Contrasting Responses to Multiple Chemotactic Stimuli in Transendothelial Migration

Heterologous Desensitization in Neutrophils and Augmentation of Migration in Eosinophils[1]

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At inflammatory sites in vivo, leukocytes may confront multiple, competing chemoattractive signals. We found significant differences between eosinophils and neutrophils in transendothelial chemotaxis to a chemoattractant diffusing from the lower chamber, when a chemoattractant that binds to another receptor is present at uniform concentration. The transendothelial migration of eosinophils to FMLP, C5a, RANTES, or MCP-3 was totally inhibited by the presence of the homologous chemoattractant, and only RANTES and MCP-3 showed mutual inhibition. C5a and to a lesser extent FMLP chemokinetically stimulated migration to RANTES and MCP-3, without stimulating random migration. Results with neutrophils contrasted. The presence of FMLP not only abrogated neutrophil transmigration to FMLP but also strongly decreased chemotaxis to C5a, IL-8, and Gro-α. Similarly, C5a inhibited neutrophil chemotaxis to IL-8 and Gro-α. IL-8 almost totally abrogated chemotaxis to Gro-α, but Gro-α only moderately inhibited chemotaxis to IL-8. Neither IL-8 nor Gro-α significantly inhibited transmigration to FMLP or C5a. Actin polymerization in eosinophils and neutrophils was desensitized by the same combinations of chemoattractants that desensitized chemotaxis. We conclude that eosinophils have at least three noninterfering receptor-signal transduction pathways for chemotaxis and actin polymerization. In contrast, the signaling pathways for FMLP, C5a, and IL-8/Gro-α in neutrophils are heterologously cross-desensitized, with a hierarchy of resistance to competing signals of FMLP > C5a > IL-8 > Gro-α, in agreement with previous results in neutrophils on the Ca^{2+}-mobilizing response. These results may have important implications for the behavior of these cell types in inflammatory sites. ***The Journal of Immunology,*** **1997, 158: 2340–2349.**

Neutrophils and eosinophils are the major classes of granulocytes that emigrate from the bloodstream and accumulate in inflammatory reactions. However, their accumulation patterns in inflamed tissue are strikingly different; neutrophils are rapidly recruited into sites of acute bacterial infection, whereas eosinophils are predominantly recruited into tissues with chronic allergic inflammation or parasitic infection (1–3). The emigration of circulating leukocytes from the bloodstream into subendothelial tissues involves multiple steps, including initial tethering and rolling on the local vascular endothelium and subsequent migration into the subendothelial space, which are dependent on selectin-carbohydrate and integrin-Ig superfamily-molecule interactions (4). Chemoattractants have been shown to play a critical role by activating integrin adhesiveness and inducing directional movement across the endothelial cell layer and are thought to also guide subsequent migration within tissues toward the inflammatory stimulus. Classical chemoattractants including FMLP, C5a, LTB_4, and PAF[3] act on multiple leukocyte subpopulations, whereas chemokines can more selectively activate specific leukocyte subsets (5–7). In general, many of the C-*X*-C chemokines are chemotactic for neutrophils, whereas C-C chemokines act on monocytes, eosinophils, basophils, and lymphocyte subpopulations. For example, the C-*X*-C chemokines IL-8 (8), neutrophil-activating peptide-2 (9), and Gro-α, -β, -γ (10) are potent attractants for neutrophils, while eosinophils are attracted strongly by the C-C chemokines RANTES (11–13), MCP-2 (13), MCP-3 (13, 14) and weakly by MIP-1α (12). Differential chemokine expression in tissues may be responsible for the selective accumulation of specific leukocyte subsets.

Inflammatory stimuli result in production of a bewildering array of chemoattractive signals. Although there may be overall differences between acute and chronic inflammation, there is also considerable overlap, and multiple chemokines are produced in individual diseases (15, 16). Classical chemoattractants will also be coexpressed in many diseases. For example, bacteria produce *N*-formylated peptides, e.g., FMLP, activate alternative and classical complement pathways resulting in C5a production, release LPS that activates a variety of cell types to produce chemokines and other cytokines and, after processing by APCs, stimulate T lymphocytes to produce further cytokines and chemokines. The released cytokines will in turn stimulate further chemokine production by many classes of tissue cells. Studies in vitro have shown that granulocytes can rapidly orient to, migrate toward, and phagocytose point sources of chemoattractant production, e.g., microorganisms that activate the alternative pathway of complement and

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[3] Abbreviations used in this paper: PAF, platelet-activating factor; MCP, monocyte chemotactic protein; MIP, monocyte inflammatory protein; HUVEC, human umbilical vein endothelial cell; MACS, magnetic cell separation system; PKA, protein kinase A; PKC, protein kinase C; F-actin, filamentous actin; LTB_4, leukotriene B_4.

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set up a surrounding C5a gradient (17). It is likely that once granulocytes enter tissues in vivo they encounter multiple chemoattractants: some present in gradients, others more constant in concentration, some produced directly by or at the surface of microorganisms, and others produced by host cells.

In this study, we have asked how neutrophilic and eosinophilic granulocytes would integrate signals from and respond to the presence of multiple chemoattractants. Previous studies have examined desensitization to one chemoattractant by another, usually by measuring transient Ca^{2+} mobilization, but also in some cases by measuring degranulation, the respiratory burst (6, 7), membrane-bound GTPase activity (18), or chemotaxis (13). Chemoattractants that bind to the same receptor induce homologous desensitization to one another. However, calcium flux studies on neutrophils have shown heterologous desensitization among peptide chemoattractant receptors, whereby FMLP, C5a, and IL-8 partially desensitize to one another (19, 20). We extend these results to neutrophil chemotaxis, and show contrasting results in eosinophils. Even chemoattractants that bind to the same receptors in neutrophils and eosinophils, C5a and FMLP, have very differing effects, inhibiting migration to other chemoattractants in neutrophils, and enhancing or having no effect on migration to other chemoattractants in eosinophils. The results are confirmed with actin polymerization assays and demonstrate that signaling pathways emanating from chemoattractant receptors are integrated quite differently in neutrophils and eosinophils.

Materials and Methods

Chemoattractants

Recombinant human RANTES, IL-8, and Gro-α were from R&D Systems (Minneapolis, MN). Human C5a and FMLP were from Sigma Chemical Co. (St. Louis, MO). Chemically synthesized MCP-1, MCP-2, and MCP-3 (21) were generous gifts from Dr. Ian Clark-Lewis (University of British Columbia, Vancouver, BC, Canada).

Cell purification

Granulocytes were isolated from citrated blood of healthy volunteers by dextran sedimentation, Ficoll-Hypaque centrifugation, and hypotonic lysis of the granulocyte cell pellet to remove RBC (22). Granulocytes were washed twice in PBS containing 1% BSA and 5 mM EDTA and were 90 to 98% neutrophils. This granulocyte preparation was used as the input neutrophil population in chemotaxis or actin polymerization assays after two further washes in PBS with 1% BSA and 5 mM EDTA, or used for purification of eosinophils by negative immunomagnetic selection using the MACS protocol (Miltenyi Biotec, Sunnyvale, CA), as described elsewhere (23). Briefly, 2×10^8 granulocytes in 2 ml were incubated with 0.2 ml of anti-CD16-conjugated microbeads. After 30 min of incubation on ice, cells were loaded onto the MACS column and eluted nonadherent cells were collected and used for the input eosinophil populations. The purity of eosinophils was examined by Wright-Giemsa staining and found to be >98%.

Transendothelial chemotaxis assay

Transendothelial migration was quantified as described previously (24). Briefly, HUVEC were cultured for 1 wk on collagen-coated, 6.5-mm-diameter Transwell inserts of 5-μm pore size (Costar, Cambridge, MA). Chemokines were diluted in assay medium (1:1 mixture of RPMI 1640 and M199 medium + 0.25% human serum albumin) to various concentrations and placed in the wells of a 24-well culture plate in a final volume of 500 μl. Inserts with HUVEC-covered filters were transferred into the plate wells. Neutrophils or eosinophils were labeled with 0.5 μg/ml 2′,7′-bis-(2-carboxyethyl)-5(and-6)-carboxyfluorescein (Molecular Probes, Eugene, OR) in RPMI 1640 medium/0.25% BSA for 15 min and washed once, and 2.5×10^5 cells in 100 μl of assay medium were added to each insert. After a 45-min (neutrophils) or 1-h (eosinophils) incubation at 37°C, in 5% CO_2, the HUVEC-covered inserts were removed. The bottom surfaces of the filters were gently scraped several times against the well edge to dislodge the cells adhering to the under surface of the HUVEC-covered filters, and to collect them in the lower chamber. In both standard chemotaxis and desensitization experiments, the number of cells bound to the under surface of the filter after the treatments described above was determined by removing cells adherent to the upper surface with EDTA, and microscopic observation of the undersurface of the filter. The number of cells bound to the under surface of the filters was less than 10% (neutrophils) or 5% (eosinophils) of the total number of cells accumulated in the lower chambers in any experiment. This percentage was not significantly affected by the presence of chemoattractants at any concentration used in this study, whether added in top, bottom, or both chambers. Thus, the percentage of migration determined by this method reflected more than 90% of the total migration in all experiments. Migrated cells were resuspended by pipeting and allowed to settle 30 min to assure homogeneous cell distributions. The number of the cells that migrated into the bottom wells was quantitated by counting three different fields on each bottom well using a 10 × 10 grid (0.1 mm^2) on an inverted phase-contrast fluorescent microscope. In desensitization experiments, 1 to 100 nM chemokine was added in assay medium to both top and bottom chambers and the chemotactic migration to another chemokine present only in the bottom chamber was measured. All experiments were performed in duplicate and data are expressed as the percentage of migrated cells.

Actin polymerization assay

Polymerized actin (F-actin) was determined by staining with FITC-labeled phalloidin (25), with some modifications. Briefly, 10^6 cells were equilibrated in 0.8 ml of prewarmed L-15 medium for 10 min at 37°C, and the first chemoattractant was added to each sample at a final concentration of 10 nM. After 15-min incubation at 37°C in a dry bath, the cells were restimulated with a 10 nM concentration of a second chemoattractant, and the amount of F-actin was examined 0, 15, 30, 60, 300, and 600 s later. At these time points, the cells were fixed, permeabilized, and stained in a single step by adding 0.2 ml of a solution containing 100 ng of FITC-conjugated phalloidin, 0.1 mg/ml L-α-lysophosphatidylcholine, and 37% formaldehyde. The stained cells were incubated for 10 min at room temperature, washed with PBS, and subjected to fluorescent flow cytometry.

Statistical analysis

All migration and actin polymerization experiments were performed in duplicate and repeated two to six times. The data obtained from all the experiments were used for calculation of p values by paired Student's t test and the differences with $p < 0.05$ were considered to be significant.

Results

We first compared a panel of chemoattractants for elicitation of transendothelial chemotaxis of neutrophils (Fig. 1*A*) and eosinophils (Fig. 1*B*). The classical chemoattractants, FMLP and C5a, and the C-*X*-C chemokines, IL-8, and Gro-α, but not the C-C chemokine RANTES, produced substantial and similar levels of neutrophil chemotaxis at 10 and 100 nM (Fig. 1*A*). C5a, MCP-3, and RANTES elicited substantial eosinophil chemotaxis at 100 nM, and MCP-2 and FMLP attracted somewhat lower numbers of eosinophils (Fig. 1*B*). C5a was the most potent chemoattractant, attracting cells at 1 nM, and RANTES was effective at 10 nM. The C-*X*-C chemokines IL-8 and Gro-α (not shown) and the C-C chemokines MIP-1α and MCP-1 were ineffective at the highest concentration tested of 100 nM.

To model physiologic situations in which granulocytes would be exposed to multiple chemoattractant gradients, we placed neutrophils or eosinophils in a top chamber containing one chemoattractant, with a bottom chamber containing a second chemoattractant, to determine which signal would predominate. The chemoattractant FMLP in the upper chamber largely inhibited transendothelial migration of neutrophils to 10 nM IL-8 (Fig. 2*A*). C5a was less inhibitory. C5a, FMLP, and IL-8 bind to distinct receptors. IL-8 binds to both IL-8RA and IL-8RB receptors, whereas Gro-α binds only IL-8RB (7). Despite this sharing, Gro-α had the least effect on chemotaxis to IL-8 (Fig. 2*A*). MCP-3 completely inhibited migration of eosinophils to RANTES (Fig. 2*B*), in agreement with sharing by these chemokines of a receptor on eosinophils. C5a and FMLP had a definite but minor effect on migration to RANTES. Similar results were obtained in the absence of an endothelial

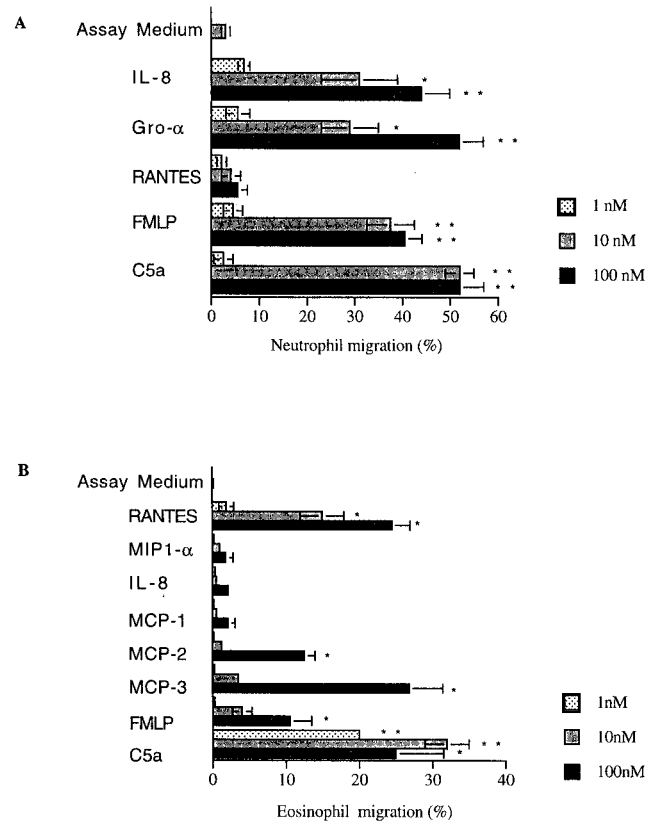

FIGURE 1. Transendothelial chemotaxis of neutrophils and eosinophils. Chemoattractants were placed in the bottom chamber only and migration was determined after 45 min for neutrophils (*A*) or 1 h for eosinophils (*B*) as described in *Materials and Methods*. Data are mean ± range of two experiments or mean ± SD of three experiments. $*p < 0.05$. $**p < 0.02$ ($n = 3$ for neutrophils and $n = 2$ for eosinophils).

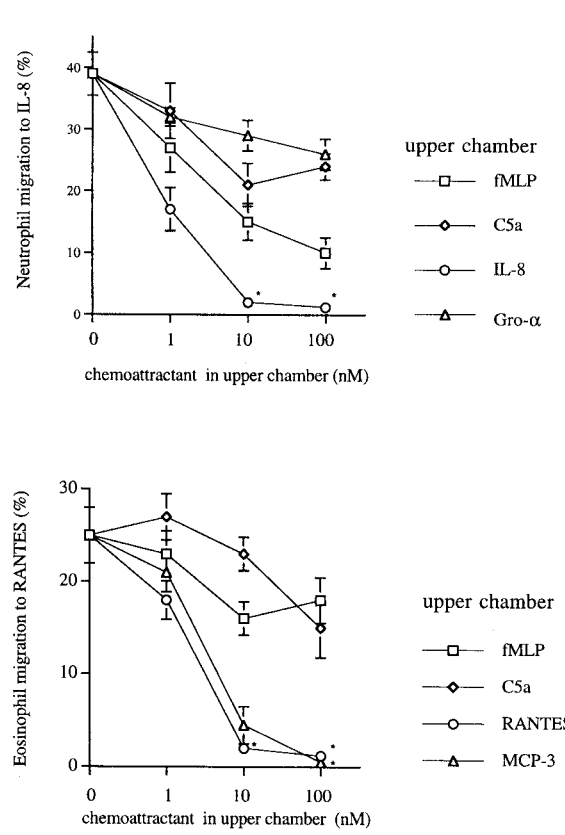

FIGURE 2. Effect of chemoattractants in the upper chamber on transendothelial chemotaxis of neutrophils to IL-8 (*A*) or eosinophils to RANTES (*B*). The indicated concentrations of chemoattractants were placed in the upper chamber with cells, and 10 nM IL-8 (*a*) or RANTES (*b*) were placed in the lower chamber. Data are mean ± range of two different experiments. $*p < 0.05$, $n = 2$.

monolayer on the filter although background migration in the absence of a chemoattractant in the lower chamber was higher (data not shown).

Inhibition in the above experiments cannot be equated with desensitization. Migration to the chemoattractant in the lower chamber may be opposed by competing attraction to the chemoattractant in the top chamber, because when a leukocyte migrates halfway through the endothelial monolayer, it would be exposed to gradients in both directions. Migration to the lower compartment will be favored, because gravity can permanently remove an emigrated leukocyte from the bottom of the filter to the lower chamber, but a nonemigrated leukocyte remains on the upper surface of the monolayer, and migration to the lower chamber continues to be possible. Therefore, we examined another system in which one chemoattractant was present in equal concentrations in both the top and bottom chambers, and a second chemoattractant was present only in the lower chamber. In this system, the first chemoattractant can inhibit only through desensitization to the second chemoattractant. When HUVEC monolayers were present on the filters, the presence of the first chemoattractant in both chambers induced little or no random migration (Figs. 3 and 4; 0 nM in lower chamber). Thus, a decrease in chemotactic migration in this system reflects desensitization by the first chemokine of the chemotactic signal from the second chemokine.

Transendothelial chemotaxis of eosinophils to FMLP, C5a, RANTES, and MCP-3 in the lower chamber were measured in the presence of 0 to 100 nM concentrations of each of these four chemoattractants in both the top and bottom chambers (Fig. 3). Chemotaxis to FMLP was strongly inhibited by the presence of FMLP, with 1, 10, and 100 nM FMLP totally abrogating chemotaxis to 1, 10, and 100 nM FMLP, respectively ($p < 0.01$, $n = 3$). (Fig. 3*A*). In contrast, the presence of C5a, RANTES, and MCP-3 produced no significant inhibition of FMLP-induced chemotaxis; in fact, each slightly enhanced FMLP-induced chemotaxis, although this was not statistically significant (Fig. 3, *B–D*). Similarly, chemotaxis to C5a was inhibited by the presence of C5a ($p < 0.02$, $n = 3$, for 10 and 100 nM C5a), and not by the other three chemokines (Fig. 3, *E–H*). In contrast, migration to RANTES was significantly decreased by MCP-3 as well as by RANTES; either one at a concentration of 100 nM almost totally abrogated the response to RANTES (Fig. 3, *K* and *L*) ($p < 0.05$ for MCP-3, $p < 0.05$ for RANTES, $n = 3$). Similarly, migration to MCP-3 was completely inhibited by both RANTES ($p < 0.05$, $n = 3$) and MCP-3 ($p < 0.05$, $n = 3$) (Fig. 3, *O* and *P*). Neither FMLP nor C5a had any inhibitory effects on eosinophil migration to RANTES or MCP-3; indeed, C5a markedly enhanced the chemotactic response to both chemoattractants over a wide range of RANTES and MCP-3 concentrations (Fig. 3, *J* and *N*). This effect of C5a was statistically significant at 10 nM in three experiments performed in duplicate ($p < 0.05$, $n = 3$, for augmentation to 10 nM RANTES, 100 nM RANTES, and 100 nM MCP-3; $p < 0.01$, $n = 3$, for augmentation to 1 and 10 nM MCP-3). FMLP (10 nM) also significantly enhanced the migration to 100 nM RANTES ($p < 0.05$, $n = 3$) and

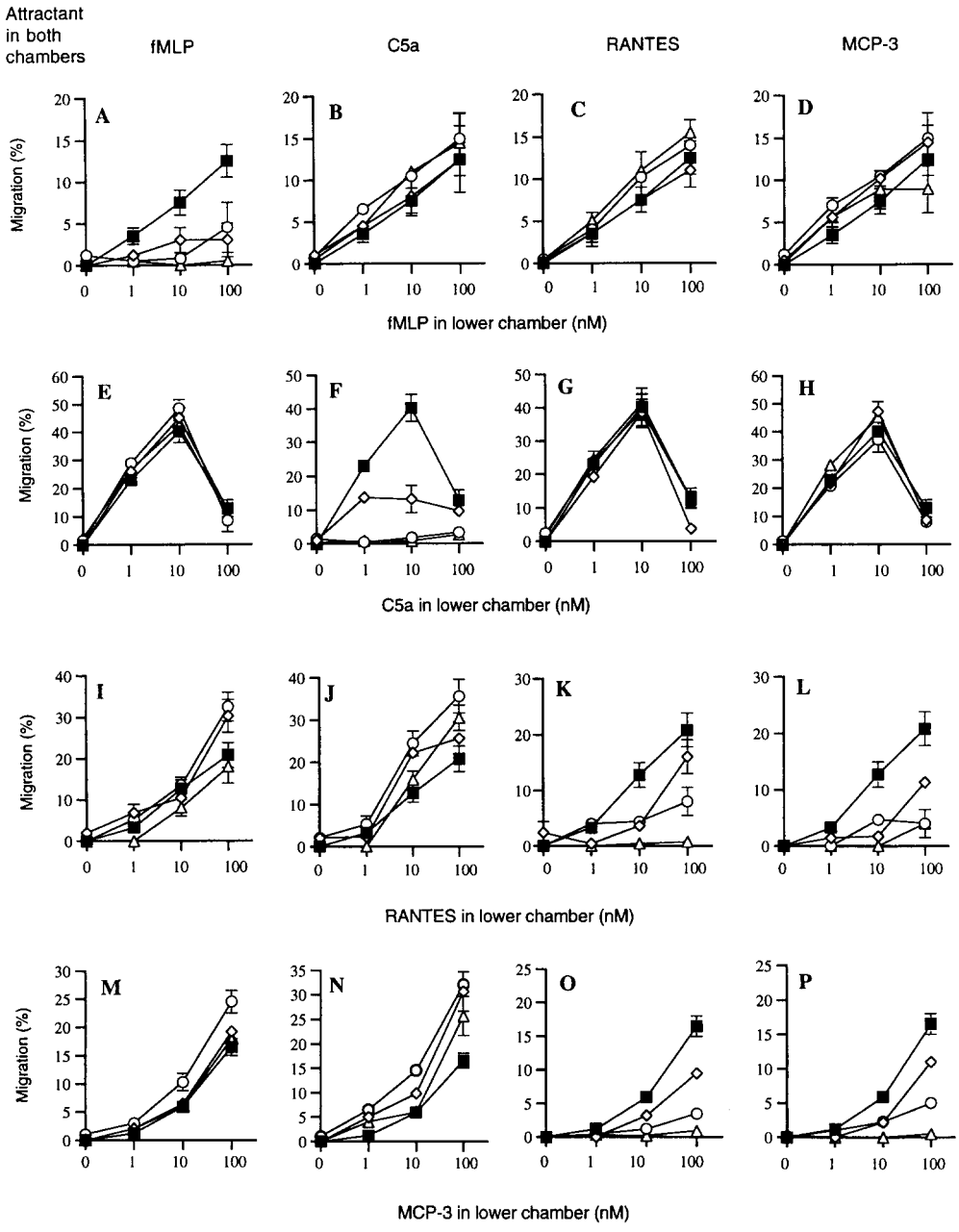

FIGURE 3. Eosinophil transendothelial chemotaxis in the presence of an additional chemoattractant in both the upper and lower chambers. FMLP (*A–D*), C5a (*E–H*), RANTES (*I–L*), and MCP-3 (*M–P*), at the indicated concentrations on the *x*-axis were present in the lower chamber; FMLP (*A, E, I,* and *M*), C5a (*B, F, J,* and *N*), RANTES (*C, G, K,* and *O*), or MCP-3 (*D, H, L,* and *P*) were absent (■) or present in both upper and lower chambers at 1 nM (◇), 10 nM (○), and 100 nM (△). The cells were placed in the top chamber and migration was examined as described in *Materials and Methods*. Data are mean ± range of duplicates in one representative experiment of three different experiments with similar results.

10 and 100 nM MCP-3, ($p < 0.05$, $n = 3$), (Fig. 3, *I* and *M*). These data suggest that eosinophils possess at least three distinct chemoattractant receptor-mediated signaling pathways for chemotaxis: one for FMLP, a second for C5a, and a third that is shared by RANTES and MCP-3. Furthermore, they show that uniform concentrations of C5a and to a lesser extent FMLP can enhance migration in a gradient of RANTES and MCP-3.

When neutrophil chemotaxis was examined with the same assay scheme, a more complicated desensitization pattern was observed (Fig. 4). As with eosinophils, the migration to FMLP in the lower chamber was inhibited by FMLP in both chambers in a dose-dependent manner but was not strongly affected by the other chemokines. However, the presence of FMLP strongly inhibited neutrophil migration to the other chemokines. Specifically, 100 nM FMLP inhibited chemotaxis induced by 10 nM C5a or IL-8 by 30 ± 13% ($p < 0.02$, $n = 6$) and 54 ± 13% ($p < 0.05$, $n = 4$), and 100 nM C5a- or IL-8-induced chemotaxis by 57 ± 8.0% ($p < 0.05$, $n = 3$) and 88 ± 1.9% ($p < 0.05$, $n = 3$), respectively (Figs. 4, *E* and *I*). The response to Gro-α was even more sensitive to this inhibition, since even the lower concentration of 1 nM FMLP inhibited 100 nM Gro-α-mediated chemotaxis by 69 ± 7.7% ($p < 0.05$, $n = 3$) (Fig. 4*M*). Similarly, the presence of 100 nM C5a not only abrogated its own chemotactic effects but also decreased the chemotactic responses to 10 nM IL-8 and Gro-α by 38 ± 13%

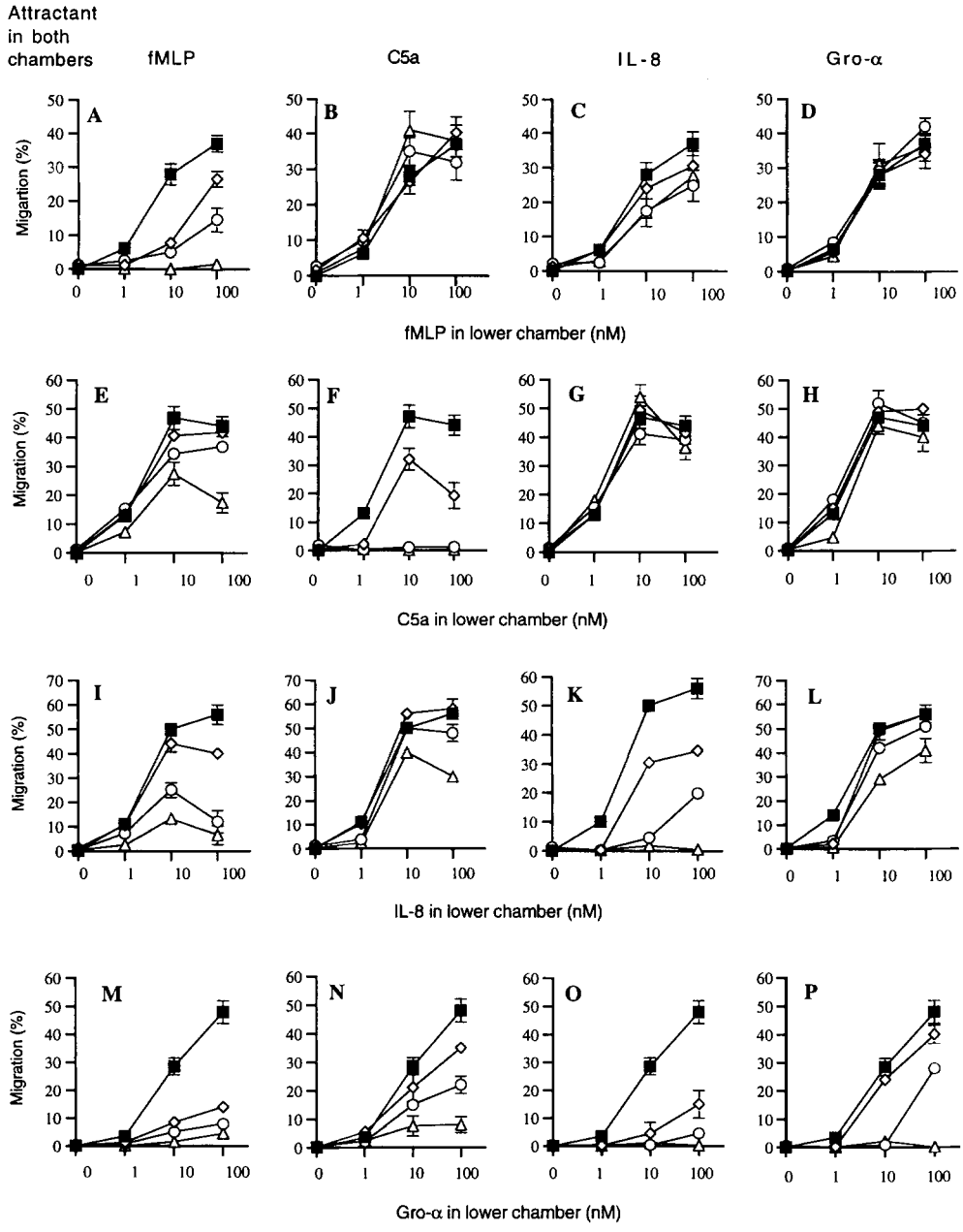

FIGURE 4. Neutrophil transendothelial chemotaxis in the presence of an additional chemoattractant in both the upper and lower chambers. FMLP (*A–D*), C5a (*E–H*), IL-8 (*I–L*) and Gro-α (*M–P*) at the indicated concentrations on the *x*-axis were present in the lower chamber; FMLP (*A, E, I,* and *M*), C5a (*B, F, J,* and *N*), IL-8 (*C, G, K,* and *O*), and Gro-α (*D, H, L,* and *P*) were absent (■) or present in both upper and lower chambers at 1 nM (◇), 10 nM (○), and 100 nM (△). The cells were placed in the top chamber and migration was examined as described in *Materials and Methods*. Data are mean ± range of duplicates in one representative experiment of three to six different experiments with similar results.

($p < 0.05$, $n = 4$) and 68 ± 10% ($p < 0.05$, $n = 3$), and those to 100 nM IL-8 and Gro-α by 44 ± 7.6% ($p < 0.05$, $n = 3$) and 85 ± 4.7% ($p < 0.02$, $n = 3$), respectively (Fig. 4, *F, J,* and *N*); however, FMLP induced-chemotaxis was not inhibited by C5a (Fig. 4*B*). IL-8 and Gro-α inhibited chemotaxis to one another. IL-8 potently inhibited chemotaxis to Gro-α; inhibition was marked at 1 nM (77 ± 5.9%, $p < 0.05$, $n = 3$, and 73 ± 4.4%, $p < 0.02$, $n = 3$, for 10 and 100 nM Gro-α, respectively), and the inhibition by 100 nM IL-8 reached 94 ± 4.0% ($p < 0.02$, $n = 3$) and 96 ± 1.5% ($p < 0.01$, $n = 3$) for 10 and 100 nM Gro-α (Fig. 4*O*). Gro-α only partially inhibited IL-8-induced chemotaxis; there was little or no inhibition at 1 and 10 nM, and 100 nM Gro-α inhibited 10 and 100 nM IL-8-induced chemotaxis by 24 ± 4.8% ($p < 0.05$, $n = 4$) and 31 ± 9.0% ($p < 0.02$, $n = 4$) (Fig. 4*L*). Neither IL-8 nor Gro-α significantly inhibited FMLP- or C5a-induced migration, although IL-8 at 10 and 100 nM slightly inhibited FMLP-induced migration (Figs. 4, *C, D, G,* and *H*). Thus, the signals to mediate neutrophil chemotaxis showed a complex pattern of heterologous and homologous cross-desensitization, with a general hierarchy in strength of FMLP > C5a > IL-8 > Gro-α. Conversely, the susceptibility of neutrophils to be desensitized occurred in reverse order for the receptors for these chemoattractants.

Since granulocyte migration is usually accompanied by activation of endothelial cells with inflammatory cytokines, the same experiment was performed with HUVEC activated with 100 U/ml TNF-α for 24 h. The presence of equal concentrations (10 nM) of

Table I. *Specific migration to a chemoattractant in the lower chamber of eosinophils through TNF-stimulated HUVEC*[a]

Chemoattractant in Medium	Background Migration	Chemoattractant in Lower Chamber			
		FMLP	C5a	RANTES	MCP-3
Control	3.3 ± 0.1	25.3 ± 1.0	50.9 ± 7.7	33.9 ± 6.7	31.7 ± 3.0
FMLP	5.0 ± 0.95	14.1 ± 4.0	59.6 ± 2.5	37.0 ± 0.1	37.0 ± 3.0
C5a	12.0 ± 2.2	26.1 ± 6.1	11.3 ± 1.1	40.2 ± 1.8	34.2 ± 5.2
RANTES	6.7 ± 2.3	28.5 ± 0.55	45.5 ± 2.0	13.9 ± 0.55	15.7 ± 4.3
MCP-3	6.9 ± 1.3	30.3 ± 1.5	48.1 ± 8.3	11.6 ± 1.8	15.8 ± 5.2

[a] HUVEC was activated with 100 U/ml TNF-α for 24 h before transmigration experiments. All the chemoattractants were used at 10 nM. Background migration represents percentage of migration to uniform concentration of chemoattractant in medium. Other values represent percentages of specific chemotaxis (% of total migration in experimental well − % of background migration in the well containing equal concentration of a chemoattractant in assay medium). Data show mean ± range of two different experiments each performed in duplicate.

Table II. *Specific migration to a chemoattractant in the lower chamber of neutrophils through TNF-stimulated HUVEC*[a]

Chemoattractant in Medium	Background Migration	Chemoattractant in Lower Chamber			
		FMLP	C5a	IL-8	Gro-α
Control	3.6 ± 0.85	47.2 ± 7.2	47.6 ± 4.2	52.0 ± 7.9	45.9 ± 4.7
FMLP	12.0 ± 1.5	28.7 ± 5.4	34.3 ± 2.3	26.2 ± 1.3	22.5 ± 5.5
C5a	9.2 ± 0.7	59.6 ± 6.0	22.3 ± 0.35	38.5 ± 2.9	27.2 ± 4.6
IL-8	11.7 ± 2.8	44.8 ± 0.35	45.6 ± 6.2	26.4 ± 1.3	25.3 ± 2.3
Gro-α	6.9 ± 1.3	60.6 ± 6.3	48.1 ± 8.3	48.7 ± 5.3	21.5 ± 3.2

[a] HUVEC was activated with 100 U/ml TNF-α for 24 h before transmigration experiments. All the chemoattractants were used at 10 nM. Background migration represents percentage of migration to uniform concentration of chemoattractant in medium. Other values represent percentages of specific chemotaxis (% of total migration in experimental well − % of background migration in the well containing equal concentration of a chemoattractant in assay medium). Data show mean ± range of two different experiments each performed in duplicate.

chemoattractants in the top and bottom chambers produced higher random migration both in eosinophils (Table I) and neutrophils (Table II), probably because the stimulation with TNF-α impaired the barrier function of the HUVEC monolayer and induced the production of additional chemoattractants from HUVEC. When the percentage of specific chemotactic migration to a chemoattractant in the lower chamber was calculated by subtracting percentage of random migration, inhibitory patterns similar to those seen with unstimulated HUVEC by uniform concentration of chemoattractant in medium were observed in both eosinophils and neutrophils (Tables I and II).

These migration experiments suggested that when more than one chemoattractant receptor is stimulated, the signaling pathways for these receptors interact differently in neutrophils and eosinophils. Since cell movement requires assembly and disassembly of cytoskeletal actin filaments, we next tested whether sequential stimulation with these combinations of chemoattractants could induce similar desensitization patterns in actin polymerization. Cells were incubated with or without a primary chemoattractant (10 nM) for 15 min, restimulated with another chemoattractant at 10 nM, and then assessed for change of F-actin content. The time point of 15 min was chosen because microscopic observation in the transendothelial chemotaxis assay revealed that most of the cells began migration by this time point. In eosinophils, all chemoattractants used for the primary stimulation showed a peak increase in F-actin content in 15 s, followed by a rapid depolymerization phase (data not shown). When restimulated with the same chemoattractants, the actin polymerization responses of eosinophils were homologously inhibited (Fig. 5). Consistent with the chemotaxis results, significant cross-inhibition was observed only between MCP-3 and RANTES ($p < 0.01$, MCP-3 for RANTES, $p < 0.05$, RANTES for MCP-3, $n = 3$) (Fig. 5, *C* and *D*, open diamonds and triangles).

In contrast, neutrophils showed more complicated cross-desensitization patterns. Stimulation with the primary chemoattractants generated a more pronounced and prolonged effect on neutrophils. Generally, the chemoattractant stimulation of neutrophils caused a 2.5- to 3.5-fold increase in F-actin, and more than 30 min was required for the F-actin content to return to baseline levels. FMLP showed the most prolonged effect, followed by C5a, IL-8, and Gro-α (Fig. 6, closed squares). Thus, the neutrophils prestimulated for 15 min with 10 nM primary chemoattractants, as was done with eosinophils, still have 1.3- to 2.0-fold higher amounts of F-actin than neutrophils without prestimulation at the time of chemoattractant restimulation (Fig. 6, time 0). When neutrophils were restimulated with 10 nM FMLP, actin-polymerization was significantly reduced by pre-exposure with FMLP ($p < 0.02$, $n = 3$) and not with any of the other chemoattractants (Fig. 6*A*). Actin polymerization after restimulation with C5a was reduced by prestimulation with C5a ($p < 0.05$, $n = 3$) and not by IL-8 or Gro-α; after prestimulation with FMLP, actin polymerization stimulated with C5a was reduced in two of three experiments (Fig. 6*B* and data not shown). The peak level of F-actin content induced by IL-8 or Gro-α was significantly reduced by prestimulation with FMLP ($p < 0.01$ or $p < 0.05$, $n = 3$, respectively) and C5a ($p < 0.05$, $n = 3$) (Figs. 6, *C* and *D*). IL-8 prestimulation strongly inhibited the Gro-α-induced response ($p < 0.02$, $n = 3$) (Fig. 6*D*), whereas prestimulation with Gro-α showed no inhibitory effects on the response to IL-8 as well as to FMLP and C5a (Figs. 6, *A* to *C*). In general, these data on actin polymerization are consistent with our chemotactic migration results, although the inhibitory effects are less pronounced in actin polymerization than in chemotaxis.

Discussion

Leukocyte chemotaxis is a complex phenomenon that includes polarization and orientation in the direction of the highest concentration of the chemoattractant, and dynamic cytoskeletal reorganizations that lead to extension of the leading edge and retraction of the trailing edge of the cellular membrane that are coordinated

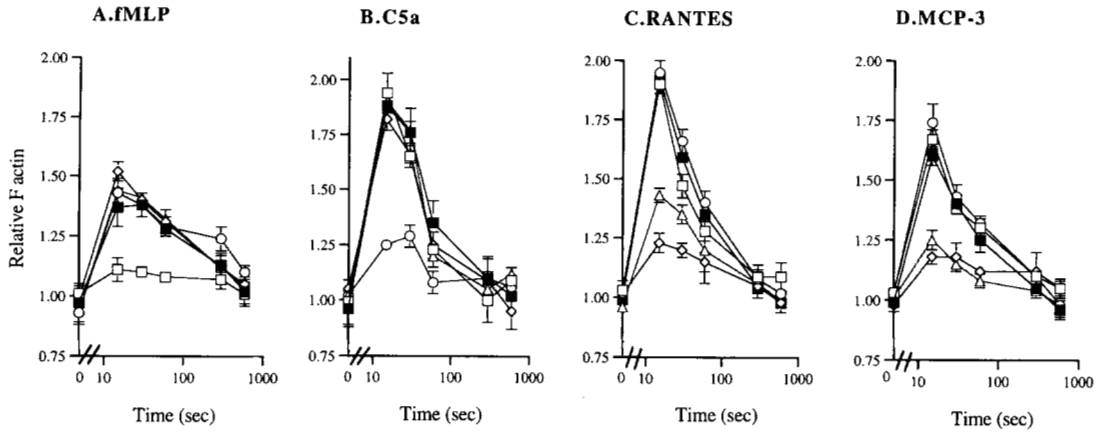

FIGURE 5. Effect of chemoattractant prestimulation on chemoattractant-induced actin polymerization in eosinophils. Eosinophils were prestimulated with medium only (■), 10 nM FMLP (□), C5a (○), RANTES (◇), or MCP-3 (△) and 15 min later restimulated with the 10 nM FMLP (*A*), C5a (*B*), RANTES (*C*), or MCP-3 (*D*). After 15, 30, 60, 300, and 600 s, F-actin content was evaluated as described in *Materials and Methods*. All experiments were performed in duplicate and relative F-actin content was calculated from mean fluorescent channels compared with eosinophils without any chemoattractant prestimulation. Each value represents the mean ± range in one of the three different experiments with similar results.

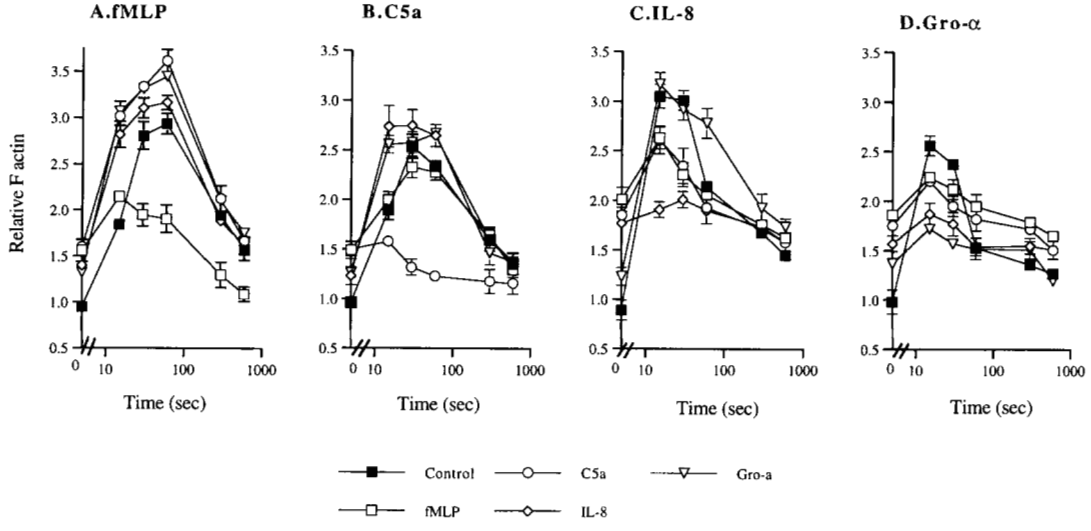

FIGURE 6. Effect of chemoattractant prestimulation on chemoattractant-induced actin polymerization in neutrophils. Neutrophils were prestimulated with media only (■), or 10 nM fMLP (□), C5a (○), IL-8 (◇), or Gro-α (△) and 15 min later restimulated with the same concentration of FMLP (*A*), C5a (*B*), IL-8 (*C*), or Gro-α (*D*). After 15, 30, 60, 300, and 600 s, relative F-actin content was evaluated as described. One representative experiment of three is shown.

with adhesion and de-adhesion (5, 26). The signal transduction pathways that mediate this complicated process are not yet fully understood. Some studies have shown that the intracellular signaling pathways for chemotaxis are distinct from those leading to superoxide production or enzyme secretion (27–30). Although there is considerable literature on desensitization to one chemoattractant by another, most previous studies have focused on assays other than chemotaxis, with Ca^{2+} mobilization by far the most popular assay. Strong random migration is stimulated by a uniform concentration of chemoattractant in filter chemotaxis assays, which obscures inhibitory effects on migration to a gradient of a second chemoattractant. However, transendothelial migration appears much more dependent on directional cues, since we found little or no random migration. We observed that the diffusion of FITC-labeled dextran or ^{125}I-labeled chemoattractants, namely, IL-8, MCP-1, and RANTES, was 8 to 10 times slower in HUVEC-covered filters than in bare filters (31). The retarded diffusion of chemoattractants may cause the low background or random migration in this assay system. Also, we found that the direct binding of the chemoattractants to filter membranes was usually less than 0.05% with no significant difference between HUVEC-covered filters and uncovered filters (31). This excludes the possibility that the differences in the ability of the chemoattractants to bind filters could be a major factor in the chemoattractant-induced modification of granulocyte transmigration. Another possibility is that the expression of adhesion molecules on HUVEC may be altered by chemoattractant stimulation, which results in the modification of granulocyte migration. However, it is also unlikely because none of the chemoattractants used in this study changes the expression of intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and E-selectin on HUVEC within 15 to 60 min (32) (J. Kitayama and T. A. Springer, unpublished observation). Therefore, we have used the transendothelial assay to simulate situations that may occur in vivo, particularly within tissues in which multiple chemoattractants may be present. We asked how neutrophils and eosinophils would respond chemotactically when confronted

with a uniform concentration of one chemoattractant and a gradient of concentration of another, and confirmed our results with studies of actin polymerization. We measured the appearance of leukocytes in the lower chamber, whereas some chemotaxis assays measure cells adherent to the lower surface of a filter. We should emphasize that counts of cells adherent to the lower surface of the filter showed that they constituted <5% of the emigrated eosinophils, and fewer than 10% of the emigrated neutrophils, and thus none of the effects reported here are influenced by the step of de-adhesion from the lower surface of the Transwell filter.

We found a clear contrast in the desensitization patterns between eosinophils and neutrophils. In eosinophils, the chemotactic movements induced by FMLP, C5a, or RANTES and MCP-3, were not reduced at any concentrations by the presence of another chemoattractant, except mutual inhibition between RANTES and MCP-3. This phenomenon can be partially explained by different usage of surface receptors by these chemoattractants. Receptors for C5a (33) and FMLP (34) have been cloned and characterized for ligand specificity; no heterologous affinity for other chemoattractants has been reported. Based on Ca^{2+} flux and competitive binding studies, eosinophils have been shown to use a common receptor for RANTES and MCP-3 (14, 35). The lack of desensitization of Ca^{2+} flux, chemotaxis, and actin polymerization among ligands that bind to three different G protein-coupled receptors on eosinophils shows that the postreceptor signaling pathways are independent and not desensitized by one another. Perhaps the most unexpected finding to emerge from this study is that a uniform concentration of one chemoattractant can augment migration to a gradient of another. Chemokinesis is defined as "a reaction by which the speed or frequency of locomotion of cells and/or the frequency and the magnitude of turning (change of direction) of cells or organisms moving at random is determined by substances in the environment" (36). Chemokinesis is distinct from, but not usually experimentally distinguished from, stimulation of random migration. In our transendothelial migration assay, however, chemokinesis was clearly distinguished from random migration. Uniform concentrations of C5a or FMLP stimulated no random migration in the absence of secondary chemoattractant; however, uniform concentrations of C5a and to a lesser extent FMLP gave statistically significant augmentation of chemotaxis to gradients of RANTES and MCP-3. C5a and FMLP thus acted in a truly chemokinetic manner, to increase "the speed or frequency of locomotion of cells" to another chemoattractant.

The detailed mechanisms of this augmentation in eosinophil migration are under current investigation. One possibility is that C5a and FMLP might impair the barrier function of HUVEC, since there is accumulating evidence that endothelial cells have receptors for C5a (37, 38). However, C5a or FMLP did not alter barrier function as measured by the diffusion of FITC-labeled dextran at time points from 15 to 60 min. Moreover, the presence of eosinophils in the upper chamber together with C5a or FMLP also had no significant effect on the permeability (data not shown).

Another possible explanation for augmentation is that C5a or FMLP present in both the upper and lower chamber could increase the initial binding of eosinophils to HUVEC and thus enhance subsequent transmigration. PAF has been shown to increase the binding of eosinophils to endothelial cells through the activation of $\beta 2$ integrin molecules (39). We examined the eosinophil adhesion to HUVEC with or without these chemoattractants and found that C5a and FMLP enhanced eosinophil adhesion to HUVEC in a 20-min assay by 2.0- and 3.2-fold at 10 nM, respectively, and no further enhancement was seen at higher concentrations (data not shown). This is largely consistent with the amount of heterologous enhancement of migration but does not prove that this is the basis for the enhancement.

Contrasting results were obtained in neutrophil chemotaxis assays. The signaling pathways for FMLP, C5a, IL-8, and Gro-α were partially desensitized by one another and a general hierarchy was observed in the potential to desensitize signals from another chemoattractant, with the rank order of FMLP > C5a > IL-8 > Gro-α. The hierarchy of IL-8 desensitizing to Gro-α, but Gro-α not desensitizing IL-8 may be explained by receptor usage. Two receptors for IL-8 have been characterized. IL-8 binds to both IL-8RA and IL-8RB, whereas Gro-α binds only to IL-8RB (40). The hierarchy of desensitization in neutrophils, except for desensitization by IL-8 of the Gro-α response, appears to be due to heterologous desensitization. A similar hierarchy among chemoattractants in the potential to cross-desensitize the signal for Ca^{2+} mobilization in neutrophils was previously reported (18–20). The peptide chemoattractants C5a, FMLP, and IL-8 were found to desensitize responses to the lipid chemoattractants LTB_4 and PAF but not to desensitize the α-adrenergic receptor in neutrophils, a phenomenon that was termed receptor class desensitization. Furthermore, cross-desensitization among the peptide chemoattractants was found, with the rank order FMLP > C5a > IL-8. However, some differences exist in the desensitization patterns observed for Ca^{2+} mobilization and chemotaxis. For example, C5a partially desensitized the FMLP-induced Ca^{2+} response but did not desensitize FMLP-induced chemotaxis at any concentration. Similarly, IL-8 somewhat attenuated C5a-induced Ca^{2+}-mobilization but had no significant effect on C5a- or FMLP-induced chemotaxis. These discrepancies are not unexpected, since there is gathering evidence that the signal for chemotaxis is distinct from that to increase cytosolic Ca^{2+} (27–30), and that neutrophil chemotaxis can occur even when the increase in cytosolic Ca^{2+} is partially suppressed (41). In the same study, Tomhave et al. reported that IL-8 did not desensitize C5a-induced GTP-γS binding to cell membrane preparations, and C5a did not desensitize FMLP-stimulated GTP-γS binding; but FMLP desensitized C5a and IL-8 stimulated GTP-γS binding, and C5a desensitized IL-8-stimulated GTP-γS binding (20). GTPase activity is also heterologously down-regulated by FMLP (18). Since the desensitization patterns in GTP-γS binding are almost totally consistent with our results on chemotactic migration, it seems probable that the signals that increase cytosolic Ca^{2+} and those that mediate chemotaxis are differently desensitized by chemoattractant exposure, and that chemotactic migration may reflect the receptor-G protein coupling more directly than Ca^{2+} mobilization. In other words, our assay method may be better suited than Ca^{2+} measurement to detect receptor-G protein interactions following chemoattractant-induced desensitization.

The mechanisms of receptor desensitization in leukocytes are unknown. Up to now, the molecular mechanisms of desensitization have been most thoroughly characterized in rhodopsin and β-adrenergic receptors (42–44). There are two types of desensitization, referred to as homologous and heterologous desensitization, and they are thought to be caused by the phosphorylation of receptor molecules at distinct sites by separate protein kinases, with G protein-coupled receptor kinases for homologous, and protein kinase A (PKA) or protein kinase C (PKC) for heterologous desensitization. Both FMLP and C5a have previously been shown to induce rapid phosphorylation of their own receptors, leading to attenuation of signaling in either HL-60 cells or cell lines transfected with receptor genes (45, 46). PKC stimulation by PMA led to phosphorylation of the C5a receptor and caused attenuation of C5a-induced GTP-γS binding, but PMA did not stimulate phosphorylation of the FMLP receptor and thus did not affect the FMLP-induced signal. This is in agreement with the resistance of

the FMLP receptor to desensitization by exposure to other chemoattractants. We found that 5 ng/ml PMA abolished transendothelial chemotaxis of both neutrophils and eosinophils to all chemoattractants studied here (data not shown). Since there are many possible explanations for this, it remains possible that PKC might account for the differences between these cell types.

Examination of cross-desensitization in actin-polymerization assays showed desensitization patterns in both cell types that were similar to those observed in chemotaxis. An interesting finding in the actin-polymerization assay was that the kinetics of actin polymerization were quite different among the chemoattractants used for the first stimulation as well as between cell types. Although the chemoattractants induced rapid actin assembly in both cell types, eosinophils showed quick depolymerization of F-actin, whereas neutrophils showed a sustained polymerization phase, which was in agreement with previous studies (47, 48). More interestingly, the potential to keep the neutrophil actin in the polymerized state was most prominent after FMLP and least after Gro-α stimulation, which was similar to the potential to desensitize another chemoattractant signal. From these data, it can be speculated that the signal to produce the sustained assembly of actin filaments might be directly involved in the mechanisms of the chemoattractant-induced desensitization.

The differences between neutrophils and eosinophils may be physiologically important. Neutrophils are the most rapidly recruited and abundant leukocyte class, and not only are essential for killing and phagocytosis of bacteria but also can damage host cells. In extreme cases, neutrophils are responsible for death of the infected host, as in toxic shock syndrome and endotoxin shock. Formylated peptides, C5a, and IL-8, in that order, are produced increasingly remotely from bacteria. Formylated peptides are produced directly by bacteria. C5a can be generated at the surface of bacteria by the alternative complement pathway and by the classical complement pathway following reaction of Ab with outer membrane or capsule Ags, or can be generated more distally following Ab reaction with secreted Ags. IL-8 is produced by host cells in response to bacterial products and in response to cytokines secreted by other host cells. The same hierarchy for heterologous desensitization of neutrophil chemotaxis may have evolved to enable neutrophils to emigrate from the bloodstream in response to chemokines such as IL-8, followed by movement within tissues toward bacteria in preference over host cells secreting IL-8. Eosinophils show independent migration to MCP-3/RANTES, formylated peptides, and C5a. Thus, in the presence of one of these attractants at uniform concentration in vivo, eosinophils are predicted to migrate up a gradient of another attractant. These different behaviors of neutrophils and eosinophils may reflect specialization for different functions in inflammation. A particularly interesting contrast is that C5a heterologously desensitizes neutrophil chemotaxis to IL-8 and Gro-α but acts chemokinetically on eosinophils to heterologously accelerate chemotaxis to RANTES and MCP-3. Further work is required to determine the mechanisms underlying the differences between neutrophils and eosinophils in how they integrate signals and prioritize responses to chemoattractants that bind to different receptors. These mechanisms may include significant differences in components of the G protein-coupled receptor-signaling pathways in these cell types.

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