Figure legends

FIG 1. Genomic organization of human *IL-12B*.   By comparing the complete genomic

sequence with the published cDNA sequence, the position of exons 1-8 and introns was

deduced.   Arrows indicate approximate positions of confirmed polymorphisms.   NP2,

15 and 16 are novel polymorphisms (underlines).   The other described NPs have been

reported in the previous study.   NP = nucleotide polymorphism

FIG 2. (a) Plasma IL-12 concentrations in asthmatic patients and controls.   The plasma

IL-12 concentrations in the asthmatic patients were significantly lower than those in the

controls (P<0.001).   (b) Relationship between *IL-12B* promoter polymorphism and

plasma IL-12 concentrations in both the controls and asthmatic patients (n=189).   The

plasma IL-12 concentrations in the subjects with a 1.1 genotype were significantly

lower than those in the subjects with a 2.2 genotype (P<0.001).   (c) Relationship

between *IL-12B* promoter polymorphism and plasma IL-12 concentrations in the

controls (n=78).   The plasma IL-12 concentrations in the controls with a 1.1 genotype

were significantly lower than those in the controls with a 2.2 genotype (P<0.05).

FIG 3. (a) IL-12 production by Derf1-stimulated PBMCs in asthmatic patients and

controls. The IL-12 production in the asthmatic patients was significantly lower than

that in the controls ($P<0.001$). (b) Relationship between *IL-12B* promoter

polymorphism and IL-12 production by Derf1-stimulated PBMCs in both the controls

and asthmatic patients ($n=189$). The IL-12 production in the subjects with a 1.1

genotype was significantly lower than that in the subjects with a 2.2 genotype ($P<0.001$).

(c) Relationship between *IL-12B* promoter polymorphism and IL-12 production by

Derf1-stimulated PBMCs in the controls ($n=78$). The IL-12 production in the controls

with a 1.1 genotype was significantly lower than that in the controls with a 2.2 genotype

($P<0.001$).

FIG 4. Effects of *IL-12B* promoter polymorphism on promoter activity. RAW 264

cells were transfected by lipofectamine with reporter-constructs and stimulated with

IFN-γ and LPS. To examine whether NP1 or NP2 affect the *IL-12B* transcriptional

activity, mutated vectors (mut-1 and mut-2) were constructed. Luciferase activity was

measured using the Dual-Luciferase reporter assay system.   The presented results are

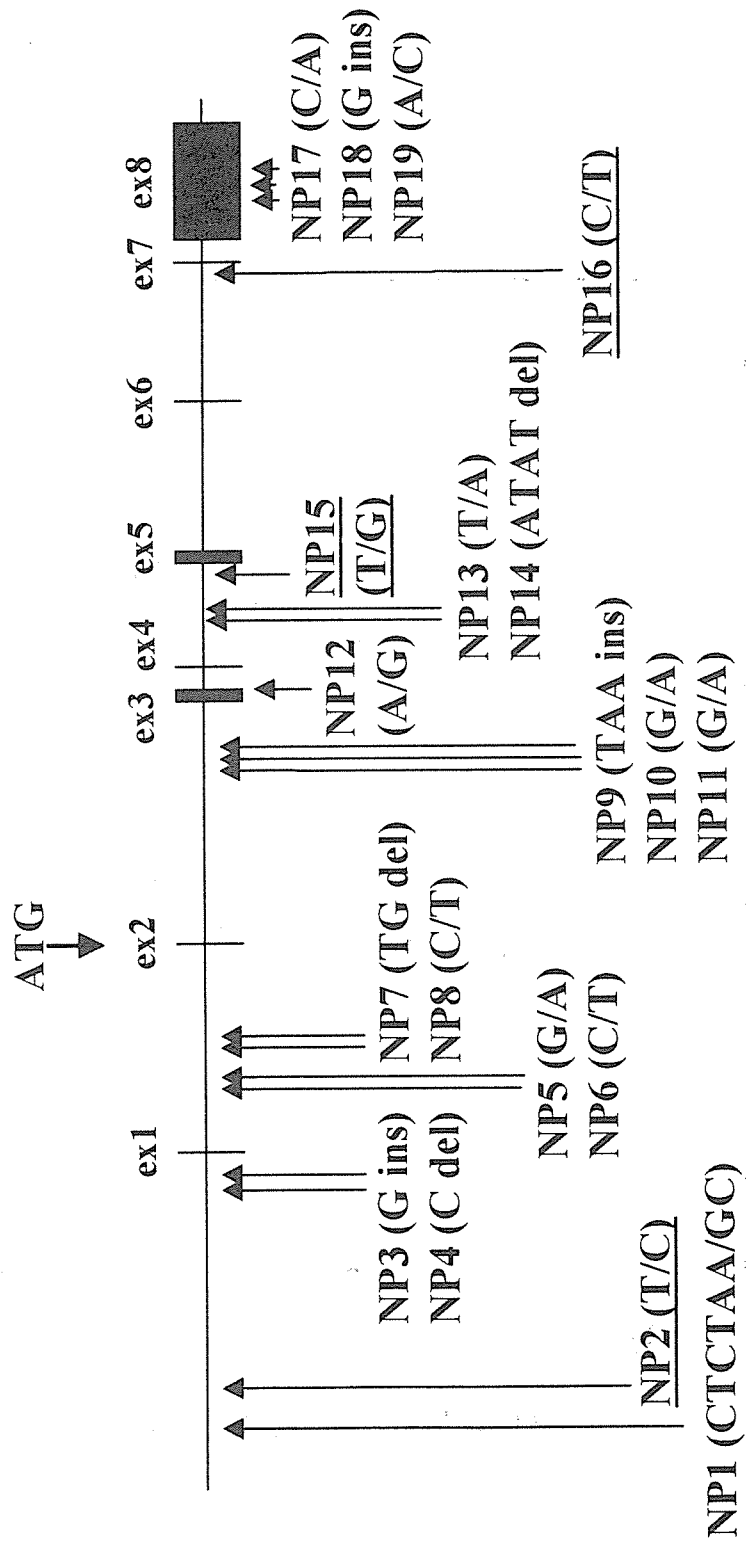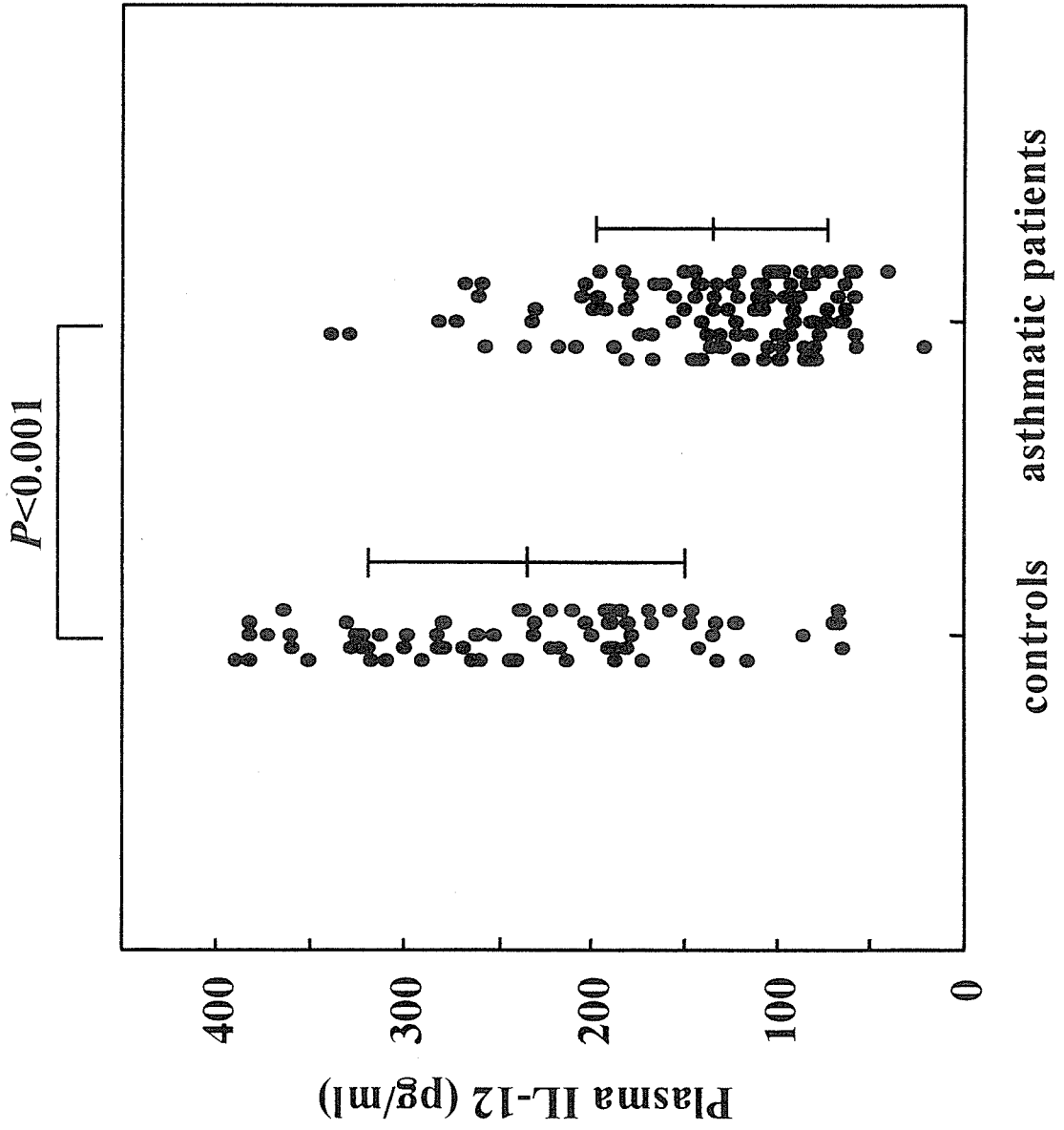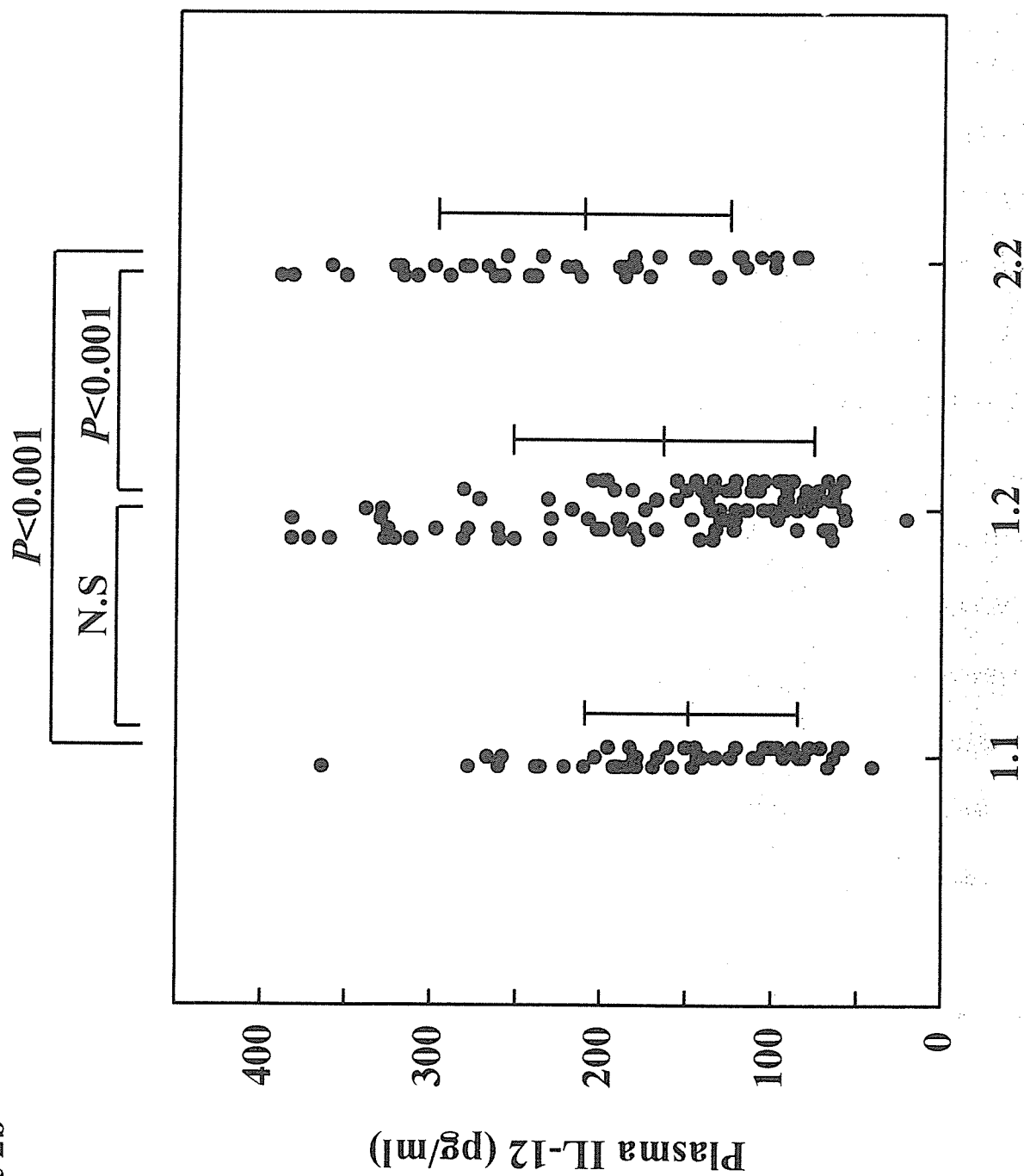mean $\pm$ SD from three independent experiments.   *$P<0.05$.

Figure 1

NP17 (C/A)
NP18 (G ins)
NP19 (A/C)

NP16 (C/T)

NP15 (T/G)

NP13 (T/A)
NP14 (ATAT del)

NP12 (A/G)

NP9 (TAA ins)
NP10 (G/A)
NP11 (G/A)

NP7 (TG del)
NP8 (C/T)

NP5 (G/A)
NP6 (C/T)

NP3 (G ins)
NP4 (C del)

NP2 (T/C)

NP1 (CTCTAA/GC)

ATG

ex1   ex2   ex3 ex4   ex5   ex6   ex7 ex8

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Figure 2a

P<0.001

Plasma IL-12 (pg/ml)

400   300   200   100   0

controls   asthmatic patients

Figure 2b

Figure 2c

Figure 3a

asthmatic patients

controls

P<0.001

IL-12(pg/ml)
by Der f II-stimulated PBMCs

1600
1400
1200
1000
800
600
400
200
0

— 145 —

Figure 3b

Figure 3c

IL-12 (pg/ml)
by Derf1-stimulated PBMCs

Figure 4

Relative activity

GC/C

CTCTAA/T

GC/T

CTCTAA/C

PGV-P2

0   0.2   0.4   0.6   0.8   1   1.2

N.S

*

*

*

N.S
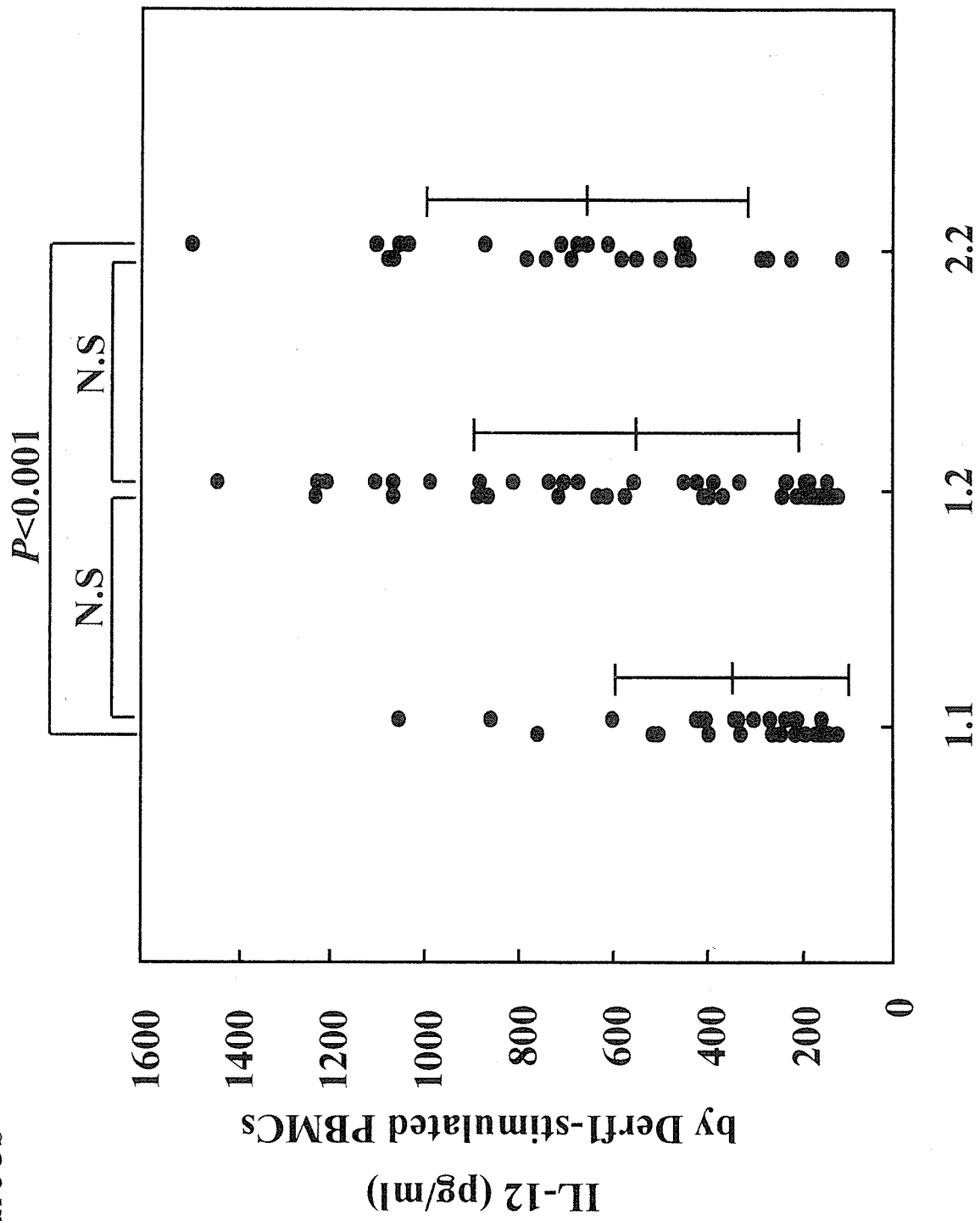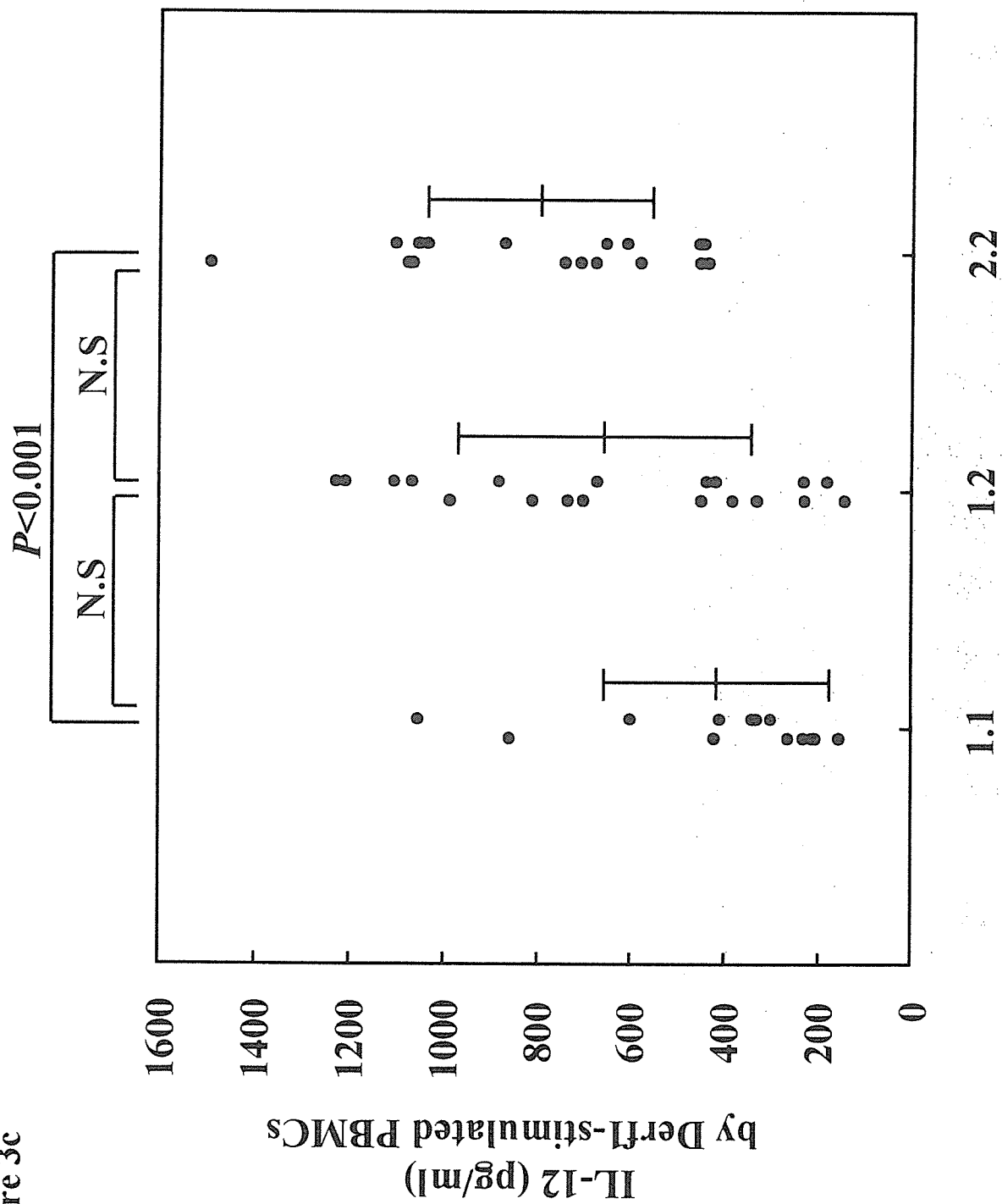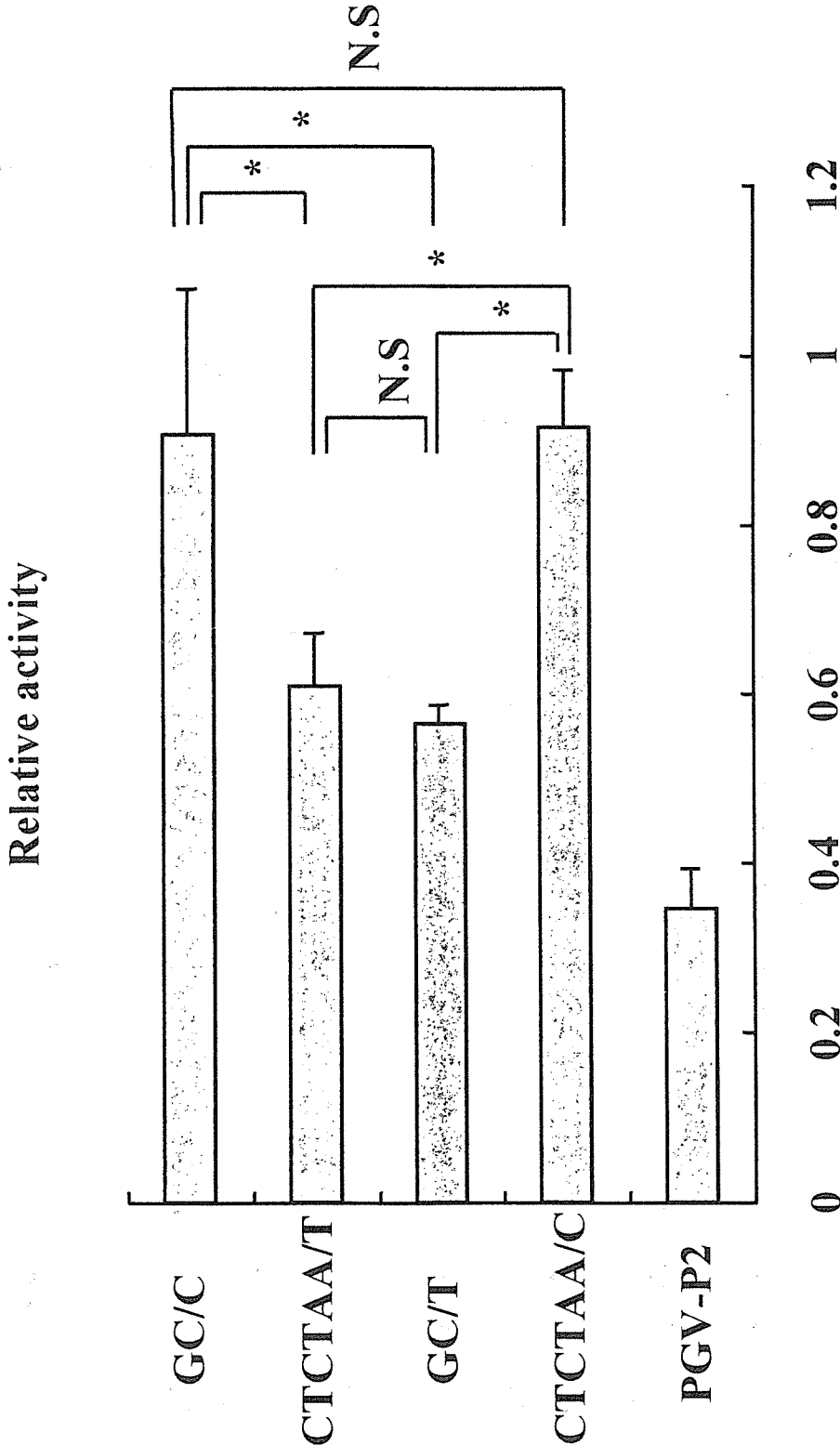
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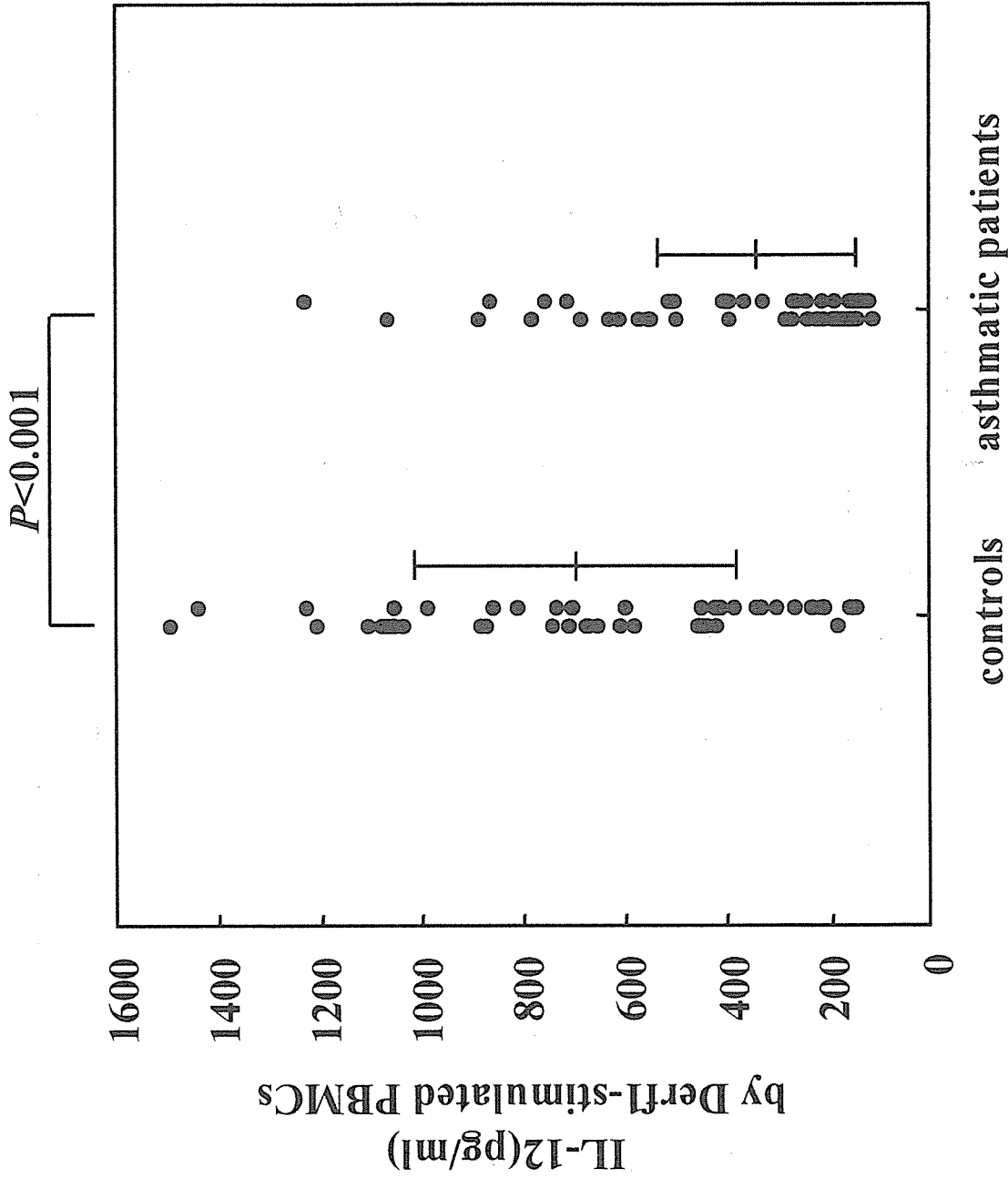
Transendothelial Migration of Human Basophils[1]

Motoyasu Iikura,[2]* Motohiro Ebisawa,[‡] Masao Yamaguchi,* Hiroshi Tachimoto,[‡] Ken Ohta,[§] Kazuhiko Yamamoto,* and Koichi Hirai[†]

During allergic reactions, basophils migrate from the blood compartment to inflammatory sites, where they act as effector cells in concert with eosinophils. Because transendothelial migration (TEM) represents an essential step for extravasation of cells, for the first time we have studied basophil TEM using HUVEC. Treatment of HUVEC with IL-1β significantly enhanced basophil TEM, which was further potentiated by the presence of a CCR3-specific ligand, eotaxin/CCL11. In addition to CCR3 ligands, MCP-1/CCL2 was also active on basophil TEM. Although stromal cell-derived factor-1/CXCL12, a CXCR4 ligand, failed to induce TEM in freshly isolated basophils, it caused strong TEM in 24-h cultured cells. IL-3 enhanced basophil TEM by increasing the chemokinetic response. Spontaneous TEM across activated HUVEC was inhibited by treatment of cells with anti-CD18 mAb, but not with anti-CD29 mAb, and also by treatment of HUVEC with anti-ICAM-1 mAb. Anti-VCAM-1 mAb alone failed to inhibit TEM, but showed an additive inhibitory effect in combination with anti-ICAM-1 mAb. In contrast, eotaxin- and IL-3-mediated TEM was significantly inhibited by anti-CD29 mAb as well as anti-CD18 mAb. These results indicate that β_2 integrins play the primary role in basophil TEM, but β_1 integrins are also involved, especially in TEM of cytokine/chemokine-stimulated basophils. In conclusion, the regulatory profile of basophil TEM is very similar to that reported for eosinophils. Our results thus support the previous argument for a close relationship between basophils and eosinophils and suggest that the in vivo kinetics of these two cell types are similar. *The Journal of Immunology,* 2004, 173: 5189–5195.

A lthough basophils are the least abundant circulating leukocytes, an increasing body of evidence has demonstrated that these cells play an active pathogenic role in allergic inflammation by releasing diverse proinflammatory mediators, including vasoactive amines, cysteinyl leukotrienes, and cytokines (1). The biological significance of basophils in allergic disorders has become more apparent with the recognition and understanding of allergic dual-phase reactions; analyses of chemical mediators have revealed increases in basophil-derived mediators at the sites of late-phase reactions (2–4).

The results of experimental allergen challenge in various organs have revealed the influx of basophils to inflammatory sites several hours after Ag exposure (5–9), indicating the existence of a mechanism for recruitment of basophils from the blood compartment to inflamed tissue sites during allergic reactions. Like other types of leukocytes, the entire process of basophil influx to inflamed tissue sites comprises three essential sequential steps: adhesion to the vascular endothelium, transendothelial migration (TEM),[3] and locomotion toward inflammatory sites in extravascular tissues. To date, a considerable number of studies have outlined the mechanisms of both the adhesion and locomotion processes in basophils

(10–14), demonstrating that adhesion molecules and/or several cytokines and chemokines are critically involved in these processes. In contrast to neutrophils, basophils express $\alpha_4\beta_1$ integrins in addition to β_2 integrins. Basophil-activating cytokines such as IL-3 are capable of up-regulating the expression of β_2 integrins (CD11b and CD18), thereby potentiating the β_2 integrin-mediated adherence of basophils to the endothelium (14). These basophil-activating cytokines also enhance basophil locomotive responses by inducing random movement (chemokinesis) (11). Regarding the basophil migration process, however, basophil-directed chemokines such as eotaxin play more critical roles by virtue of inducing strong directional movement (chemotaxis) (15, 16).

The molecular aspects of both the adhesion and migration processes of basophils have thus become increasingly clear, but those of basophil TEM, another essential process that links the adhesion process and the locomotion process, have not been studied to date. Given the importance of the TEM process for the recruitment of basophils to allergic inflammatory sites, for the first time we have studied basophil TEM using vascular endothelial cell monolayers.

Materials and Methods

Reagents and mAbs

Murine mAbs with blocking activity against adhesion molecules were used. Anti-CD49d mAb (IgG1, clone HP2/1) and anti-CD29 mAb (IgG1, clone 4B4) were purchased from Coulter Immunotech (Marseille, France). Anti-CD11a mAb (IgG1, clone TS1/22) was obtained from Endogen (Woburn, MA). Anti-CD49e mAb (IgG1, clone IIA1), anti-CD11b mAb (IgG1, clone 44), and anti-CD18 mAb (IgG1, clone L130) were purchased from BD Pharmingen (San Diego, CA). The F(ab')$_2$ of anti-CD54 mAb (IgG2a, clone MEM-111) and anti-CD106 mAb (IgG1, clone 1G11B1) were purchased from Caltag Laboratories (Burlingame, CA). The F(ab')$_2$ of mouse IgG was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Mouse IgG1 (MOPC-21) with irrelevant specificity was purchased from Sigma-Aldrich (St. Louis, MO). Murine anti-CCR1 mAb (IgG1, clone 141) and anti-CCR3 mAb (IgG1, clone 444) were donated by Dr. H. Kawasaki (Institute of Medical Science, University of Tokyo, Tokyo, Japan) (17). Other reagents used in the experiments were previously described (13).

Cell separation and culture conditions

Human basophils were isolated from venous blood obtained from consenting volunteers with no history of atopic diseases. Basophils were semipurified by Percoll density gradient centrifugation as previously described (18). The purity of semipurified basophil preparations was $12.5 \pm 0.13\%$ ($n = 60$). A vast majority of the contaminating cells were lymphocytes and monocytes, and the preparations usually contained no eosinophils. Their viability, measured by the trypan blue exclusion test, was consistently >95%. In some experiments basophils were further purified (purity, ~98%) by negative selection with MACS beads (Basophil Isolation kit; Miltenyi Biotec, Auburn. CA) as described previously (13).

HUVEC were isolated and cultured according to the previously described methods (19). The culture medium was medium 199 (Invitrogen Life Technologies, Grand Island, NY) supplemented with 20% FCS (Invitrogen Life Technologies), 2 mM L-glutamine (Invitrogen Life Technologies), antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin; Invitrogen Life Technologies), endothelial cell growth supplement (20 μg/ml; Collaborative Research, Bedford, MA), and heparin sodium (90 μg/ml; Sigma-Aldrich). HUVEC ($2-5 \times 10^4$ cells) were cultured on 2% gelatin-coated Transwell culture inserts (6.5-mm diameter polycarbonate membranes with 5-μm pores; Costar, Cambridge, MA) for 2–5 days. To confirm the confluence of the HUVEC on the membrane, sample monolayers were routinely stained with Diff-Quick (International Reagents, Kobe, Japan) before use in each experiment.

TEM assay

Unless otherwise indicated. HUVEC monolayers grown in Transwell inserts were pretreated for 4 h with human rIL-1β (5 ng/ml; Diaclone Research, Besaçn, France) and washed twice with PIPES ACM buffer (25 mM PIPES, 119 mM NaCl. 5 mM KCl. 2 mM Ca^{2+}, 0.5 mM Mg^{2+}, and 0.03% human serum albumin). Percoll-separated basophils ($3 \times 10^4/100$ μl) suspended in PIPES ACM buffer were added to the upper wells, and samples to be tested (600 μl) were placed in the lower wells. After incubation for 3 h at 37°C. the cells that had migrated to the lower wells were collected and washed twice in PBS supplemented with 3% FCS and 0.1% NaN_3. For analysis of the numbers of migrated basophils, the cells were stained with FITC-conjugated goat anti-human IgE (BioSource International. Camarillo, CA) at 4°C for 30 min. After washing the cells, the numbers of FITC-positive cells were counted by flow cytometry as previously described (13). Basophil TEM was expressed as a percentage of the number of inoculated basophils.

The effects of integrins and CCRs were examined by treating cells with blocking mAbs or controls at 37°C for 30 min. Without washing, treated cells were placed in the upper wells. We used F(ab')$_2$ for blocking CD54 (ICAM-1) and CD106 (VCAM-1) on HUVEC to avoid the undesired effects of the Fc portion of immobilized mouse mAb on basophil FcγR. HUVEC were pretreated with the F(ab')$_2$ of each mAb or controls at 37°C for 30 min before assay. The inhibition by mAbs was calculated using the following formula: % inhibition = (migration by isotype-matched control-treated cells − migration by blocking mAb-treated cells)/(migration by isotype-matched control-treated cells) × 100.

Statistics

All data are expressed as the mean ± SEM, and differences between values were analyzed by the one-way ANOVA. When this test indicated a significant difference, Fisher's protected least significant difference test was used to compare individual groups.

Results

Effects of IL-1 and eotaxin on basophil migration across endothelial cells

A basophil TEM assay was performed using Percoll-separated basophils and Transwell systems with cultivated HUVEC on the surface. In accordance with the previous reports of eosinophil TEM assays (19), HUVEC were activated by stimulation with IL-1β for 4 h before assays. As shown in Fig. 1A, only marginal numbers of basophils had transmigrated across a layer of unstimulated HUVEC even after 3 h of incubation. In contrast, stimulation of HUVEC with IL-1β significantly enhanced basophil TEM at later time points. During the initial 2 h, TEM across IL-1-activated HUVEC showed a similar time course of accumulation as TEM across unstimulated HUVEC. After 3-h incubation, however, the
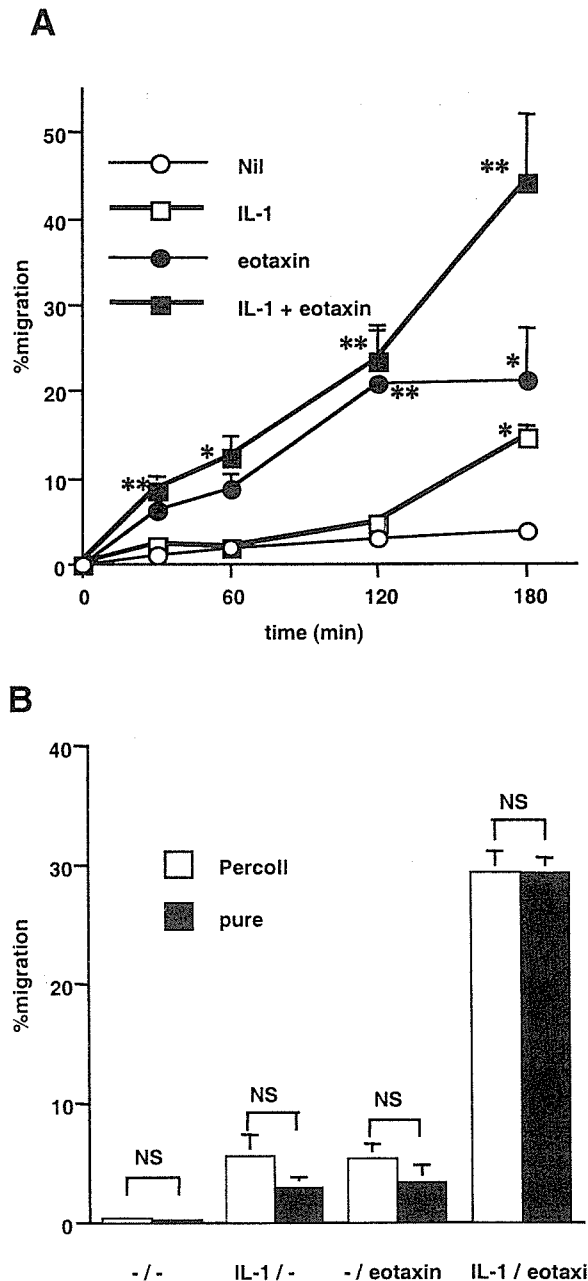
FIGURE 1. Time course of TEM of basophils. A, HUVEC monolayers in Transwell inserts were pretreated with (■ and □) and without (● and ○) IL-1-β (5 ng/ml) at 37°C for 4 h. Percoll-separated basophils were added to the upper wells, and the chambers were incubated at 37°C in the presence (■ and ●) and the absence (□ and ○) of eotaxin (5 nM) in the lower wells. After the indicated time periods, the cells in lower wells were collected, and the number of IgE-positive cells was determined by flow cytometry. Bars represent the SEM ($n = 6-8$). *, $p < 0.05$; **, $p < 0.01$ (vs spontaneous migration (○) at corresponding time point). B, TEM through HUVEC by highly purified basophils was indicated. Basophils from a sample of Percoll-separated cells were further enriched by negative selection with MACS beads. TEM assays were performed for 3 h, as described above, using Percoll- (□) and MACS-separated (■) basophils. The purities of Percoll- and MACS-separated basophils were 19.6 ± 5.7 and $98.3 \pm 0.4\%$, respectively. Bars represent the SEM ($n = 5$). NS, no significant difference between Percoll- and MACS-separated basophils.

number of transmigrated cells was significantly increased in cultures stimulated with IL-1β (3.9 ± 1.2 and $14.7 \pm 2.7\%$, for TEM across unstimulated and IL-1-stimulated HUVEC, respectively). Furthermore, basophil TEM was markedly enhanced by the presence of
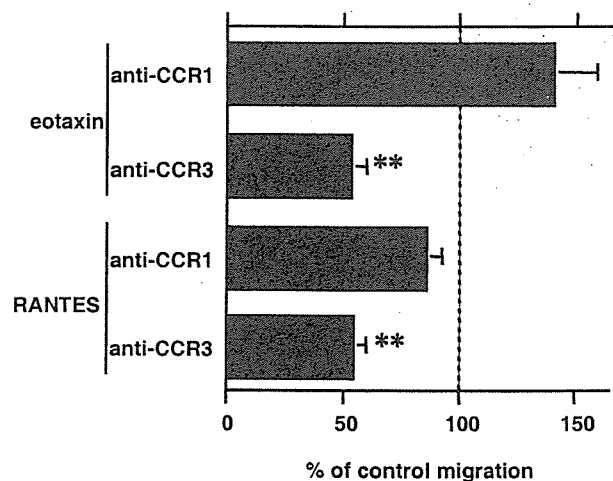
A

B
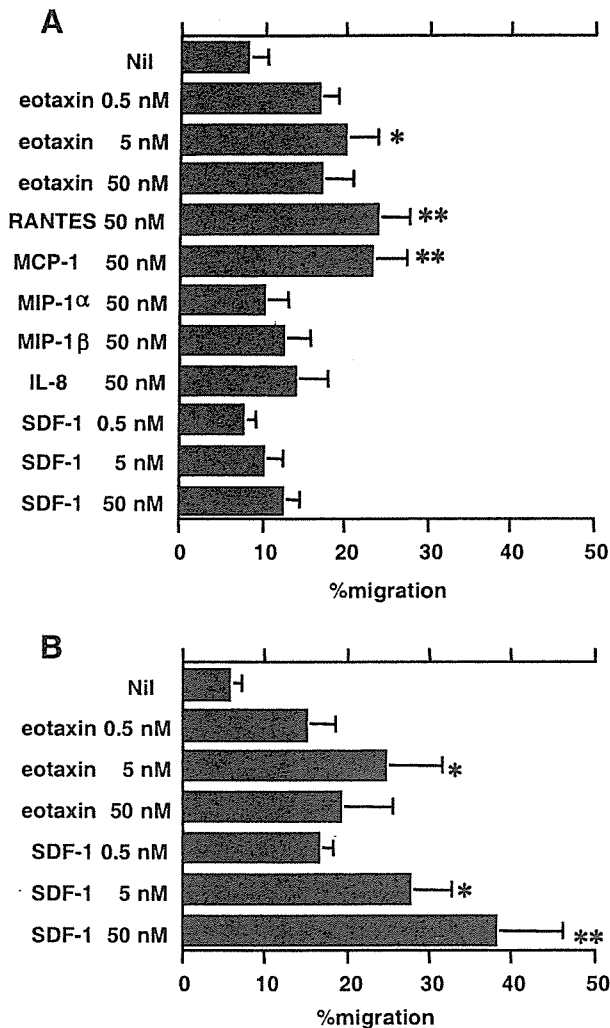
FIGURE 2. Effects of chemokines on basophil TEM. *A*, HUVEC were pretreated with IL-1-β (5 ng/ml) at 37°C for 4 h. A basophil TEM assay was performed in the presence of various chemokines in the lower wells. Basophils were separated by Percoll and immediately added to the upper wells. After 3 h of incubation at 37°C, the cells in the lower wells were collected, and the number of IgE-positive cells was determined by flow cytometry. *B*, Percoll-separated basophils were cultured in RPMI 1640 supplemented with 10% FCS and antibiotics at 37°C for 24 h. The cultured cells were added to the upper wells, and TEM assay was performed as described above. Bars represent the SEM ($n = 4-7$). *, $p < 0.05$; **, $p < 0.01$ (vs migration in the absence of chemokines (Nil)).

a chemoattractant, eotaxin/CCL11. Compared with spontaneous TEM, chemokine-induced TEM showed a faster time course of accumulation. Even with unstimulated HUVEC, the number of trans-migrated cells increased linearly until 2 h and then plateaued. Although a similar time course of accumulation was observed with activated HUVEC until 2 h of incubation, the number of transmigrated cells was significantly increased at 3 h of incubation (Fig. 1*A*). The basophil preparations used in these experiments were Percoll-separated and consisted of ~90% contaminating cells. To explore whether these contaminating cells indirectly affect basophil TEM, we further purified the Percoll-separated cells by negative selection with MACS beads (purity, >98%) and compared the TEM between Percoll- and MACS-separated basophils. As shown in Fig. 1*B*, no significant difference in TEM was observed between the two preparations, indicating that contaminating cell populations do not significantly affect the basophil TEM.

Basophil TEM by chemokines

The results of our and other laboratories have demonstrated the expression of multiple chemokine receptors in basophils (13, 16, 20), and our previous functional survey demonstrated that CCR3 ligands are mainly responsible for the migratory responses of resting basophils (13). Fig. 2 depicts the effects of various chemokines on basophil TEM. Transmigration was determined after 3 h of incubation using IL-1-activated HUVEC. CCR3 ligands, eotaxin, and RANTES/CCL5 induced strong basophil TEM. Eotaxin binds specifically and exclusively to CCR3, whereas RANTES binds to CCR1 with higher affinity than to CCR3. To determine the chemokine receptors responsible for RANTES-induced basophil TEM, we performed blocking experiments using receptor-specific mAbs of an antagonistic nature. As shown in Fig. 3, eotaxin-induced TEM was almost completely inhibited by anti-CCR3 mAb. Although RANTES-induced TEM was also markedly blocked by anti-CCR3 mAb, treatment with anti-CCR1 mAb exerted virtually no effect on the migratory response.

Our previous study using a HUVEC-free Transwell system demonstrated that CXCR1/CXCR2 ligand IL-8/CXCL8 and CCR2 ligand MCP-1/CCL2 also induced significant, but weak, migration of basophils (13). However, no statistically significant TEM was observed in cells stimulated with IL-8. In contrast, MCP-1 was capable of inducing TEM as strongly as eotaxin. Furthermore, a CXCR4-specific ligand, stromal cell-derived factor-1 (SDF-1)/CXCL12, was also capable of inducing strong basophil TEM under certain conditions. In our previous report we demonstrated that the expression of functional CXCR4 is induced on the basophil surface by 24 h of culture, and SDF-1 induced significant migratory responses in 24-h cultured basophils (13). The same situation was observed in TEM; although freshly isolated basophils failed to exhibit TEM in response to SDF-1, strong TEM, comparable to that induced by eotaxin, was observed with 24-h cultured basophils (Fig. 2*B*).

FIGURE 3. Effects of chemokine receptor blocking on chemokine-induced basophil TEM. HUVEC were pretreated with IL-1-β (5 ng/ml) at 37°C for 4 h. Percoll-separated basophils were preincubated with anti-chemokine receptor mAbs (20 μg/ml) or isotype control mAb (mouse IgG1, 20 μg/ml) at 37°C for 30 min. TEM assay was performed in the presence and the absence of eotaxin (15 nM) or RANTES (15 nM) in the lower wells for 3 h. Migrations of control Ab-treated basophils toward eotaxin and RANTES were 29.5 ± 4.6 and 28.5 ± 5.7%, respectively. Spontaneous migration without any chemokine was 9.2 ± 2.5%. Bars represent the SEM ($n = 9$). **, $p < 0.01$ (vs percentage of control migration).

Basophil TEM by IL-3

Results from our and other laboratories have established that IL-3 stimulates various aspects of basophil functions, such as mediator release (21, 22) and in vitro survival (23, 24). Furthermore, our earlier study using Boyden chamber systems demonstrated that IL-3 potently enhances in vitro locomotive responses by basophils (11). The migratory response induced by IL-3 was chemokinetic rather than chemotactic. When serially diluted IL-3 was added to the upper wells of a Transwell, we observed that the number of transmigrated basophils increased in a dose-dependent fashion (Fig. 4). A significant increase in basophil TEM was observed when the cell suspension in the upper wells contained as little as 3 pM IL-3. When the IL-3 gradient was diminished by adding the same concentration of IL-3 to both the upper and lower wells, the basophil TEM was not significantly affected. In contrast, elimination of the eotaxin gradient mostly inhibited the increase in basophil TEM, indicating that eotaxin-induced TEM was mainly chemotactic (Fig. 4). Furthermore, IL-3 showed an additive effect on eotaxin-induced TEM; the number of transmigrated cells induced by eotaxin was significantly increased in the presence of IL-3 (17.7 ± 4.4 and $23.4 \pm 4.6\%$, eotaxin-induced TEM for the absence and the presence of IL-3, respectively; $n = 4$; $p < 0.05$).

Effects of adhesion molecules on basophil TEM

Adhesion molecules and their counter-receptors play a critical role in the TEM of various cell types. Previous studies by others revealed that basophils express both β_1 (CD49d, CD49e, and CD29) and β_2 (CD11a, CD11b, and CD18) integrins on their surface (25). When the effects of β_1 and β_2 integrins on basophil TEM across IL-1-stimulated HUVEC were studied using blocking mAbs, significant inhibition of spontaneous TEM was observed in cells treated with anti-CD18 mAb, but not in those treated with anti-

CD29 mAb, in both Percoll-separated and MACS-separated basophils (Fig. 5A). No significant inhibition was observed in cells treated with anti-CD11a or anti-CD11b mAb alone, but treatment

FIGURE 5. Effects of adhesion molecules on spontaneous basophil TEM across activated HUVEC. *A*, HUVEC were pretreated with IL-1-β (5 ng/ml) at 37°C for 4 h. Percoll–separated ($\square$) and MACS-separated ($\square$) basophils were preincubated with anti-adhesion molecule mAb (10 μg/ml) or isotype control mAb (mouse IgG1) at 37°C for 30 min. The TEM assay was performed at 37°C for 3 h. Bars represent the SEM ($n = 4$). The purities of Percoll- and MACS-separated basophils were 17.6 ± 3.7 and $98.8 \pm 0.9\%$, respectively. Control migrations of Percoll-separated and highly purified basophils pretreated with control mAb were 4.6 ± 1.8 and $3.3 \pm 1.6\%$, respectively. *, $p < 0.05$; **, $p < 0.01$ (vs control migration). NS, no significant difference between Percoll-separated and highly purified basophils. *B*, HUVEC were pretreated with IL-1β (5 ng/ml) at 37°C for 4 h. Percoll-separated basophils were preincubated with anti-adhesion molecule mAb (10 μg/ml) or isotype control mAb (mouse IgG1) at 37°C for 30 min. The TEM assay was performed at 37°C for 3 h. Bars represent the SEM ($n = 8–10$). Control migration of basophils pretreated with control mAb was $7.3 \pm 1.1\%$. *, $p < 0.05$; **, $p < 0.01$ (vs control migration). *C*, IL-1-stimulated HUVEC were pretreated with the F(ab')$_2$ of anti-VCAM-1 mAb (3 μg/ml), anti-ICAM-1 mAb (10 μg/ml), or control Ab (mouse IgG, 10 μg/ml) at 37°C for 30 min. The TEM assay was performed using Percoll-separated basophils at 37°C for 3 h. Bars represent the SEM ($n = 6$). Control migration across HUVEC precultured with the F(ab')$_2$ of control Ab was $9.5 \pm 1.8\%$. *, $p < 0.05$; **, $p < 0.01$ (vs control migration). †, $p < 0.05$ (vs migration after pretreatment with anti-ICAM-1 mAb).

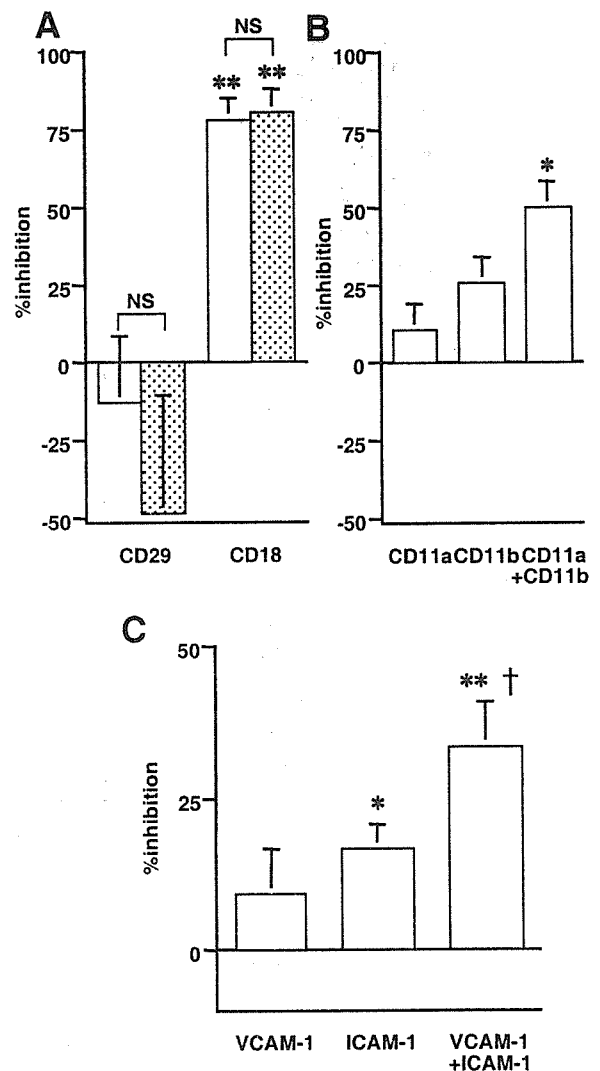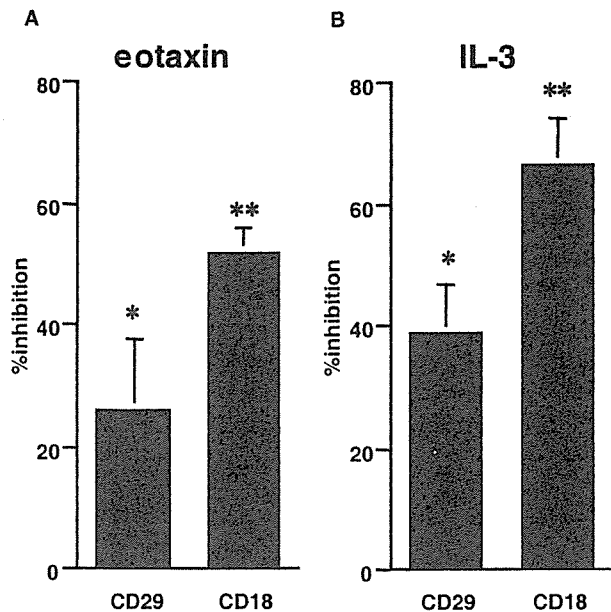|	IL-3				eotaxin	
Upper	-	30 fM	3 pM	300 pM	300 pM	-	5 nM
Lower	-	-	-	-	300 pM	5 nM	5 nM

FIGURE 4. Effects of IL-3 on basophil TEM. HUVEC were treated with IL-1-β (5 ng/ml) at 37°C for 4 h. IL-3 and eotaxin (5 nM) were added to the upper and/or lower wells as indicated. Basophils were separated by Percoll, and TEM assay was performed at 37°C for 3 h. Bars represent the SEM ($n = 8$). *, $p < 0.05$; **, $p < 0.01$ (vs spontaneous migration without IL-3 or eotaxin).

FIGURE 6. Effects of adhesion molecules on eotaxin-mediated (A) and IL-3-mediated (B) basophil TEM across activated HUVEC. HUVEC were pretreated with IL-1-β (5 ng/ml) at 37°C for 4 h. A, Percoll-separated basophils were treated with anti-CD18 mAb (10 µg/ml), anti-CD29 mAb (10 µg/ml), or isotype control mAb (10 µg/ml) at 37°C for 30 min. TEM assay was performed at 37°C for 3 h in the presence of eotaxin (5 nM) in the lower wells. B, Percoll-separated basophils were preincubated with IL-3 (300 pM) at 37°C for 30 min plus anti-CD18 mAb (10 µg/ml), anti-CD29 mAb (10 µg/ml), or isotype control mAb (10 µg/ml) at 37°C for 30 min. Basophil TEM was performed at 37°C for 3 h. Bars represent the SEM (n = 6–8). Control migrations induced by eotaxin and IL-3 in the case of basophils pretreated with isotype control mAb were 22.7 ± 7.6 and 22.4 ± 3.4%, respectively. Spontaneous TEM without eotaxin or IL-3 was 8.6 ± 1.7%. *, $p < 0.05$; **, $p < 0.01$ (vs control migration).

with a combination of both mAbs resulted in significant inhibition of basophil TEM (Fig. 5B). In contrast, as expected, neither anti-CD49d nor anti-CD49e mAb inhibited TEM (−14.6 ± 24.3 and −37.5 ± 26.8%, inhibitory effects of anti-CD49d and anti-CD49e mAb, respectively; n = 7). Fig. 5C depicts the effects of counter-receptors of endothelial cells on spontaneous TEM. When IL-1-stimulated HUVEC were pretreated with F(ab′)$_2$ of anti-CD106 (VCAM-1) or anti-CD54 (ICAM-1) mAb, we found that TEM of fresh basophils was significantly inhibited by anti-ICAM-1 mAb alone. Although anti-VCAM-1 mAb alone failed to exert a significant inhibitory effect, the combination of anti-VCAM-1 and anti-ICAM-1 mAbs produced enhanced inhibition compared with anti-ICAM-1 alone, indicating an additive inhibitory effect of anti-VCAM-1 mAb. Taken together, these results indicate that the β_2 integrin/ICAM-1 system is mainly responsible for TEM of fresh basophils across activated HUVEC, in which the β_1 integrin/VCAM-1 system may also play an additive role.

In contrast to spontaneous TEM, eotaxin-induced TEM was significantly inhibited not only by anti-CD18 mAb, but also by anti-CD29 mAb (Fig. 6A). The inhibition by anti-CD29 mAb was significantly less prominent than that by anti-CD18 mAb. TEM of IL-3-primed basophils was also inhibited by anti-CD29 mAb, although again less potently than with anti-CD18 mAb. These results indicate that β_1 integrins are more actively involved in eotaxin- or IL-3-induced basophil TEM, in contrast to spontaneous TEM.

Discussion

Participation of basophils in allergic inflammation has been strongly inferred from both the increase in the number of these

cells and the presence of basophil-derived mediators at inflammatory sites during allergic reactions (2–6, 9). TEM represents an essential step for movement of leukocytes from the circulation to inflamed tissue sites, and numerous reports have extensively studied TEM of various types of leukocytes (26). In contrast, to our knowledge no studies have dealt with basophil TEM to date. In the present study, using well-established assay systems, for the first time we have investigated basophil migration across vascular endothelial cell monolayers.

The chemokine family has been highly implicated in the pathogenesis of inflammation of various etiologies (27). In addition, several lines of evidence have demonstrated that chemokines play an important role in TEM of various leukocytes (28–31). The same situation was observed in TEM of basophils. We previously studied the expression profile and functions of chemokine receptors in human basophils (13). CCR3 is constitutively expressed on the basophil surface, and CCR3 ligands such as eotaxin induced the most potent migratory responses in freshly isolated basophils. In contrast, although surface expression of CXCR4 is hardly detectable in freshly isolated basophils, it becomes gradually apparent during culture, and SDF-1 induces a strong migratory response, comparable to that induced by eotaxin (13). In parallel to these previous findings, our present results show the involvement of both CCR3 and CXCR4 in basophil TEM; strong TEM was induced by CCR3 ligands in freshly isolated cells and by SDF-1 in 24-h cultured basophils (Fig. 2B). The physiological role of CXCR4 on basophils is uncertain at present. However, as discussed in our previous paper (13), constitutive and ubiquitous expression of SDF-1 in many tissues suggests that CXCR4 is involved, rather, in baseline trafficking of basophils. In the present study we found that CCR2, which is strongly expressed on basophils (13, 16), is also actively involved in basophil TEM. Our earlier report showed that whereas CCR2 causes the most potent degranulation of basophils (13), its migration-inducing ability is only marginal. Based on the numbers of migrated basophils, MCP-1 is ∼5-fold less potent than eotaxin (13). In contrast to those findings, the present study demonstrated that MCP-1 elicited strong basophil TEM, comparable to that induced by eotaxin. This observation is of potential importance, because CCR2 is virtually undetectable on human eosinophils (32), and MCP-1 fails to induce eosinophil TEM at all (29). As discussed below, many of the regulatory mechanisms of TEM of basophils are shared with eosinophils. Therefore, MCP-1-mediated basophil TEM may represent a unique mechanism for selective migration of human basophils. In contrast, we failed to observe any significant direct effects of CCR1, CCR2, CCR5, CXCR1, and CXCR2 on basophil TEM. Thus, although we cannot exclude possible additional effects of these receptors, it is reasonably concluded that basophil TEM is regulated mainly by both CCR2 and CCR3, and by CXCR4 under certain circumstances.

HUVEC are capable of liberating various chemokines in response to diverse stimuli (33–35). Therefore, it should be noted that HUVEC-derived chemokines might disturb the chemotactic gradient and thereby affect the TEM results. Furthermore, more complicated mechanisms may exist regarding the CXCR4/SDF-1 system; Murdoch et al. (36) reported that HUVEC expressed CXCR4 and induced a rapid calcium influx in response to SDF-1. Inasmuch as CXCR4 on vascular endothelial cells is up-regulated by activating stimuli, the presence of SDF-1 may induce and/or modulate cytokine/chemokine production by activated HUVEC. Even though we stimulated HUVEC for only a short time (4 h) and eliminated possible soluble factors from HUVEC by washing before performing TEM assays, there remains the possibility that HUVEC-derived mediators might affect the TEM results, as reported for eosinophil TEM (34).

We and others have demonstrated that IL-3 represents the most potent basophil-activating cytokine and stimulates various aspects of basophil functions, including mediator release (21, 22), survival (23, 24), motility (11), adhesiveness to the endothelium (14), and expression of activation markers (14). Our present finding that IL-3 potentiated basophil TEM broadens the current concept of the capacity of IL-3 to modulate allergic inflammatory responses by up-regulating basophil functions. However, it seems unlikely that IL-3 is directly involved in basophil accumulation at allergic inflammatory sites, because checkerboard analyses clearly showed that IL-3-mediated TEM was chemokinetic rather than chemotactic. Nevertheless, the additive effect of IL-3 seen in eotaxin-induced TEM strongly suggested that IL-3, and potentially IL-5 and GM-CSF as well, plays a cooperative role in basophil TEM with these basophil-directed chemokines by modulating locomotive movement.

In addition to basophil-directed cytokines and chemokines, adhesion molecules play a critical role in basophil TEM. Although basophils express both β_1 and β_2 integrins (12), we found that β_2 integrins represent the first line of adhesion molecules that account for basophil TEM. TEM of fresh basophils across activated HUVEC was significantly attenuated by treatment with anti-CD18 mAb. Cotreatment with anti-CD11a and anti-CD11b mAbs showed additive inhibitory effects, indicating the involvement of both the α_L and α_M chains in basophil TEM. In line with these observations, pretreatment of activated HUVEC with mAb against ICAM-1, a counter-receptor for both CD11a and CD11b, significantly inhibited basophil TEM. Furthermore, basophil TEM induced by IL-3 or eotaxin was also suppressed by anti-CD18 mAb. An in vitro study by others showed up-regulation of the expression of a β_2 integrin (CD11b) by IL-3 (14). We found that CD11b and CD18 were also up-regulated by IL-3, and that eotaxin exerted the same effect to a lesser extent (M. Iikura and K. Hirai, unpublished observation). Thus, the enhanced TEM observed in IL-3- and eotaxin-treated cells can be attributed at least in part to strengthened interaction with β_2 integrin caused by up-regulated expression of these molecules.

Our results indicated an additional role of β_1 integrins in basophil TEM. Spontaneous TEM across activated HUVEC was not inhibited by anti-CD49d, anti-49e, or anti-CD29 mAb (Fig. 5A), but anti-CD29 mAb significantly inhibited TEM of eotaxin- and IL-3-stimulated basophils (Fig. 6). The increase in the involvement of β_1 integrin in these cells is potentially mediated by functional up-regulation of β_1 integrin. Sung et al. (37) reported that short term incubation of eosinophils with eotaxin or GM-CSF (30 min) up-regulates the binding of cells to VCAM-1 without affecting the expression level of VLA-4 protein. Although not proven, a conformational change in the VLA-4 receptor is likely to be responsible for the increased involvement of β_1 integrin in basophils stimulated with either eotaxin or IL-3. Because β_1 integrin is specifically expressed on basophils and eosinophils, but not neutrophils (38), this molecule is strongly implicated in the selective accumulation of basophils as well as eosinophils at allergic inflammatory sites.

The results of blocking experiments showed a discrepancy in the efficacy between inhibition of α and β subunits as well as counter-receptors and leukocyte integrins. This discrepancy strongly suggests additional involvement of untested adhesion molecules in basophil TEM. Although our present results indicated essential roles for α_L and α_M in basophil TEM, the blockade of the β subunit (CD18) was more effective than that of a combination of CD11a plus CD11b (Fig. 5, A and B). The lower efficacy of blockade of α subunits indicates possible roles for other members of α subunits of β_2 integrin, i.e., CD11c (α_X) and α_d, which have been shown to be constitutively expressed on basophils (39, 40). A similar situation was postulated for counter-receptors of Ig superfamily members. We found that anti-VCAM-1 mAb showed an additive inhibitory effect in combination with anti-ICAM-1 mAb on TEM of fresh basophils regardless of the inefficacy of anti-CD29 treatment (Fig. 5, A and C). Because $\alpha_d\beta_2$ integrins use VCAM-1 as one of their counter-receptors (41), an additive role for VCAM-1 may also support the involvement of $\alpha_d\beta_2$ integrins in spontaneous basophil TEM. Furthermore, our finding that blockade of counter-receptors is markedly less effective than that of leukocyte integrins (Fig. 5, A and C) may reflect the involvement of other untested Ig superfamily members, such as ICAM-2. Additional study is required to elucidate the possible roles of these molecules in basophil TEM.

The increased TEM observed in IL-1-stimulated HUVEC indicated the importance of endothelial cell activation in basophil TEM. Throughout our experiments we used HUVEC stimulated with IL-1β for 4 h, which up-regulates mainly ICAM-1 expression (42). However, the expression profile of counter-receptors on endothelial cells varies in a stimulus- and time-dependent fashion. Furthermore, phenotypic and functional heterogeneity of vascular endothelial cells is observed among different tissues. For example, IL-4 selectively induces VCAM-1 expression in HUVEC (43) or nasal polyp-derived microvascular endothelium (44), but not in microvascular endothelial cells from skin (45), intestine (46), or lung (47). In contrast, VCAM-1 expression of pulmonary microvascular endothelial cells was selectively up-regulated by long term (24-h) stimulation with TNF-α (47). Thus, it is highly likely that basophil TEM across endothelial cells obtained from other organs and/or stimulated with other cytokines would show a different profile from our present results derived using IL-1-activated HUVEC. To elucidate the precise mechanisms of basophil TEM in various allergic diseases, basophil TEM across these endothelial cells is an important issue meriting additional investigation.

Substantial evidence to date has clearly indicated a close relationship between basophils and eosinophils (48). Both cell types share a majority of their cell surface structures, including cytokine/chemokine receptors and adhesion molecules. The activating hemopoietins for both cells, i.e., IL-3, GM-CSF, and IL-5, completely overlap, and CCR3 induces strong migratory responses of basophils as well as eosinophils. Furthermore, in contrast to neutrophils, β_1 integrins in addition to β_2 integrins are specifically expressed on the surface of both cells. The results of the present study clearly show that the regulatory profile of basophil TEM is very similar to that reported for eosinophils (19, 29, 31, 49, 50). In addition to regulatory cytokines and chemokines, adhesion molecules are also involved in TEM of basophils in a quite similar fashion as in TEM of eosinophils. These common aspects of TEM determined in vitro strongly suggest similarity of the in vivo kinetics of these two cell types.

In summary, for the first time we have studied the profile and regulation of basophil TEM using IL-1-activated HUVEC. Basophil-activating hemopoietin IL-3 and basophil-directed chemokines, such as CCR2 and CCR3 ligands, are critically involved in TEM of basophils. Although β_2 integrin/ICAM-1 plays a major role in basophil TEM, β_1 integrin/VCAM-1 clearly plays an additional role. These profiles of the regulation of basophil TEM are very similar to those of eosinophil TEM, except for that by CCR2 ligand MCP-1. Our results thus support the previous argument for a close relationship between basophils and eosinophils, and suggest similar in vivo kinetics for these two cell types.

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ELSEVIER

A prospective clinical study of theophylline safety in 3810 elderly with asthma or COPD

Ken Ohta[a,*], Yoshinosuke Fukuchi[b], Lawrence Grouse[c], Ryuji Mizutani[d], Klaus F Rabe[e], Stephen I Rennard[f], Nan-Shan Zhong[g]

[a]Department of Medicine, School of Medicine, Teikyo University, 2-11-1 Kaga, Itabashi-Ku, Tokyo, Japan
[b]Juntendo University, Tokyo, Japan
[c]University of Washington, Seattle, Washington, USA
[d]Mitsubishi Pharma Corporation, Osaka, Japan
[e]Leiden University, Leiden, The Netherlands
[f]Nebraska University, Omaha, Nebraska, USA
[g]Guangzhou University of Medicine, Guangzhou, China

Summary A large-scale prospective study was conducted in 3810 Japanese elderly (≥65 years old) patients with asthma or chronic obstructive pulmonary disease (COPD) who had been treated with sustained-release theophylline tablets (THEODUR[R]) at a dose of 400 mg/day for 1–6 months, in principle.

Among 3798 protocol-complying patients (mean age: 73.8 ± 0.10 years, 1997 with COPD), 261 theophylline-related adverse events were observed in 179 (4.71%) patients. The 5 most frequently observed adverse events were "nausea" (40 episodes, 1.05%), "loss of appetite" (22 episodes, 0.56%), "hyperuricemia" (16 episodes, 0.42%), "palpitation" (15 episodes, 0.39%), and "increased alkaline phosphatase" (11 episodes, 0.28%). No convulsions were reported. Six patients had serious adverse events.

The incidence of theophylline-related adverse events was higher in patients with hepatic disease (odds ratio: 1:1.81) and in patients with arrhythmia (odds ratio: 1:1.88). Blood drug concentration measurements in 736 patients indicated that the drug levels were ≤15 µg/ml in 641 patients (87.1%), and no correlation was noted between dose and theophylline-related adverse events.

These results suggest that sustained-release theophylline can be used safely in elderly patients with asthma or COPD.
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Introduction

Theophylline has been used for many years for the treatment of asthma and chronic obstructive

pulmonary disease (COPD). The control of blood theophylline concentrations has become easier since the development of sustained-release preparations. In addition, the anti-inflammatory effect of theophylline has also been documented.[1–4] Sustained-release theophylline plays an important role in the pharmacotherapy for asthma and COPD.

The toxic range of blood theophylline concentrations is generally considered to exceed 15 µg/ml.[5] Following oral administration of sustained-release

theophylline at the usual dose (in Japan, 400 mg/day), blood theophylline concentrations in excess of 15 µg/ml and theophylline-related adverse events are rarely observed. Most serious adverse events of theophylline have been observed when the drug was overdosed.[5] However, adverse events of theophylline may develop also in patients who are taking the usual dose because the clearance of theophylline is modified by various factors, including aging,[6–13] a reduction in physiological hepatic function[14–20] and drug interactions with other medications.[21,22] To investigate the incidence and severity of such adverse events, we studied elderly patients who received sustained-release theophylline in the present large-scale prospective study.

Patients and methods

Japanese elderly (≥65 years old) with bronchial asthma (BA) or chronic obstructive pulmonary disease (COPD), who received sustained-release theophylline (THEODUR® 100- or 200-mg Tablets, Nikken-Chemicals, Tokyo, Japan) between January 1, 1999 and March 31, 2000, were registered in order of their visits as subjects according to the continuous entry method.

Patients with a history of serious adverse events caused by xanthine-derived drugs were excluded.

A serious adverse event was defined to be any untoward medical occurrence that at any dose:

- results in death,
- is life-threatening,
- requires inpatient hospitalization or prolongation of existing hospitalization,
- results in persistent or significant disability/incapacity,
- is a congenital anomaly/birth defect, or
- does not meet any of the above criteria for seriousness but may jeopardize the patient or subject or may require medical or surgical intervention to prevent one of the outcomes listed above.

As a general rule, the daily dose was 400 mg for sustained-release theophylline which was administered orally twice a day in the morning and at night in accordance with the approved regimen in Japan. However, the dose was adjusted according to the patient's status. Adverse events which developed during the study period of 4 weeks or longer were recorded. Patients were examined up to 6 months after the onset of the present study.

The date of and reason for discontinuation or withdrawal from the present study were recorded, if any, and the clinical course of the relevant patients was monitored when conductible. All adverse events reported during the study period were recorded. Regarding all observed adverse events, the date, symptoms, severity, drugs other than theophylline and outcomes were recorded. Causality of an adverse event with theophylline was categorized as follows: (1) related; (2) probably related; (3) probably unrelated; and (4) undeterminable. The severity of an adverse event was categorized as follows: (1) died; (2) injured; (3) potential risk of death or injury; (4) hospitalized for treatment or increased the hospitalization period; (5) severe; (6) moderate; and (7) mild. Categorization was made by the attending physician. Serious adverse events were defined as those which were categorized to 1–5.

Blood theophylline concentration at the time of adverse event development was also recorded when conductible. This study was conducted in compliance with the Good Post-Marketing Surveillance Practice which had been implemented by the Ministry of Health and Welfare in April 1994.

Bonferroni–Dunn's test was performed for parametric data, whilst χ^2 test was performed for nonparametric data, both at a P-value of 0.05, two-tailed.

Results

Subjects

The present study included a total of 3810 patients. However, 12 patients were excluded from the present study due to protocol violation. Therefore, 3798 patients (mean age: 73.8 years) remained as patients who were eligible for the analysis of safety (Table 1).

Among 3798 patients, 2720 showed one or more concurrent diseases which totaled to 5690 in number, 2227 of which were related to the cardiovascular system and 184 to the liver system. The present study was terminated within 4 weeks after the onset of the present study in 148 patients (3.90%). The most frequent causes were as follows: failures to visit the hospital after previous visit(s) and to follow-up the patient in 52 patients (1.37%); adverse events in 30 patients (0.79%); improvement of symptoms in 16 patients (0.42%); and development or aggravation of complications in 15 patients (0.39%).