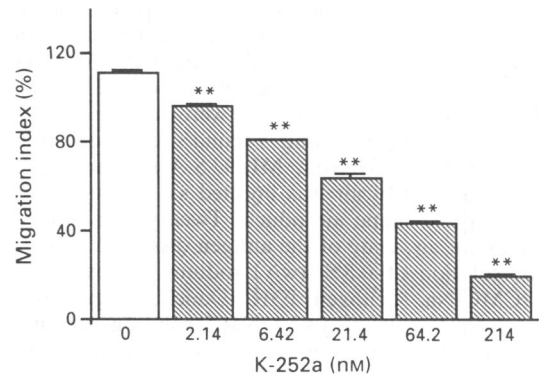

Figure 3 Effects of K-252a on chemotactic factor production by infiltrated leucocytes. Leucocytes (4×10^7 cells) in the pouch fluid were incubated at 37°C for 4 h in 4 ml of the medium containing indicated concentrations of K-252a. A concentration of K-252a at 1 ng ml^{-1} corresponds to 2.14 nM. Neutrophil chemotactic activity in the conditioned medium was determined after 20-fold dilution. Values are the means from four samples with s.e.mean shown by vertical bars. Statistical significance: $^{**}P < 0.001$ vs the control. The results were confirmed by three separate experiments.

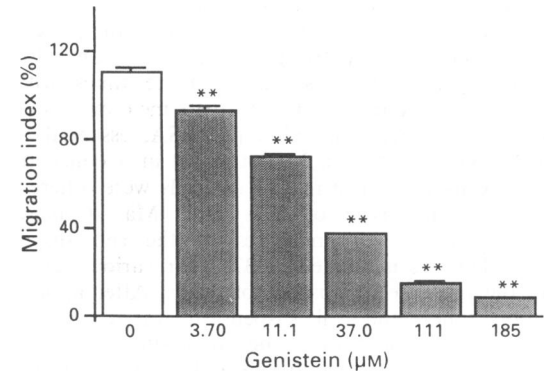

Figure 4 Effects of genistein on chemotactic factor production by the infiltrated leucocytes. Leucocytes (4×10^7 cells) in the pouch fluid were incubated at 37°C for 4 h in 4 ml of the medium containing indicated concentrations of genistein. A concentration of genistein at 1 μg ml^{-1} corresponds to 3.70 μM. Neutrophil chemotactic activity in the conditioned medium was determined after 20-fold dilution. Values are the means from four samples with s.e.mean shown by vertical bars. Statistical significance: $^{**}P < 0.001$ vs the control. The results were confirmed by three separate experiments.

kinase inhibitor genistein decreased neutrophil chemotactic activity in the conditioned medium in a concentration-dependent manner with a half-maximal effect at 5.6 $\mu g\ ml^{-1}$ (20.7 μM) (Figure 4). Similar results were obtained by the more selective protein kinase C inhibitor H-7 with a half-maximal effect at 8.9 $\mu g\ ml^{-1}$ (24.4 μM) (Figure 5). In contrast, treatment with the cAMP-dependent protein kinase inhibitor H-89 did not inhibit neutrophil chemotactic factor production at concentrations up to 1000 $ng\ ml^{-1}$ (2240 nM) (Figure 6). Each drug showed no direct effect on neutrophil chemotaxis toward 10 nM PAF (data not shown).

Effects of dexamethasone on chemotactic factor production by infiltrated leucocytes

When infiltrated leucocytes (4×10^7 cells) were incubated for 4 h in 4 ml of the medium containing various concentrations of dexamethasone, the neutrophil chemotactic activity in the conditioned medium was decreased in a concentration-dependent manner, with a half-maximal effect at 28 nM (Figure 7). Time-course experiments revealed that the inhibitory effect by dexamethasone was apparent even 30 min after incubation at 10 and 100 nM dexamethasone (Figure 8).

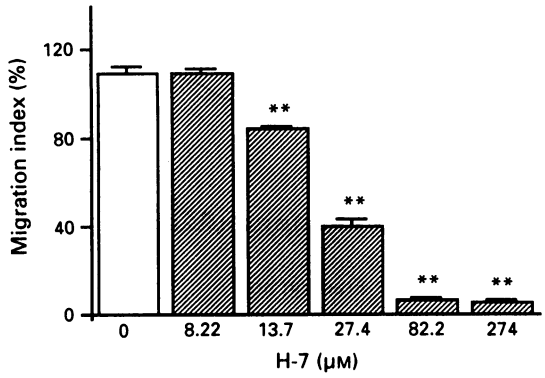

Figure 5 Effects of H-7 on chemotactic factor production by infiltrated leucocytes. Leucocytes (4×10^7 cells) in the pouch fluid were incubated at 37°C for 4 h in 4 ml of the medium containing indicated concentrations of H-7. A concentration of H-7 at 3 $\mu g\ ml^{-1}$ corresponds to 8.22 μM. Neutrophil chemotactic activity in the conditioned medium was determined after 20-fold dilution. Values are the means from four samples with s.e.mean shown by vertical bars. Statistical significance: $^{**}P < 0.001$ vs the control. The results were confirmed by three separate experiments.

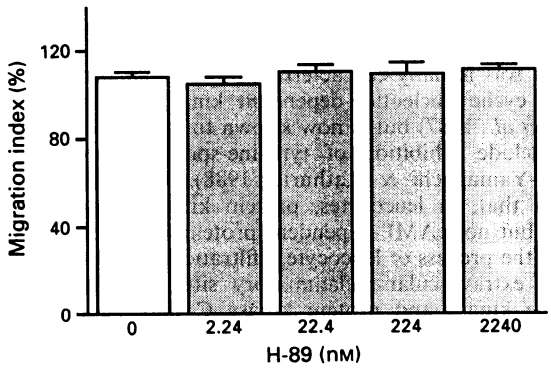

Figure 6 Effects of H-89 on chemotactic factor production by infiltrated leucocytes. Leucocytes (4×10^7 cells) in the pouch fluid were incubated at 37°C for 4 h in 4 ml of the medium containing indicated concentrations of H-89. A concentration of H-89 at 1 $ng\ ml^{-1}$ corresponds to 2.24 nM. Neutrophil chemotactic activity in the conditioned medium was determined after 20-fold dilution. Values are the means from four samples with s.e.mean shown by vertical bars. The results were confirmed by three separate experiments.

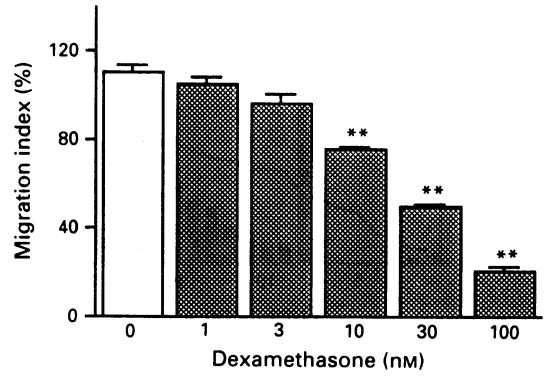

Figure 7 Effects of dexamethasone on chemotactic factor production by infiltrated leucocytes. Leucocytes (4×10^7 cells) in the pouch fluid were incubated at 37°C for 4 h in 4 ml of the medium containing indicated concentrations of dexamethasone. A concentration of dexamethasone at 0.39 $ng\ ml^{-1}$ corresponds to 1 nM. Neutrophil chemotactic activity in the conditioned medium was determined after 20-fold dilution. Values are the means from four samples with s.e.mean shown by vertical bars. Statistical significance: $^{**}P < 0.001$ vs the control. The results were confirmed by three separate experiments.

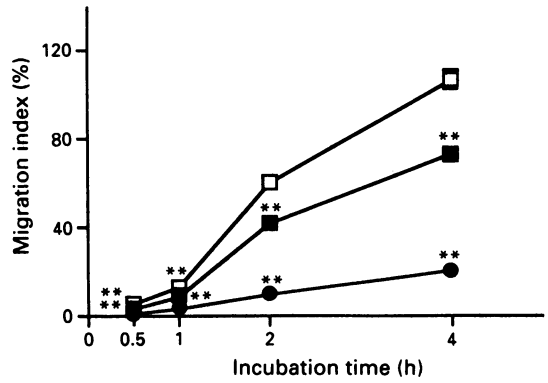

Figure 8 Time-course of effects of dexamethasone on neutrophil chemotactic factor production by infiltrated leucocytes. Leucocytes (4×10^7 cells) in the pouch fluid were incubated at 37°C for 4 h in 4 ml of the medium containing dexamethasone at 0 nM (□), 10 nM (■) and 100 nM (●). Neutrophil chemotactic activity in the conditioned medium was determined after 20-fold dilution. Values are the means from four samples with s.e.mean shown by vertical bars. Where vertical bars are not depicted, s.e.mean is inside the symbol. Statistical significance: $^{**}P < 0.001$ vs the control. The results were confirmed by three separate experiments.

Dexamethasone alone at these concentrations showed no direct effect on neutrophil chemotaxis towards 10 nM PAF (data not shown).

Effects of drugs on production of LDNCF-1 and LDNCF-2 by infiltrated leucocytes

Four hours after incubation of the infiltrated leucocytes in the medium with or without drugs, conditioned medium was obtained and the chemotactic activity for neutrophils in the conditioned medium was separated by isoelectric focusing. As shown in Figure 9a, neutrophil chemotactic activity was separated into two peaks: the first one is a sharp peak with a pI value around 6 (leucocyte-derived neutrophil chemotactic factor 1, LDNCF-1) and the second one is LDNCF-2 with a pI value above 8. Gradient analysis for neutrophil migration with LDNCF-1 or LDNCF-2 demonstrated that movement of neutrophils was not chemokinetic but chemotactic, because migrations in positive gradient were more effective than those in negative gradient (data not shown). When the

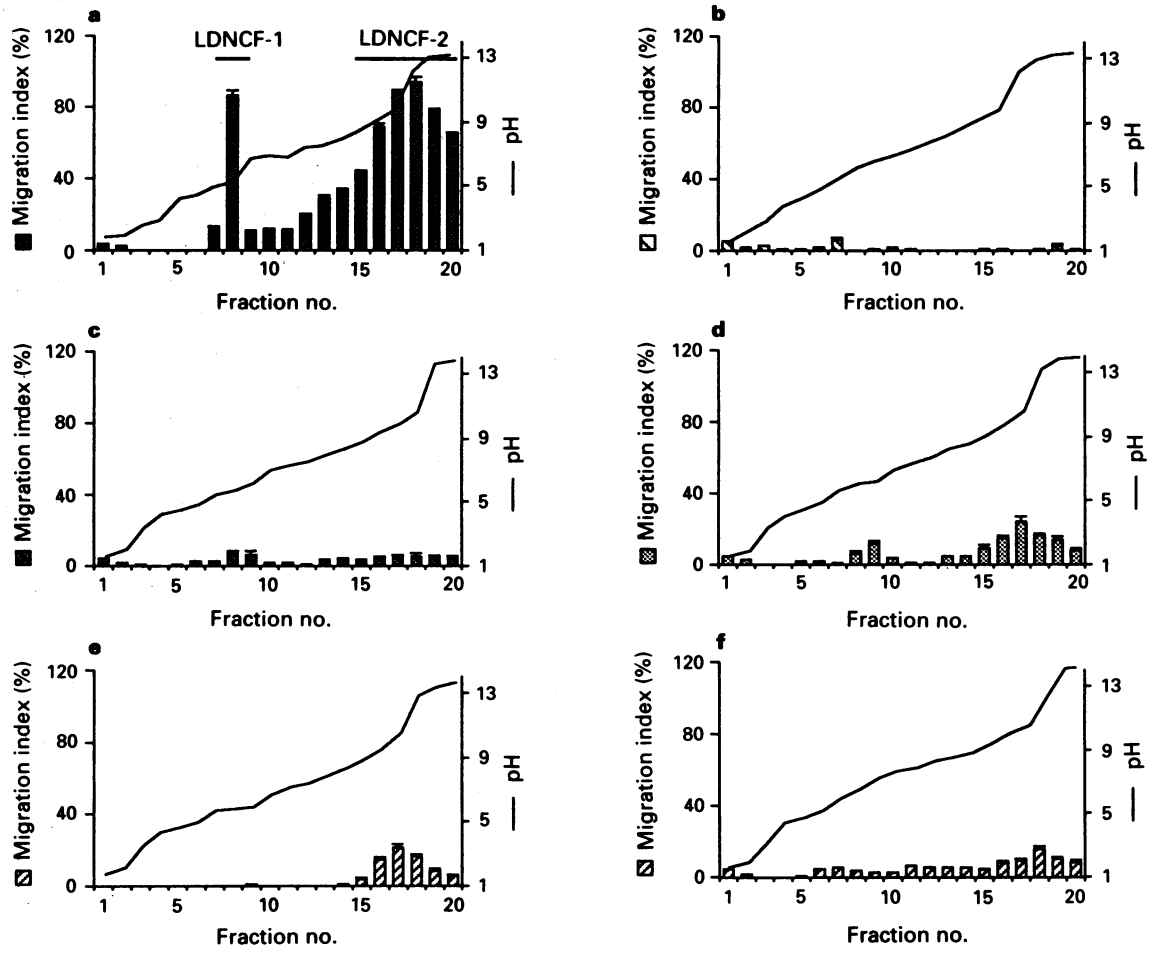

Figure 9 Separation profiles obtained by isoelectric focusing of the chemotactic activity for neutrophils in the conditioned medium. Leucocytes (5×10^8 cells) in the pouch fluid were incubated at 37 °C for 4 h in 50 ml of the medium containing (a) nothing, (b) cycloheximide (5 μg ml^{-1}, 17.8 μM), (c) K-252a (100 ng ml^{-1}, 214 nM), (d) genistein (50 μg ml^{-1}, 185 μM), (e) H-7 (30 μg ml^{-1}, 82.2 μM) or (f) dexamethasone (39.3 ng ml^{-1}, 100 nM). Neutrophil chemotactic activity in the conditioned medium was separated by isoelectric focusing. Chemotactic activity in each fraction was determined after 10-fold dilution. Values are the means with s.e.mean from four assays. In each group, one representative profile out of four separate experiments is shown.

infiltrated leucocytes were incubated in the presence of cycloheximide (5 μg ml^{-1}, 17.8 μM), K-252a (100 ng ml^{-1}, 214 nM), genistein (50 μg ml^{-1}, 185 μM), H-7 (30 μg ml^{-1}, 82.2 μM) or dexamethasone (39.3 ng ml^{-1}, 100 nM), production of both LDNCF-1 and LDNCF-2 was inhibited simultaneously (Figure 9b–f).

Discussion

Neutrophil chemotactic activity in the conditioned medium of leucocytes infiltrated into the inflammatory locus was decreased by treatment with the protein synthesis inhibitor cycloheximide (Figure 1). This treatment also inhibited production of LDNCF-1 and LDNCF-2 (Figure 9b). Therefore, the chemotactic factors in the conditioned medium are proteinaceous factors produced by the infiltrated leucocytes during the incubation period. The aim of the present investigation was to clarify the mechanism of the production of proteinaceous neutrophil chemotactic factors by leucocyte infiltrated into the inflammatory sites to gain further insight into the inflammatory reactions.

This study shows that production of chemotactic factor for neutrophils by leucocytes infiltrated into the inflammatory locus is inhibited by the non-selective inhibitor of protein kinases K-252a (Elliott *et al.*, 1990; Nye *et al.*, 1992), the more selective protein kinase C inhibitor H-7 (Hidaka *et al.*, 1991) and the tyrosine kinase inhibitor genistein (Tremblay *et al.*, 1992). However, the cAMP-dependent protein kinase inhibitor H-89 (Chijiwa *et al.*, 1990) did not inhibit the production of neutrophil chemotactic factor at concentrations great enough to inhibit the cAMP analogue-induced intercellular adhesion of JY cells (Haverstick & Gray, 1992). K-252a was initially characterized as a potent protein kinase C and cyclic nucleotide-dependent kinase inhibitor *in vitro* (Kase *et al.*, 1987) but is now known to have broader actions that include inhibition of tyrosine-specific protein kinases (Fujita-Yamaguchi & Kathuria, 1988). The present results suggest that, in leucocytes, protein kinase C and tyrosine kinase but not cAMP-dependent protein kinase are activated during the process of leucocyte infiltration from blood-stream to the extravascular inflammatory site. The activation of tyrosine kinase and protein kinase C may be induced by leucocyte–vascular endothelial cell interactions or by cytokines produced by inflammatory stimuli. Signalling cascades that initiate with tyrosine phosphorylations eventually also result in the serine/threonine phosphorylation of downstream molecules owing to the activation of serine/threonine kinases, such as Raf, protein kinase C and ribosomal S6 protein kinase (Ullrich & Schlessinger, 1990). For example, if tyrosine kinase in platelet-derived growth factor (PDGF) receptor is activated by the ligand, phospholipase C-γ associated with the PDGF receptor is activated (Kaplan *et al.*, 1990) and stimulates the canonical phosphatidyl inositol

turnover pathway, leading to elevation of cytosolic calcium and activation of protein kinase C (Whitman & Cantley, 1988). Other than GM-CSF, the association of tyrosine kinase with such receptors for cytokines as IL-2, IL-3, IL-5 and IL-6 has also been reported (Kishimoto *et al.*, 1994). On the other hand, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), an activator of protein kinase C, induces protein tyrosine phosphorylation in neutrophils (Huang *et al.*, 1990). Actually, activation of protein kinase C of the infiltrated leucocytes by incubating the cells for 4 h with TPA at concentrations of $1-100\ ng\ ml^{-1}$ (1.62–162 nM) further stimulated the production of both LDNCF-1 and LDNCF-2 (J. Tanabe *et al.*, unpublished observations). This observation also indicates that production of LDNCF-1 and LDNCF-2 is mediated by activation of protein kinase C. Furthermore, the adhesion molecule component β1-integrin, which plays significant roles in leucocyte–endothelial cell and leucocyte–leucocyte interactions, is reported to induce protein tyrosine phosphorylation of $pp125^{FAK}$ (Kornberg *et al.*, 1992).

As to the characteristics of the neutrophil chemotactic factors produced by the leucocytes infiltrated into the inflammatory locus, we have reported that the major components of the neutrophil chemotactic factor are LDNCF-1 and LDNCF-2, of which the pI values are approximately 4 and >8 respectively (Watanabe *et al.*, 1994). During 4 h incubation of the leucocytes, leukotriene B_4 was produced, but the amount was less than the concentration needed to contribute to the chemotactic activity in the conditioned medium (Watanabe *et al.*, 1994). Determination of PAF in the conditioned medium after partial purification using immunoaffinity mini-columns (Watanabe *et al.*, 1992) and by radioimmunoassay revealed that the amount of PAF in the conditioned medium at 4 h was less than the detectable amount (<0.56 nM). Consequently, the proteinaceous chemotactic factors LDNCF-1 and LDNCF-2 are the major chemotactic factors produced by leucocytes infiltrated into the inflammatory locus. Further studies are necessary to clarify which type of cell (Table 1) is responsible for the production of LDNCF-1 and LDNCF-2. Production of both LDNCF-1 and LDNCF-2 was simultaneously inhibited by treatment with the non-selective protein kinase inhibitor K-252a, the more selective protein kinase C inhibitor H-7 and the tyrosine kinase inhibitor genistein, but was not inhibited by the cAMP-dependent protein kinase inhibitor H-89 (Figure 9), as was the case for the neutrophil chemotactic activity in the conditioned medium (Figures 3, 4, 5 and 6). These results indicate that the production of LDNCF-1 and LDNCF-2 is mediated by activation of tyrosine kinase and protein kinase C but not by cAMP-dependent protein kinase.

Production of LDNCF-1 and LDNCF-2 by the infiltrated leucocytes was inhibited by dexamethasone treatment (Figure 9). The time-course experiment revealed that the significant decrease in neutrophil chemotactic activity in the conditioned medium was observable even 30 min after incubation with dexamethasone at 10 and 100 nM (Figure 8). Dexamethasone binds to its receptor protein in the cytosol, moves into the nucleus, and the activated receptor–dexamethasone complex acts as the transcriptional factor, finally showing its biological effects under ongoing protein synthesis and RNA synthesis (Tobler *et al.*, 1992) or by suppression of gene expression (Jonat *et al.*, 1990; Schüle *et al.*, 1990; Yang-Yen *et al.*, 1990). In the present investigation, because the inhibitory effect of dexamethasone has appeared shortly after incubation (30 min), dexamethasone's inhibitory effect might not be mediated by induction of certain protein synthesis. As shown in Figures 1 and 9, production of the neutrophil chemotactic factor was inhibited by treatment with the protein synthesis inhibitor cycloheximide, so it is hard to prove that dexamethasone can inhibit the production of neutrophil chemotactic factors when protein synthesis is inhibited. Alternatively, dexamethasone might inhibit neutrophil chemotactic factor production by suppressing gene expression for LDNCF-1 and LDNCF-2. Inhibition by dexamethasone of IL-2 production in Jurkat T77 cells (Vacca *et al.*, 1990) and of IL-8 production in lipopolysaccharide-stimulated neutrophils (Weltheim *et al.*, 1993) is also reported to be mediated by the inhibition of gene expression.

Our recent work on the determination of amino acid sequence of LDNCF-1 and LDNCF-2 has revealed that the latter resembles murine macrophage inflammatory protein 2 (Wolpe *et al.*, 1989; Tekamp-Olson *et al.*, 1990) but not cytokine-induced neutrophil chemoattractant in rat (Watanabe *et al.*, 1989). Judging from the pI value, LDNCF-1 might be a new proteinaceous chemoattractant produced by leucocytes infiltrated into the inflammatory locus.

The mechanism of activation of protein kinase C and tyrosine kinase to produce LDNCF-1 and LDNCF-2 and the types of leucocytes that produce these factors remain to be elucidated. Further purification and characterization of LDNCF-1 are under investigation in our laboratories.

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