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PAF-Induced Eosinophil Chemotaxis Increases during an Asthmatic Attack and Is Inhibited by Prednisolone *in Vivo* and *in Vitro*

Kunihiko Shindo,[1] Kohei Koide, and Motonori Fukumura
First Department of Internal Medicine, Yokohama City University School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236, Japan

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We investigated platelet-activating factor (PAF)-induced migration in eosinophils obtained from asthmatic patients who were treated with or without intravenous prednisolone. The migration of asthmatic eosinophils in remission and during an attack was significantly greater than that in healthy volunteers. The migration of asthmatic eosinophils exposed to prednisolone *in vivo* and *in vitro* was significantly inhibited, compared to asthmatic eosinophils not exposed to prednisolone. These findings suggest that an intracellular factor causes asthmatic eosinophils to migrate, and that prednisolone inhibits PAF-induced eosinophil migration.

Eosinophil migration into the bronchial lumen plays a major role in the pathophysiology of allergic asthma [1,2]. Migration of eosinophils into inflammatory sites is dependent on the release of local chemoattractants and involves eosinophil adhesion and migration through the endothelial wall. Although the complex adhesive interactions related to transendothelial migration are undergoing thorough investigation [3,4], little is known concerning eosinophil-epithelial migration. There is possibility of further study concerning eosinophil migration. Especially, there has been no report concerning migration of eosinophils obtained from asthmatics. Prednisolone is effective in treating bronchial asthma [5], and effectively reduces bronchial hyperresponsiveness [6,7]. In addition, steroids cause a reduction in the number of mast cells and eosinophils in patients with allergic rhinitis [8,9]. However, the clinical efficacy of prednisolone in bronchial asthma has not necessarily been paralleled by firm evidence of its anti-inflammatory action in vivo and has not been evaluated objectively and sufficiently.

[1] To whom correspondence should be addressed. Fax: 45-787-2509.

The present study, therefore, was designed to examine the migration of eosinophils during the clinical course of bronchial asthma compared to healthy donors, as well as the migration of eosinophils in response to platelet activating factor (PAF) in asthmatics exposed to prednisolone in vivo and in vitro.

MATERIALS AND METHODS

Materials. PAF (1-*O*-Octadecyl-2-0-acetyl-*sn*-glycero-3-phosphoryl-choline;C-18), Hanks' balanced salt solution (HBSS), 10% fetal calf serum (FCS), type II bovine serum albumin (BSA) and piperazine-*N*,*N*′-bis-(2-ethane-sulfonic acid) (Pipes) were purchased from Sigma Chemical Japan (Tokyo). 6% Dextran T70 and Percoll solution were purchased from Pharmacia Fine Chemicals (Piscataway, NJ, USA). Budget-Solve was purchased from Research Products International Corp., Mount Prospect, IL). GF/C glass microfiber filters was purchased from Whatman, Ltd., Maidstone, England. Binding assay was performed in a modified Hanks' balanced salt solution (1.4 mM calcium chloride, 0.7 mM magnesium chloride, pH 7.4) [10].

Subjects. Eosinophils were collected from 19 healthy men 29 to 57 years of ages, and from 37 asthmatic men 30 to 51 years of age. Patients were divided into two groups: Group 1 comprised 20 patients who were treated with intravenous prednisolone after admission for asthma attack, and Group 2 comprised 17 patients who were treated without intravenous prednisolone after admission for asthma attack. The 19 healthy men had normal pulmonary function and had no history of bronchial asthma or of allergies; none were taking medication. All subjects gave informed consent for participation prior to the study. All asthmatic subjects met the criteria for atopic asthma proposed by the American Thoracic Society [11].

Blood sampling and medication. Blood was obtained from each patient on three occasions: (1) during remission; (2) on admission to the hospital during an asthma attack; and (3) 48 hrs after admission and treatment with intravenous prednisolone (water-soluble, 500 mg/day) accompanied by anti-asthmatic drugs including inhaled prednisolone (400 ug/day)(Group 1: 20 cases); or 48 hrs after admission and treatment with anti-asthmatic drugs including inhaled prednisolone (400 μg/day) without intravenous prednisolone (Group 2: 17 cases). To evaluate the patients' biochemical status and to harvest eosinophils, 20 mL of heparinized venous blood were sampled from each patient at some time during a remission and from healthy subjects. The second 20-mL blood sample was collected to evaluate the patients' biochemical status and eosinophils just after admission and prior to treatment. The third blood sampling was performed 48 hrs

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TABLE 1

Characteristics of Subjects

Characteristics	Healthy	Asthmatics* Group 1		Asthmatics* Group 2	
Subjects, n	19	20		17	
Age (range)	40 (31-52)	44 (34-57)		46 (31-56)	
Sex, M	19	20		17	
Smoking	3	4		3	
Atopic, n*	0	20		17	
		Timepoint (1)	Timepoint (3)	Timepoint (1)	Timepoint (3)
FEV1 (range), % pred**	107 (98-110)	89 (80-97)	80 (70-86)	85 (79-93)	76 (66-80)
FEV1/VC (range), %	84 (80-87)	75 (66-85)	71 (66-77)	75 (64-88)	68 (64-78)
PC20 (GSD), mg/ml^	>16	3.79 (2.22)		3.52 (2.4)	
delta FEV1 (range), %^^	−1.4 (0.4-2.8)	−3.5 (0.2-5.7)	−3.2 (0.2-4.6)	−3.6 (0.3-6.7)	−4.6 (0.0-7.7)
On inhaled steroid^^^	—	10		9	

Data are expressed as mean and 95% confidence interval.

* Atopic means one or more positive allergy skin prick tests, n indicates the number of atopic subjects.

** FEV1 predicted values from Crapo (1) or previous best in the last 2 yr.

^ Methacholine PC20 geometric means; GSD is the geometric standard deviation.

^^ deltaFEV1 after sputum induction on Day 1.

^^^ Inhaled steroid was bechlomethasone dipropionate or budesonide.

(1) Crapo, R. O., Morris, A. H., and Gardner, R. M. (1987). Reference spirometric values using techniques and equipment that meets ATS recommendations. *Am. Rev. Respir. Dis.* **123,** 659–694.

after initiation of treatment in both groups. A cumulative dose(> 1000 mg/2 days) of water-soluble prednisolone was administered before the third blood sampling. In addition, the patients all who have received intravenous prednisolone were clinically improved on the third occasion (3), compared to patients that did not (table 1).

Leukocytes were obtained by the following method. Five volumes of blood were mixed with 1 volume of 6% Dextran T70 and suspended in normal saline for 1 hr at 37 °C. The dextran-plasma was collected and centrifuged at 450 × g for 8 min. Cells were washed once in saline and suspended in Percoll solution (density 1.070 g/mL) with 5% FCS. The concentration of cells was adjusted to 1×10^8/mL.

Percoll density gradient and eosinophil separation. Percoll density gradients were prepared in accordance with the method of Gartner [12]. Nine parts of Percoll were mixed with one part of HBSS 10× (10 times physiologic concentration), i.e., "heavy solution". One part of this heavy solution was mixed with nine parts of HBSS 1×; this mixture was termed "light solution". These heavy and light solutions were then combined to obtain mixtures of the following densities: 1.100, 1.090, 1.085, 1.080, and 1.070 g/mL, in accordance with Days formula [13,14]. Starting with the most dense solution, solutions of decreasing density were layered in a 16-mL poly-carbonate tube using a peristaltic pump at a low speed. Gradient densities were 1.100 g/mL (1.5 mL), 1.090 g/mL (3 mL), 1.085 g/mL (3 mL), and 1.080 g/mL (3 mL). Cells suspended in Percoll 1.070 g/mL supplemented with 5% FCS were layered on top of the gradients(1×10^8 cells in a 2-mL volume per gradient). The tubes were then centrifuged at 1600 × g for 20 min at room temperature. Cells were harvested from the gradients by 1 mL fractions from the bottom of the tubes. The density of each fraction was determined, and each was washed twice in 25 mM Pipes buffer, 110 mM NaCl, 5 mM KCl, 40 mM NaOH, and 5.4 mM glucose (pH 7.4) [15]. Cells were then counted in a hemocytometer. Cytocentrifuge smears were prepared from each fraction for differential counts and stained with Wright's stain. The fractions with the highest purity of eosinophils were pooled. The viability of cells exceeded 95% in all experiments, as confirmed by trypan blue dye exclusion [12,13]. Cells were immediately suspended in RPMI-1640 medium supplemented with 10% FCS. The cells were diluted to 2×10^6 cells/mL so that they were ready for use in the chemotactic assay.

TABLE 2

Characteristics of Eosinophils at Three Time Points in the Study Groups

Group	Mean blood eosinophilia (cells/mm^3) control	(1)	(2)	(3)
Asthmatics (G-1)		622	1976	1845
Asthmatics (G-2)		603	1888	1923
Healthy Donors (G-3)	213			

(1) in remission; (2) on admission to the hospital with an asthma attack; and (3) 48 hrs after admission and open treatment with (G-1) or without (G-2) intravenous prednisolone.

Migration assay. Migration toward PAF C-18 (5×10^{-5} mol/L) was measured. Migration (chemotaxis + chemokinesis) was assessed using a modified Boyden chamber, as previously described [16]. In addition, the eosinophil migration assay was done with reference to that previously detailed for neutrophil migration [17-21]. One milliliter of cell suspension in 0.4% ovalbumin was placed in the upper chamber separated from 0.8 mL of chemoattractant (PAF C-18) in the lower chamber by an 8-μM nitrocellulose filter. The solution of PAF was evaporated over nitrogen and redissolved in RPMI + 0.25% bovine serum albumin (BSA). Diluent controls were included in each assay. The filters were then washed in normal saline and fixed in saturated mercuric chloride/ethanol (50:50) for a minimum of 45 min prior to being stained with hematoxylin and chromotope 2R. The number of cells that had migrated through to the underside of the filters were counted within the area of a graticule on the 40 × objective. Results were expressed as the number of cells per 10 high-

TABLE 3

Eosinophil Migration in Response to PAF

Group	Eosinophil locomotion cells/10 hpf	FEV1(L)	Treatment			% eosinophils	% neutrophils
			IV-Pred	IH-Pred	Others/day		
Asthmatics (n=20): G-1							
(1) n=10	152 ± 7	3.11 ± 0.56	(−)	(−)	(−)	92.3 ± 2.6	9.5 ± 0.9
(2) n=10	553 ± 12	1.11 ± 0.19	(−)	(−)	(−)	89.5 ± 4.1	9.9 ± 1.8
(3) n=10	172 ± 5*	2.95 ± 0.27	(+)	(+)	salbutamol 24mg	89.2 ± 3.1	10.1 ± 2.3
Asthmatics (n=17): G-2							
(1) n=9	167 ± 4	3.42 ± 0.37	(−)	(−)	(−)	93.2 ± 6.2	9.0 ± 1.4
(2) n=9	585 ± 9	1.23 ± 0.22	(−)	(−)	(−)	90.1 ± 3.7	9.4 ± 1.2
(3) n=9	497 ± 6	2.79 ± 0.43	(−)	(+)	salbutamol 24mg	89.6 ± 3.3	11.2 ± 3.7
Healthy donors (n=19): G-3							
intact cells (n=10)	49 ± 1	3.79 ± 0.27	(−)	(−)	(−)	92.7 ± 3.6	9.1 ± 2.1

(1) during remission; (2) on admission to the hospital with an asthma attack; and (3) 48 hrs after admission; with intravenous prednisolone (G-1), without intravenous prednisolone (G-2). IV-Pred: treated with(+) or without(−) intravenous prednisolone; IH-Pred: treated with(+) or without(−) inhaled prednisolone.

FEV1 (L); forced expiratory volume, 1 second. (*) significantly differs from the eosinophil migration in (3) of group 2.

power fields (hpf). The intra-assay coefficient of variation was 11%, and duplicates did not differ from the mean by >11.9%. The cells were washed as described above and were placed in the upper part of the chamber at a concentration of 4×10^6 cells/mL separated by a nitrocellulose filter from the lower half. The chamber was incubated for 1.5 h in a CO_2 incubator, and the filter was stained and counted as above. Each assay was performed in triplicate [22, 23].

Binding assay. 1×10^7 eosinophils, 200 ul of a fraction from Percoll gradients, was suspended with a final volume of 0.9 ml Hanks' buffer in a 1.5-ml conical polyethylene microcentrifuge tube. Suspensions were then simultaneously exposed to [^{3}H]PAF C-18 (50 μl in Hanks' buffer containing BSA) plus unlabeled PAF (50 μl in BSA-Hanks' buffer) or BSA-Hanks' buffer (50 μl) in the presence or absence of prednisolone. All studies were performed at 4 °C in a final volume of 1 ml containing 250 ug BSA, 1.4 mM Ca^{2+}, and 0.7 mM Mg^{2+}. Following incubation (10 min) [24], suspensions were suctioned through premoistened GF/C filters. The microfuge tubes were washed five times with 1.5 ml of Hanks' buffer (4 °C) and each wash was suctioned through the same filter. Filters were air dried, placed in the bottom of 10-ml plastic vials, overlaid with 1 ml of methanol for 5 min, and mixed with 7 ml of Budget-Solve. Vials were counted for 4 min with a Beckman LS 1801 scintillation counter (Research Products International Corp., Mt. Prospect, IL). All test samples were paired with an identically treated control sample. Binding was defined as the difference between the disintegrations per minute retained by filters loaded with test and control samples. The difference (resulting value) is called by bound [^{3}H]PAF in the present study. The resulting value was corrected for the bound [^{3}H]PAF in tandem assays of test and control samples containing excess PAF to obtain specifically bound [^{3}H]PAF [24,25].

Statistical analysis. Migration of eosinophils to PAF was compared using the two-tailed paired Student's t test. Data were expressed as mean ± standard error [SEM]. Comparison of the effect of chemokinesis and chemotaxis was performed using a three-way analysis of variance. A level of $p < 0.05$ was accepted as statistically significant.

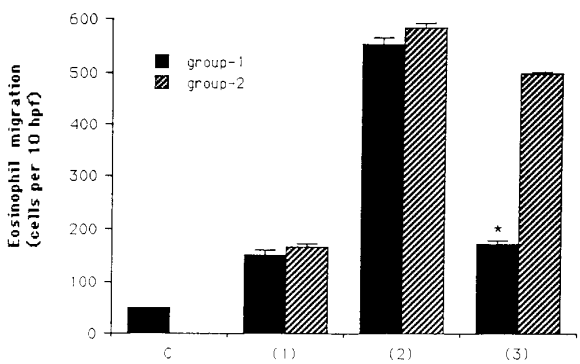

FIG. 1. Histogram of PAF (5×10^{-5} M)-induced eosinophil migration from healthy donors and from asthmatics at three time points: (1) during remission; (2) on admission to hospital with an asthma attack; and (3) 48 hrs after admission and open treatment with intravenous prednisolone and anti-asthmatic drugs including inhaled prednisolone (Group 1), or 48 hrs after admission and open treatment with antiasthmatic drugs including inhaled prednisolone without intravenous prednisolone (Group 2). Data are from table 3. * significantly differs from eosinophil migration in (3) of group 2.

RESULTS

Tables 1, 2, and 3 summarize the characteristics of the study groups, eosinophils, and eosinophil migra-

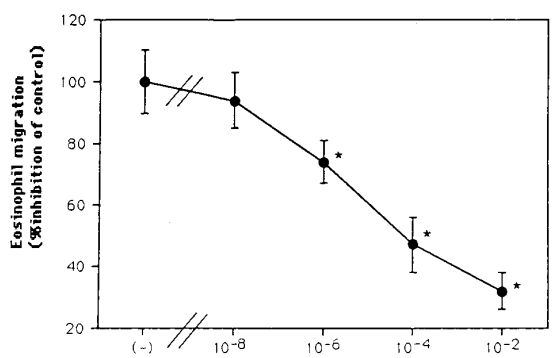

FIG. 2. In vitro effects of prednisolone on asthmatic eosinophil migration. * significantly differs from the eosinophil migration in without incubation (−).

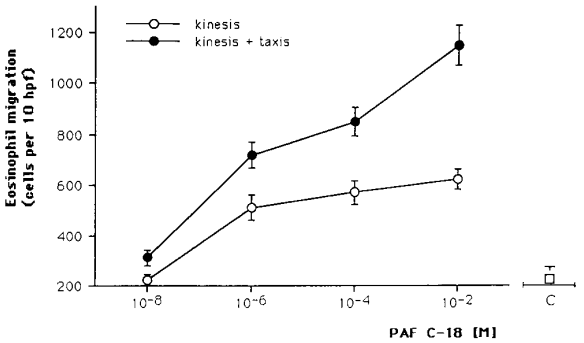

FIG. 3. Analysis of the relative contributions of chemokinesis (○) and chemotaxis + chemokinesis (●) to PAF-induced eosinophil migration. (□) Diluent control. n = 4. Eosinophil migration was expressed as the number of cells per 10 hpf (± SEM). The combination of chemotaxis and chemokinesis was significantly greater than the chemokinetic effect alone ($p < 0.05$).

tion. The number of eosinophils/mm^3 blood was calculated indirectly from the total eosinophil counts in leukocyte-rich plasma. The highest purity of eosinophils from the asthmatics was found at the 1.090 to 1.095 g/mL layer. Pooled fractions within the range of 1.085 to 1.100 g/mL showed 85% to 90% eosinophils with a recovery of 40% to 55% of the eosinophils applied to the gradient. The highest purity of eosinophils from the controls was found at the 1.090 to 1.095 g/mL layer. Pooled fractions within the range of 1.086 to 1.095 g/mL showed 92.7 ± 3.6% eosinophils with a recovery of 40% to 56% of the eosinophils applied to the gradient.

The mean numbers of eosinophils during an attack both before and after prednisolone treatment were significantly higher than the mean number of eosinophils during remission. There were no significant differences between the mean numbers before and after prednisolone treatment, and between Groups 1 and 2 (Table 2).

In asthmatics during an attack, the eosinophil migration rate (cells/10 hpf) toward PAF C-18 (5×10^{-5} mol/L) was significantly ($p < 0.05$) greater than that in asthmatics in remission or healthy donors. The eosinophil migration that occurred in asthmatics during an attack following prednisolone treatment was significantly ($p < 0.05$) less than the eosinophil migration during an attack without prednisolone treatment (Table 3 and Fig. 1).

In this in vitro experiment (Fig. 2), the prednisolone-induced inhibition on eosinophil migration occurred in a dose-dependent manner. The observed IC_{50} was approximately 10^{-4} M.

There was a significant difference ($p < 0.05$) between the two modes (chemokinesis and chemotaxis) of PAF-induced migration in asthmatic eosinophils during an acute attack (Fig. 3).

The dose response of PAF-induced nonasthmatic eosinophil migration was demonstrated in Figure 4. The eosinophil migration occurred in a dose-dependent manner at PAF concentrations of 5×10^{-5} to 1×10^{-7} M.

After 10-min incubation, uptake of [^{3}H]PAF was 90% of initial added radioactivity. Eosinophils obtained from asthmatics during an attack before treatment of prednisolone were greater in the uptake of [^{3}H]PAF than the cells obtained from asthmatics in remission and healthy donors (Fig. 5). The uptake of [^{3}H]PAF by the eosinophils obtained from asthmatics during an attack before treatment of prednisolone was reduced in a dose-dependent manner at prednisolone concentrations of 10^{-3} to 1×10^{-7} M (Fig. 6).

DISCUSSION

PAF-induced migration of eosinophils obtained from asthmatics during an attack was significantly greater than that in asthmatics in remission or healthy donors and was significantly inhibited by exposure to prednisolone in vivo and in vitro.

Prin [26] reported that the number of hypodense eosinophils (< 1.082 g/mL) increased in the blood of patients with bronchial asthma. The role of hypodense eosinophils in bronchial asthma [15] is not clear. Little and Casale [27] reported dose-response and time-course experiments that indicated no significant differences in PAF-induced hypodense versus normodense eosinophil chemotactic responses. We couldn't isolate both hypodense and normodense eosinophils in pure form from the asthmatic patients in the present study. Thus, we used only eosinophils within the range of 1.090 to 1.100 g/mL.

The in vitro inhibitory effect of prednisolone on eosinophil migration occurred in a dose-dependent manner. The observed IC50 was approximately 10^{-4}. The IC_{50} is lower than the plasma concentration 2 hours after intravenous administration of prednisolone (1000 mg/day) [28,29]. Thus, these findings suggest that the reduced eosinophil migrations may actually have been

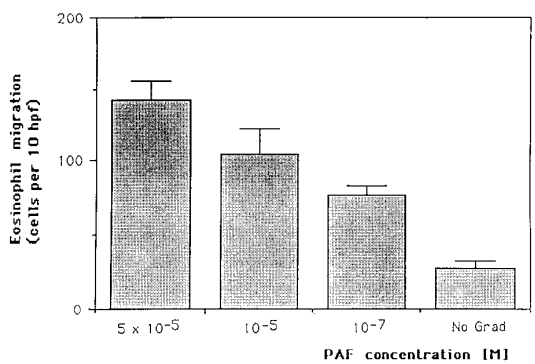

FIG. 4. Dose response of PAF-induced asthmatic eosinophil migration. In one subset of experiments (no Grad), PAF was present in both the upper and lower chambers at a concentration of 10^{-5} mol/L. Data are expressed as mean ± SEM (n=3).

caused by the direct action of prednisolone on asthmatic eosinophils in vivo.

The Boyden chamber technique does not distinguish between random motility (chemokinesis) and directional movement (chemo-taxis). To separate the relative contributions of chemokinesis and chemotaxis to PAF-induced eosinophil migration, we compared migra-tion observed with equal concentrations of PAF above and below the filter (when any migration observed will be due to chemokinesis) with migration observed with PAF only in the lower well (where migration will be due to a combination of chemokinesis and chemotaxis). Our results suggest that PAF C-18 has both chemokinetic and chemotactic effects.

Although it is possibile that neutrophil contamination affected the results of eosinophil migration, we confirmed that the proportion of eosinophils to neutrophils were equal in the upper and lower chambers.

It is not clear whether the PAF level used in this study, i.e., 50 μM, is optimal. Eosinophil migration in response to three molecular species of PAF has been reported by Erger and Casale [30]. In their report, all three PAF species (C16:0, C18:0, and C18:1) induced equivalent eosinophil migration. The eosinophils tested were obtained from healthy human donors. In contrast to our study, eosinophil migration in response to PAF C16:0 and C18:0 declined at high concentration of 10 μM. Unfortunately, we dont have sufficient data to explain the reason clearly, although we found that the migration of eosinophil from asthmatics during an attack increased as the concentration of PAF increased.

The results of binding of [^{3}H]PAF obtained with whole cells suggested that asthmatic eosinophils during an attack have higher affinity to PAF and the affinity was reduced by the increase of prednisolone. Location of the binding sites remains undecided in the present study. However, our technique measures only filterable specific binding sites. These sites appear to be associated with cell surface and membrane markers. At all events, these results may explain the fact that PAF-induced migration of eosinophils obtained from asthmatics during an attack was significantly greater and was significantly inhibited by exposure to prednisolone in vivo and in vitro.

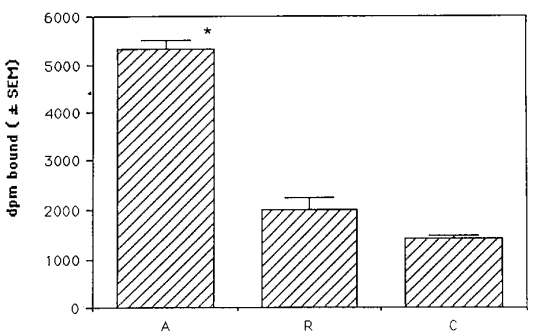

FIG. 5. Binding of [^{3}H]PAF to eosinophils at 4 °C. 1×10^7 eosinophils in 1 mL of Hanks' buffer were incubated with [^{3}H]PAF (200 pM) for 10 min and then assayed for uptake of radiolabel. Eosinophils obtained from asthmatics during an attack before treatment of prednisolone(A) and in remission (R), and healthy donors(C) were used for the binding assay. Each histogram is mean ± SEM of experiments done on five different donor cell preparations. * significantly differs from R and C.

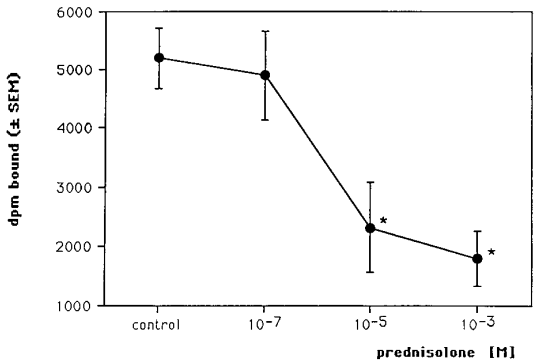

FIG. 6. Effect of prednisolone on the binding of [^{3}H]PAF to asthmatic eosinophils during an attack without treatment of prednisolone. The binding was reduced with the concentration of PAF in a dose dependent manner. Each histogram is mean ± SEM of experiments done on four different donor cell preparations. Control: no treatment of prednisolone.

The eosinophil migration demonstrated in the present study does not necessarily reflect the migration that has been demonstrated using cultured epithelial monolayers [19,20,31] or transendothelial migration of eosinophils in asthmatic lungs, our findings suggests that the migration of eosinophils into inflammatory sites is not only dependent on the release of local chemoattractants, but also may be dependent on an intracellular factor. Furthermore, prednisolone inhibits increased PAF-induced migration in asthmatic eosinophils during an acute attack both in vivo and in vitro.

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