Pharmacological analysis of neutrophil chemotactic factor production by leucocytes and roles of PAF in allergic inflammation in rats

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1 The role of platelet activating factor (PAF) in neutrophil infiltration in air pouch type allergic inflammation in rats was investigated.

2 Neutrophil infiltration into the pouch fluid 8 h after injection of the antigen (azobenzene arsonate-conjugated acetyl bovine serum albumin) solution into the air pouch of immunized rats was inhibited dose-dependently by treatment with PAF antagonists (CV-3988, L-652,731 and Y-24,180) in parallel with the decrease in neutrophil chemotactic activity in the pouch fluid.

3 Four hours after injection of the antigen solution into the air pouch of immunized and non-immunized rats, there was no significant difference between the two groups in the number of total leucocytes, neutrophils, mononuclear cells and eosinophils in the pouch fluid. However, when the infiltrated leucocytes were incubated in medium, chemotactic factor production by leucocytes from immunized rats was greater than that from non-immunized rats.

4 When leucocytes from non-immunized rats were preincubated for various periods in the medium containing 10 or 50 nM of PAF, washed, and further incubated in the medium containing no PAF, chemotactic factor production was not stimulated.

5 The increase in the chemotactic activity in the conditioned medium was not suppressed by the 5-lipoxygenase inhibitor, AA861. In addition, the chemotactic activity in the conditioned medium was not inhibited by the PAF antagonists.

6 Incubation of the infiltrated leucocytes in the medium containing the protein synthesis inhibitor, cycloheximide, inhibited chemotactic factor production in a concentration-dependent manner in parallel with the decrease in uptake of [³H]-leucine into the acid-insoluble fraction of leucocytes.

7 Separation of the chemotactic activity in the conditioned medium by isoelectric focusing revealed that the leucocyte infiltrated into the pouch fluid produce two kinds of factors, viz. leucocyte-derived neutrophil chemotactic factor-1 (LDNCF-1) and LDNCF-2 of which pI values are 4–5 and above 8, respectively.

8 The results indicate that PAF has no significant role in leucocyte activation to produce chemotactic factors, and that neutrophil chemotactic factors produced by infiltrated leucocytes are not PAF or leukotriene B₄ but are produced through a protein synthesis mechanism.

9 The mechanism of action of PAF antagonists on neutrophil infiltration into the inflammatory locus is discussed.

Keywords: Allergic inflammation; leucocytes; neutrophils; chemotactic factor; platelet activating factor; leukotriene B₄; CV-3988; L-652,731; Y-24,180; AA861

Introduction

Using an air pouch type allergic inflammation model in rats, we have demonstrated (Watanabe *et al.*, 1990) that neutrophil infiltration into the allergic inflammatory site is inhibited in a dose-dependent fashion by treatment with receptor antagonists of platelet activating factor (PAF). Inhibition of leucocyte infiltration by PAF antagonists is also reported in interleukin-1-induced inflammation in rabbit eyes (Rubin & Rosenbaum, 1988). Eosinophil infiltration into bronchial walls after the administration of antigen to passively sensitized guinea-pigs (Lellouch-Tubiana *et al.*, 1987) is also inhibited by treatment with PAF antagonists (Lellouch-Tubiana *et al.*, 1988), as is an antigen-induced bronchial hyper-reactivity model in guinea-pigs (Coyle *et al.*, 1988). These observations suggest that PAF has a significant role in leucocyte infiltration into the inflammatory site. During a series of experiments to clarify the role of PAF in allergic inflammation, we found that treatment with PAF antagonists suppressed neutrophil chemotactic activity in the pouch fluid

(the present paper) in parallel with the decrease in neutrophil infiltration into the pouch fluid (Watanabe *et al.*, 1990). This finding encouraged us to extend our research to obtain further insight into the role of PAF in allergic inflammation. The present investigation was designed to determine whether infiltrated leucocytes produce neutrophil chemotactic factors, to determine whether PAF that might be produced in the inflammatory site stimulates neutrophil chemotactic factor production by infiltrated leucocytes, and to characterize pharmacologically the chemotactic factors produced by infiltrated leucocytes. The mechanism of action of PAF antagonists on neutrophil infiltration into the inflammatory locus is also discussed.

Methods

Induction of allergic inflammation

The immunization and induction of allergic inflammation of the air pouch type in rats were carried out as described

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previously (Tsurufuji *et al.*, 1982) with a slight modification. Male Sprague-Dawley rats, specific pathogen-free weighing 140–150 g (Charles River Japan Inc., Kanagawa, Japan) were used. An antigen, azobenzenearsonate-conjugated acetyl bovine serum albumin (ABA-AcBSA) was prepared according to the procedure described by Tabachnick & Sobotka (1962). The rats were immunized by i.d. injection of the antigen and Freund's complete adjuvant (Difco Laboratories, Detroit, MI, U.S.A.). Nine days after immunization, 8 ml of air was injected s.c. on the dorsum to form an air pouch in the shape of an ellipsoid. 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 carboxymethyl-cellulose (CMC-Na, Cellogen F3H; Daiichi Kogyo Seiyaku, Niigata, Japan) in 0.9% NaCl solution was injected into the air pouch to provoke allergic inflammation. A group of rats that were injected i.d. with Freund's complete adjuvant emulsion containing no antigen was challenged in the same way and served as the 'non-immunized rats'. The CMC-Na solution was supplemented with penicillin G potassium and dihydrostreptomycin sulphate (Meiji Seika Co., Tokyo, Japan) each at 0.1 mg ml^{-1} concentration.

In vivo treatment with PAF antagonists

Four hours after the antigen challenge, 0.5 ml of saline solution containing various doses of PAF antagonists were injected into the pouches. Rats were then killed 8 h after the antigen challenge by cutting the carotid artery under diethyl ether anaesthesia, and total pouch fluid was collected and measured. A portion of the pouch fluid was used for counting the number of infiltrated leucocytes. The rest of the pouch fluid was diluted four fold with RPMI 1640 medium (Nissui Seiyaku Co., Tokyo, Japan) containing 0.25% (w/v) bovine serum albumin (BSA, essentially fatty acid-free, Sigma Chemical Co., St. Louis, MO, U.S.A.) and centrifuged at $1,500 g$ and 4°C for 5 min. The supernatant fraction was collected and finally diluted six fold with RPMI 1640 medium containing 0.25% (w/v) BSA, and used for measurement of chemotactic activity for neutrophils. The precipitate was suspended in a small volume of the medium and smeared onto a slide. Differential cell counts were performed microscopically after May-Giemsa staining.

Leucocyte collection from the pouch fluid

Four hours after the antigen solution was injected into the air pouch, rats were killed by cutting the carotid artery under diethyl ether anaesthesia and the entire pouch fluid was collected and measured. The pouch fluid was diluted four fold with RPMI 1640 medium containing 0.25% (w/v) BSA and centrifuged at $1,500 g$ and 4°C for 5 min. The precipitate was washed three times with the medium and finally suspended in RPMI 1640 medium containing 0.25% (w/v) BSA at $1 \times 10^7 \text{ cells ml}^{-1}$. A portion of the cell suspension was smeared onto a slide and a differential cell count performed as described above.

Leucocyte culture

Four millilitres of the cell suspension (4×10^7 cells) was incubated at 37°C for various times in the presence or absence of drugs. After appropriate times of incubation, the cell suspension was centrifuged at $1,500 g$ and 4°C for 5 min. The supernatant fraction was obtained, finally diluted eight fold with RPMI 1640 medium containing 0.25% (w/v) BSA, and used for the assay of chemotactic activity for neutrophils.

Measurement of chemotactic activity for neutrophils

Chemotactic activity of the diluted pouch fluid or conditioned medium was assayed in a modified Boyden chamber

(Watanabe *et al.*, 1985). The upper and lower chambers of multiwell-type Boyden chambers were separated by a polycarbonate filter with pores $2 \mu\text{m}$ in diameter (Nucleopore Corp., Pleasanton, CA, U.S.A.). Rat peritoneal neutrophils were harvested 12–15 h after injection of 20 ml of Ca^{2+} -free Krebs-Ringer solution containing 1% (w/v) casein (casein from milk, vitamin-free, Wako Pure Chemical Ind., Osaka, Japan), which had been sterilized by autoclaving at 120°C for 15 min. The peritoneal neutrophils were washed twice and suspended in RPMI 1640 medium containing 0.25% (w/v) BSA at $1 \times 10^7 \text{ cells ml}^{-1}$; 300 μl of the cell suspension was applied to the upper chamber. In the lower chamber, 400 μl of the diluted pouch fluid or conditioned medium was added and incubated at 37°C for 80 min in a humidified atmosphere containing 5% CO_2 . After incubation, the total number of neutrophils that migrated into the lower chamber was counted with a Coulter counter (Coulter Electronics Ltd., Luton, Beds.). The migration rate was calculated as follows: migration rate (%) = (number of neutrophils in the lower chamber/number of neutrophils applied in the upper chamber) $\times 100$. As a positive control, 400 μl of RPMI 1640 medium containing 0.25% (w/v) BSA and 10 nM PAF (a mixture of C_{16} and C_{18} forms, Avanti Polar Lipid Inc., Birmingham, AL, U.S.A.) was added in the lower chamber, and the migration rate was measured in each experiment. The migration index for each sample was obtained from the following equation and used as an index of chemotactic activity: Migration index (%) = (migration rate for each sample/migration rate for 10 nM PAF) $\times 100$.

Measurement of protein synthesis

Protein synthesis was determined by measuring the incorporation of L-[4,5- ^3H]-leucine ($55.9 \text{ Ci mmol}^{-1}$, Du Pont/New England Nuclear, Boston, MA, U.S.A.) into an acid-insoluble fraction (Ohuchi *et al.*, 1981). Four millilitres of leucocyte suspension ($1 \times 10^7 \text{ cells ml}^{-1}$) in RPMI 1640 medium containing 0.25% (w/v) BSA, 5 μCi of [^3H]-leucine and various concentrations of cycloheximide (Wako Pure Chemical Ind.) was incubated at 37°C for 4 h, and the cell suspension was centrifuged at $1,500 g$ and 4°C for 5 min. The supernatant fraction was obtained, diluted eight fold with the medium and used for measurement of chemotactic activity for neutrophils. The precipitate was suspended in 1 M perchloric acid (PCA) solution and centrifuged at $2,500 g$ and 4°C for 15 min; the precipitate was washed three times with 1 M PCA solution. Then the precipitate was dissolved in 0.1 M NaOH solution and transferred into scintillation vials for quantification of radioactivity.

Drug treatment

For *in vivo* treatment, PAF antagonists CV-3988 ((*RS*)-2-methoxy-3-(octadecylcarbamoyloxy)propyl-2-(3-thiazolio) ethyl phosphate) (Terashita *et al.*, 1983), L-652,731 (*trans*-2,5-bis-(3,4,5-trimethoxyphenyl)tetrahydrofuran) (Hwang *et al.*, 1985) and Y-24,180 (4-(2-chlorophenyl)-2-[2-(4-isobutyl-phenyl)ethyl]-6,9-dimethyl-6H-thieno[3,2-f][1,2,4]triazolo[4,3-a][1,4]diazepine) (Terasawa *et al.*, 1990) were dissolved in ethanol and diluted with saline solution (final 5% (v/v) ethanol); 0.5 ml of the solution were injected into the pouch. For *in vitro* experiments, the 5-lipoxygenase inhibitor AA861 (2,3,5-trimethyl-6-(12-hydroxy-5,10-dodecadienyl)-1,4-benzoquinone) (Yoshimoto *et al.*, 1982), PAF antagonists CV-3988, L-652,731 and Y-24,180, and the protein synthesis inhibitor cycloheximide were dissolved in ethanol and an aliquot of the ethanol solution was added into RPMI 1640 medium containing 0.25% (w/v) BSA. The final ethanol concentration in the medium was adjusted to 0.1% (v/v).

Measurement of leukotriene B₄ in the conditioned medium

Leukotriene B₄ levels in the conditioned medium were radioimmunoassayed with a leukotriene B₄ ³H-RIA kit (Du Pont/New England Nuclear). Three millilitres of the conditioned medium to which was added 1×10^{-3} μ Ci of [³H]-leukotriene B₄ (210 Ci mmol⁻¹, Du Pont, New England Nuclear) were applied to an octadecylsilyl silica cartridge (SEP-PAK C₁₈, Waters Associates, Inc., Milford, MA, U.S.A.). The cartridge was then washed with 10 ml of H₂O, and lipophilic components were eluted with 10 ml of methanol. After evaporation of methanol under reduced pressure, the residue was dissolved in 100 μ l of the assay buffer, and an aliquot was used for radioimmunoassay. The rest of the solution was used for the correction of recoveries during the extraction procedure by measuring radioactivity of [³H]-leukotriene B₄.

Separation of chemotactic activity for neutrophils in the conditioned medium by isoelectric focusing

Leucocytes in the pouch fluid from eight immunized rats were collected and combined 4 h after the antigen challenge, and incubated for 4 h at 37°C in RPMI 1640 medium containing 0.25% (w/v) BSA at 1×10^7 cells ml⁻¹ concentration. After incubation, the cell suspension was centrifuged at 1,500 g and 4°C for 5 min. The supernatant fraction (49 ml) was dialyzed against 10 mM NaCl and mixed with 1 ml of Bio-Lyte ampholytes (pH range 3–10, Bio-Rad Lab., Richmond, CA, U.S.A.). The mixture was loaded into the focusing chamber of the Rotofor cell (Bio-Rad Lab.). Isoelectric focusing was carried out at 12 W constant power for 3 h at 4°C. Twenty fractions were harvested and their pH values measured. Each fraction (2.5 ml) was dialyzed against 1 M NaCl to remove ampholytes, and further dialyzed against phosphate-buffered saline (pH 7.4) at 4°C. Chemotactic activity in each fraction was then determined after six fold dilution with RPMI 1640 medium containing 0.25% (w/v) BSA.

Table 1 Effects of PAF antagonists on the number of infiltrated neutrophils and neutrophil chemotactic activity in the pouch fluid at 8 h

Treatment	No. of neutrophils ($\times 10^{-8}$ cells)	Migration index (%)
Non-immunized	$1.35 \pm 0.11^{**}$	$25.6 \pm 2.2^{**}$
Immunized	2.91 ± 0.15	76.2 ± 2.5
CV-3988 30 μ g	$2.21 \pm 0.12^*$	$58.9 \pm 1.9^{**}$
100 μ g	$2.05 \pm 0.11^{**}$	$53.1 \pm 2.1^{**}$
300 μ g	$1.72 \pm 0.09^{**}$	$48.2 \pm 1.8^{**}$
L-652,731 3 μ g	$2.41 \pm 0.08^*$	$62.5 \pm 3.1^{**}$
10 μ g	$2.15 \pm 0.15^{**}$	$55.1 \pm 2.1^{**}$
30 μ g	$1.81 \pm 0.12^{**}$	$46.4 \pm 1.9^{**}$
Y-24,180 0.03 μ g	$2.35 \pm 0.07^*$	$60.1 \pm 2.2^{**}$
0.1 μ g	$1.90 \pm 0.11^{**}$	$53.2 \pm 1.9^{**}$
0.3 μ g	$1.70 \pm 0.13^{**}$	$45.2 \pm 1.7^{**}$

Chemotactic activity for neutrophils in the pouch fluid was measured after six fold dilution. Values are the means $\pm$ s.e.mean from 5–6 rats. Statistical significance: * $P < 0.01$, ** $P < 0.001$ vs. immunized control.

Table 2 Total number of leucocytes in the pouch fluid collected 4 h after injection of the antigen solution

	Pouch fluid (ml)	Total leucocytes	Total number of cells ($\times 10^{-8}$ cells per pouch)		
			Neutrophils	Mononuclear cells	Eosinophils
Immunized	3.25 ± 0.14	1.57 ± 0.11	1.32 ± 0.04	0.17 ± 0.02	0.07 ± 0.01
Non-immunized	$2.54 \pm 0.22^*$	1.49 ± 0.22	1.28 ± 0.05	0.18 ± 0.01	0.07 ± 0.01

Values are the means $\pm$ s.e.mean from 6 rats. Statistical significance: * $P < 0.05$ vs. immunized group.

Statistical analysis

Results were analysed for statistical significance by Dunnett's test for multiple comparison or Student's *t* test for unpaired observations.

Results

Effects of PAF antagonists on neutrophil infiltration and neutrophil chemotactic activity in the pouch fluid at 8 h

When PAF antagonists were injected into the pouch of immunized rats 4 h after injection of the antigen solution, neutrophil infiltration into the pouch fluid at 8 h was suppressed in a dose-dependent manner (Table 1). In parallel with the decrease in neutrophil infiltration, neutrophil chemotactic activity in the pouch fluid was decreased (Table 1). Gradient analysis for neutrophil migration with the diluted pouch fluid demonstrated that movement of neutrophils was not chemokinetic but chemotactic, since migrations in a positive gradient were more effective compared with those in a negative gradient (data not shown). Among the three PAF antagonists, Y-24,180 was the most potent. Because the number of infiltrated mononuclear cells and eosinophils was much smaller than that of neutrophils, the effects of PAF antagonists on the infiltration of these cells were inconclusive (data not shown). When the PAF antagonists were injected into the pouch at the time of antigen challenge, neutrophil infiltration into the pouch fluid at 4 h was not suppressed at all either in immunized or non-immunized rats (number of neutrophils [$\times 10^{-8}$ cells per pouch]: 1.43 ± 0.06 for control, 1.38 ± 0.05 for CV-3988 (100 μ g)-treated group, 1.46 ± 0.06 for L-652,731 (10 μ g)-treated group, and 1.35 ± 0.06 for Y-24,180 (0.1 μ g)-treated group, means $\pm$ s.e.mean from 5–7 rats).

Total number of leucocytes in pouch fluid at 4 h

There was no significant difference between immunized and non-immunized rats in total number of leucocytes, neutrophils, mononuclear cells and eosinophils in the pouch fluid collected 4 h after injection of the antigen solution (Table 2). Pouch fluid volume in the immunized rats was significantly higher than that in the non-immunized rats (Table 2).

Neutrophil chemotactic factor production by infiltrated leucocytes

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 and non-immunized rats were incubated at 37°C in 4 ml of RPMI 1640 medium containing 0.25% (w/v) BSA, chemotactic activity for neutrophils in the conditioned medium was increased in a time-dependent manner in both groups (Figure 1). Chemotactic factor production by leucocytes from immunized rats was significantly higher than that from non-immunized rats. The difference between the two groups was apparent even at 0.5 h incubation (Figure 1). Gradient analysis of neutrophil migration with the diluted conditioned medium demonstrated that movement was not chemokinetic but chemotactic, since migrations in a positive gradient were more effective than those in a negative gradient (data not shown).

Effects of PAF on chemotactic factor production

To clarify whether leucocytes in the pouch fluid of non-immunized rats are stimulated to produce neutrophil chemotactic factors by PAF treatment, the following experiments were performed. Leucocytes (4×10^7 cells) in the pouch fluid collected 4 h after injection of the antigen solution into the air pouch of non-immunized rats were preincubated at 37°C for 5 to 30 min in 4 ml of the medium containing various concentrations of PAF, washed three times with the medium containing no PAF and further incubated for 4 h in 4 ml of RPMI 1640 medium containing 0.25% (w/v) BSA; chemotactic activity for neutrophils in the conditioned medium was then measured. As shown in Table 3, chemotactic factor production by leucocytes from non-immunized rats was not stimulated by pretreatment with

Figure 1 Neutrophil chemotactic factor production by leucocytes in the pouch fluid. Leucocytes were collected from 28 immunized rats (■) and 28 non-immunized rats (□) 4 h after injection of the antigen solution. Leucocytes (4×10^7 cells) from each rat were incubated at 37°C for indicated periods in 4 ml of the medium. Chemotactic activity for neutrophils in the conditioned medium was determined after eight fold dilution. Values are the means $\pm$ s.e.mean from 5 to 6 rats. Statistical significance: ** $P < 0.001$ vs. corresponding non-immunized rats.

Table 3 Effects of PAF on chemotactic factor production by leucocytes in the pouch fluid of non-immunized rats and immunized rats

Leucocytes	Pretreatment with PAF	Migration index (%)
Non-immunized	None	28.1 $\pm$ 2.1
	10 nM, 5 min	29.3 $\pm$ 3.1
	10 min	28.8 $\pm$ 2.5
	30 min	27.9 $\pm$ 3.5
	50 nM, 5 min	30.1 $\pm$ 2.2
	10 min	28.9 $\pm$ 3.5
Immunized	30 min	27.5 $\pm$ 2.4
	None	76.3 $\pm$ 4.3*
	10 nM, 5 min	74.9 $\pm$ 3.5*
	10 min	75.5 $\pm$ 3.9*
	30 min	71.6 $\pm$ 4.3*
	50 nM, 5 min	73.2 $\pm$ 3.6*
	10 min	77.5 $\pm$ 4.2*
	30 min	76.9 $\pm$ 5.0*

Leucocytes (4×10^7 cells) from 7 non-immunized rats or 7 immunized rats were preincubated at 37°C for indicated period in 4 ml of the medium containing 10 or 50 nM PAF. Cells were then washed and incubated for 4 h in the medium containing no PAF. Chemotactic activity for neutrophils in eight fold diluted conditioned medium was assayed. Values are the means $\pm$ s.e.mean from 4 samples. Statistical significance: * $P < 0.001$ vs. non-immunized control.

PAF at concentrations of 10 or 50 nM. Failure of stimulation of chemotactic factor production by PAF also was observed in leucocytes from immunized rats (Table 3). In another experiment, leucocytes from non-immunized rats were incubated in the medium containing 10 or 50 nM PAF for 30 min, 1, 2 and 4 h, then neutrophil chemotactic activity in the conditioned medium was measured. In this case, chemotactic activity was measured after addition of the PAF antagonist, L-652,731 to the conditioned medium at a concentration of 20 μ M, at which concentration, PAF (50 nM)-induced neutrophil chemotaxis was almost completely suppressed. The results also indicated that PAF treatment did not induce chemotactic factor production (data not shown).

Effects of AA861 on chemotactic factor production

Neutrophil chemotactic factor production by leucocytes from immunized rats was not inhibited when various amounts of AA861, a 5-lipoxygenase inhibitor, were added to the incubation medium (Figure 2). Measurement of leukotriene B_4 levels in the conditioned medium by radioimmunoassay revealed that AA861 at these concentrations suppressed leukotriene B_4 production almost completely (leukotriene B_4 levels: 14.2 ± 0.5 pg ml $^{-1}$ for control, compared with less than the detectable amount for AA861-treated groups, mean $\pm$ s.e.mean from five samples). Similarly, AA861 failed to suppress chemotactic factor production by leucocytes from non-immunized rats (migration index: 37.5 ± 2.2 for control, 38.9 ± 1.9 for AA861 30.6 μ M, mean $\pm$ s.e.mean from six samples). AA861 by itself at concentrations up to 30.6 μ M showed no effect on neutrophil chemotaxis (data not shown).

Effects of PAF antagonists on neutrophil chemotactic activity in the conditioned medium

If PAF is produced by leucocytes and released into the conditioned medium, the newly synthesized PAF might account for the increased chemotactic activity in the conditioned medium. However, when various amounts of PAF antagonists were added to the diluted conditioned medium of leucocytes from immunized rats and chemotactic activity for neutrophils measured, no significant inhibition was observed at concentrations sufficient to inhibit PAF (10 nM)-induced neutrophil chemotaxis (Figure 3). Among the three PAF antagonists, Y-24,180 was the most potent in inhibiting PAF (10 nM)-induced neutrophil chemotaxis (Figure 3). The addi-

Figure 2 Effects of AA861 on chemotactic factor production by leucocytes in the pouch fluid. Leucocytes in the pouch fluid at 4 h were collected and combined from 7 immunized rats (+) and 4 non-immunized rats (-), respectively; 4×10^7 cells from each group were incubated at 37°C for 4 h in 4 ml of the medium containing the indicated concentrations of AA861. Chemotactic activity for neutrophils in the conditioned medium was determined after eight fold dilution. Values are the means from 4 to 5 samples with s.e.mean. Statistical significance: ** $P < 0.001$ vs. immunized control.

tion of PAF antagonists to the conditioned medium of leucocytes from non-immunized rats also failed to inhibit neutrophil chemotaxis (% of control: Y-24,180 at 10^{-8} M; 98.9 ± 1.1 , L-652,731 at 10^{-5} M; 99.2 ± 2.2 , CV-3988 at 10^{-4} M; 101.2 ± 2.5 , mean $\pm$ s.e.mean from 5–6 samples).

Chemotactic activity in the lipid fraction of the conditioned medium

Four hours after incubation of the leucocytes from immunized and non-immunized rats, total lipids were ex-

Figure 3 Effects of PAF antagonists on neutrophil chemotaxis induced by PAF and conditioned medium of leucocytes. Leucocytes in the pouch fluid at 4 h were collected and combined from 10 immunized rats, and incubated at 37°C for 4 h in the medium at a concentration of 4×10^7 cells per ml. The conditioned medium was diluted eight fold with fresh medium containing various concentrations of each PAF antagonist, and measured neutrophil chemotactic activity (open symbols). Migration index by eight fold diluted conditioned medium without PAF antagonist is expressed as 100%. Chemotactic activity of the medium containing 10 nM PAF and indicated concentrations of each PAF antagonist also was determined (solid symbols): Y-24,180 (O, ●); L-652,731 (Δ, ▲); CV-3988 (□, ■). Migration index for 10 nM PAF is taken as 100%. Values are the means from 4 samples; s.e.means are not shown since they are less than 3% of the mean value.

Figure 4 Chemotactic activity of the lipid fraction in the conditioned medium. Leucocytes in the pouch fluid at 4 h were collected and combined from 5 immunized rats (+) and 5 non-immunized rats (-), respectively; 4×10^7 cells from each group were incubated at 37°C for 4 h in 4 ml of the medium, and chemotactic activity was measured after eight fold dilution (a). Total lipids in the conditioned medium were extracted (Bligh & Dyer, 1959), reconstituted in the medium, finally diluted eight fold, and chemotactic activity was measured (b). Recoveries of [3 H]-leukotriene B_4 and [3 H]-PAF during the extraction procedure were more than 90% and 85%, respectively. Values are the means with s.e.mean from 4 to 5 samples. Statistical significance: * $P < 0.001$ vs. corresponding non-immunized control. # $P < 0.001$ vs. corresponding conditioned medium.

tracted from the conditioned medium and reconstituted in the medium. Chemotactic activity of the lipid fraction in the conditioned medium of leucocytes from immunized and non-immunized rats was much lower than that of the original conditioned medium (Figure 4). These results also indicate that participation of lipophilic chemoattractants such as PAF and leukotriene B_4 in the chemotactic activity of the conditioned medium is very low.

Effects of cycloheximide on chemotactic factor production

When leucocytes in the pouch fluid at 4 h were incubated at 37°C for 4 h in the medium containing various concentrations of cycloheximide, chemotactic factor production was inhibited in a concentration-dependent manner both in immunized and non-immunized rats (Figure 5). The inhibitory effect of cycloheximide was maximum at 1.78 μ M.

Effects of cycloheximide on uptake of [3 H]-leucine into acid insoluble fraction of leucocytes

Uptake of [3 H]-leucine into the 1 M perchloric acid-insoluble fraction of leucocytes was also inhibited by cycloheximide in a concentration-dependent manner (Figure 6). The time course of the inhibition of chemotactic factor production by cycloheximide at a concentration of 1.78 μ M is shown in Figure 7. Almost complete inhibition of neutrophil chemotactic factor production was observed at 0.5 to 4 h of incubation, both in immunized and non-immunized groups.

Separation of chemotactic activity of neutrophils in the conditioned medium by isoelectric focusing

Chemotactic activity of neutrophils in the conditioned medium was separated into two peaks (Figure 8). One is a sharp peak of which the pI value is around 5.5, and the other is rather broad and the pI value is around 9.0. The first one is named leucocyte-derived neutrophil chemotactic factor-1 (LDNCF-1) and the second one is LDNCF-2. pI values of each factor varied from experiment to experiment, ranging 4 to 6 for LDNCF-1 and above 8 for LDNCF-2. When leucocytes were incubated in the presence of cycloheximide

Figure 5 Effects of cycloheximide on chemotactic factor production by leucocytes in the pouch fluid. Leucocytes in the pouch fluid at 4 h were collected and combined from 7 immunized rats (hatched columns) and 7 non-immunized rats (open columns), respectively; 4×10^7 cells from each group were incubated at 37°C for 4 h in 4 ml of the medium containing indicated concentrations of cycloheximide. Chemotactic activity for neutrophils in the conditioned medium was determined after eight fold dilution. Values are the means with s.e.mean from 4 to 5 samples. Statistical significance: * $P < 0.01$ and ** $P < 0.001$ vs. corresponding non-immunized control. † $P < 0.001$ vs. corresponding non-immunized group.

Figure 6 Effects of cycloheximide on [^3H]-leucine uptake into 1 M perchloric acid-insoluble fraction of leucocytes in the pouch fluid. Leucocytes in the pouch fluid at 4 h were collected and combined from 7 immunized rats (hatched columns) and 7 non-immunized rats (open columns), respectively; 4×10^7 cells from each group were incubated at 37°C for 4 h in 4 ml of the medium containing 5 μCi of [^3H]-leucine, and indicated concentrations of cycloheximide. Values are the means with s.e.mean from 4 to 5 samples. Statistical significance: $**P < 0.001$ vs. corresponding control.

(1.78 μM) for 4 h, both peaks of chemotactic activity were diminished (not shown in Figure 8).

Discussion

Treatment with PAF antagonists suppresses neutrophil infiltration into the allergic inflammatory locus in parallel with the decrease in neutrophil chemotactic activity in the pouch fluid (Table 1). Leucocytes that had infiltrated into the allergic inflammatory locus produced neutrophil chemotactic factors much more prominently than leucocytes that had infiltrated into the non-allergic inflammatory locus (Figure 1). These results suggested that leucocytes in the pouch fluid of immunized rats might have been stimulated to produce neutrophil chemotactic factors by a certain chemical mediator, possibly produced by the antigen challenge in the inflammatory locus. Since our previous work (Watanabe *et al.*, 1990) and the present work demonstrated that treatment with PAF antagonists suppresses neutrophil infiltration into the pouch fluid in the allergic inflammation, it was possible that PAF produced in the inflammatory locus might stimulate leucocytes to produce neutrophil chemotactic factors. PAF levels in the pouch fluid collected 4 h after the antigen challenge in the immunized rats, however, were less than the amount detectable by aggregation of guinea-pig platelets (Watanabe *et al.*, 1987; 1988). This result need not imply that PAF is not synthesized at the inflammatory locus, since PAF is easily metabolized into lyso-PAF in the pouch fluid (Watanabe *et al.*, 1987). However, when leucocytes in the pouch fluid from non-immunized rats were preincubated in the medium containing 10 or 50 nM PAF, neutrophil chemotactic factor production was not stimulated at all (Table 3). Pretreatment of leucocytes from the non-immunized rats with PAF at a concentration of 100 nM for 1 h also failed to stimulate chemotactic factor production (migration index (%), 30.2 ± 2.7 for control, 31.5 ± 2.1 for PAF-treated group, means $\pm$ s.e.mean from 4 samples). Consequently, it is concluded that the PAF that might be produced in the inflammatory locus does not participate in stimulation of leucocytes to produce neutrophil chemotactic factors.

As to the characteristics of neutrophil chemotactic factors produced by infiltrated leucocytes, although leukotriene B_4 is a potent chemotactic substance derived from arachidonic

Figure 7 Time course of chemotactic factor production by leucocytes in the pouch fluid and effects of cycloheximide. Leucocytes in the pouch fluid at 4 h were collected and combined from 7 immunized rats (a) and 7 non-immunized rats (b), respectively; 4×10^7 cells were incubated at 37°C for indicated times in 4 ml of the medium with (open symbols) or without (solid symbols) cycloheximide (1.78 μM). Chemotactic activity was determined after eight fold dilution. Values are the means from 3 to 4 samples; s.e.means are not shown since they are within the symbols. Statistical significance: $**P < 0.001$ vs. corresponding control.

Figure 8 Separation of chemotactic activity for neutrophils in the conditioned medium by isoelectric focusing. Migration index in each fraction was measured after focusing proteins in the conditioned medium of infiltrated leucocytes from immunized rats. Values are the means with s.e.mean from 4 assays. The results were confirmed by four separate experiments.

acid (Ford-Hutchinson *et al.*, 1980; Smith *et al.*, 1980), it was demonstrated that the factor is unlikely to be leukotriene B_4 since neutrophil chemotactic factor production was not inhibited by AA861, a 5-lipoxygenase inhibitor (Figure 2). PAF is also reported to be a potent lipophilic chemoattractant for neutrophils (Shaw *et al.*, 1981). However, neutrophil chemotactic activity in the conditioned medium was not suppressed by the PAF antagonists, CV-3988, L-652,731 and

Y-24,180 (Figure 3). Consequently, neutrophil chemotactic activity in the conditioned medium of infiltrated leucocytes is probably not attributable to PAF. In fact, preliminary experiments revealed that levels of PAF in the conditioned medium measured by bioassay were less than the levels that induce neutrophil chemotaxis *in vitro* (0.1 nM). Furthermore, the finding that the lipophilic fraction obtained from the conditioned medium showed little chemotactic activity (Figure 4) also indicates the minor significance of leukotriene B₄ and PAF as chemotactic factors produced by infiltrated leucocytes. In summary, the present investigation demonstrated that (1) the infiltrated leucocytes are not activated by PAF to produce neutrophil chemotactic factors, and (2) chemoattractants produced by leucocytes are not PAF or leukotriene B₄. Consequently, it still remains to be clarified why PAF antagonists inhibit neutrophil infiltration into the inflammatory locus *in vivo* (Watanabe *et al.*, 1990). In the air pouch type allergic inflammation in rats, no significant infiltration of eosinophils into the pouch fluid is induced. This might be due to the effect of Freund's complete adjuvant used for the sensitization of rats. Consequently, this model is not suitable for determining the effect of PAF antagonists on eosinophil infiltration. The most plausible explanation for the inhibition by PAF antagonists of leucocyte infiltration might be as follows: PAF antagonists would inhibit leucocyte adherence to microvascular endothelial cells in the inflammatory locus, thus inhibiting the succeeding processes such as diapedesis and chemotaxis of leucocytes. This explanation is based on our findings (Watanabe *et al.*, 1991) that the PAF antagonists, CV-3988, L-651,731 and Y-24,180 inhibit neutrophil adherence to cultured vascular endothelial cells from human umbilical vein stimulated by histamine or thrombin.

When pouch fluid collected 8 h after the antigen challenge from the immunized rats was fractionated into lipophilic and hydrophilic components, chemotactic activity for neutrophils in the lipophilic component was very small. Almost all the chemotactic activity in the pouch fluid was attributable to the hydrophilic component (Omata *et al.*, 1990). These results suggest that neutrophil infiltration into the pouch fluid at 8 h is not mediated by lipophilic substances such as PAF and leukotriene B₄ but by hydrophilic substances that might be produced by infiltrated leucocytes. When the number of peripheral leucocytes was decreased by treatment with cyclophosphamide, neutrophil infiltration into the pouch fluid in immunized rats was lowered in parallel with the decrease in neutrophil chemotactic activity in the pouch fluid (Omata *et al.*, 1991). These observations also suggest that infiltrated leucocytes are responsible for neutrophil chemotactic factor

production. *In vivo* treatment with the protein synthesis inhibitor, cycloheximide, inhibits neutrophil infiltration in the air pouch type allergic inflammation in rats (Ohuchi *et al.*, 1982), and eosinophil accumulation in compound 48/80-induced rat pleurisy (Martins *et al.*, 1990) and in PAF-induced rat pleurisy (e Silva *et al.*, 1991). When the infiltrated leucocytes were incubated in the medium containing cycloheximide, neutrophil chemotactic factor production was inhibited (Figure 5) in parallel with the decrease in protein synthesis (Figure 6). These results strongly suggest that infiltrated leucocytes produce neutrophil chemoattractants through protein synthesis mechanisms. It is possible that the chemoattractants in the conditioned medium might be of the interleukin-8 (IL-8) family, since IL-8 is a polypeptide produced by mononuclear phagocytes (Schröder *et al.*, 1987; Walz *et al.*, 1987; Yoshimura *et al.*, 1987; Peveri *et al.*, 1988), macrophages (Rankin *et al.*, 1990), polymorphonuclear leucocytes (Bazzoni *et al.*, 1991) and lymphocytes (Gregory *et al.*, 1988; Schröder *et al.*, 1988). As to the proteinaceous neutrophil chemotactic factor, Watanabe *et al.* (1989) reported that rat kidney epithelioid cell line, NRK-52E, produces a cytokine-induced neutrophil chemoattractant (CINC), a superfamily of IL-8, of which the pI value is above 8. Analysis of chemotactic activity in the conditioned medium by isoelectric focusing revealed that leucocytes infiltrated into the pouch fluid produce two different factors viz. leucocyte-derived chemotactic factor-1 (LDNCF-1) and LDNCF-2 of which the pI values are 4–5 and above 8, respectively (Figure 8). Since pI values of CINC and other proteinaceous chemoattractant, murine macrophage inflammatory protein 2 (MIP-2) (Wolpe *et al.*, 1989), are more than 8, LDNCF-2 might contain CINC, MIP-2 or both. It should be stressed, however, that LDNCF-1 might be a new proteinaceous chemoattractant produced by leucocytes infiltrated into inflammatory lesions.

The mechanism of leucocyte activation in allergic inflammation and the types of leucocytes that produce neutrophil chemotactic factors remain to be elucidated. Further purification and characterization of LDNCF-1 and LDNCF-2 produced by infiltrated leucocytes are under investigation in our laboratories.

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