The border-crossing behavior of eosinophils and neutrophils in the lung

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CHAPTER III

Neutrophils Enhance Eosinophil Migration across Monolayers of Lung Epithelial Cells

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Neutrophils Enhance Eosinophil Transepithelial Migration
Neutrophils Enhance Eosinophil Migration across Monolayers of Lung Epithelial Cells

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During the late-phase asthmatic response eosinophils and neutrophils infiltrate the lungs and cause severe damage. In this study, we investigated in vitro the migration of eosinophils, in the absence and presence of neutrophils, across a monolayer of lung H292 epithelial cells. The migration of eosinophils towards the complement fragment 5a (C5a) was increased when neutrophils were added to the upper compartment of the Transwells, and decreased when neutrophils were added to the lower compartment. Moreover, neutrophils exclusively stimulated eosinophil migration towards C5a, and not towards other chemoattractants such as RANTES, IL-8 or PAF. Neutrophils and eosinophils differed in that neutrophils, but not eosinophils, rapidly inactivated C5a, suggesting that neutrophils in the upper compartment remove part of the active C5a that has diffused into the upper compartment. Indeed, we found that the addition of other C5a-degrading agents, such as human serum or carboxypeptidase B, also enhanced eosinophil migration when added to the upper compartment and decreased migration when added to the lower compartment. Taken together, these results indicate that the presence of neutrophils influences the migratory behavior of eosinophils in vitro. The neutrophils presumably maintain a proper C5a chemotactic gradient in the transmigration model, which results in enhanced eosinophil chemotaxis.

Introduction

In vivo, granulocytes have been shown to massively infiltrate the bronchial alveolar cavities of patients suffering from asthma (1-4). The influx of eosinophils in the airways is a hallmark of allergic asthma, but the influx of neutrophils has also been proven to be implicated in the pathogenesis of asthma (1-4). Besides their role in the pathogenesis of asthma, neutrophils also play a dominant role in respiratory viral infection, the major cause of common colds and asthma exacerbations (5;6). For infiltration of the airway wall, the granulocytes must extravasate, i.e. the cells initially roll on the endothelium, followed by firm adhesion to and migration across the endothelium. Subsequently, the granulocytes migrate through the interstitial matrix and across the lung epithelium. Granulocyte infiltration is followed by release of toxic granule proteins, which cause tissue damage, and by generation of lipid mediators, which can influence the behavior of surrounding vasculature and smooth-muscle cells (1).

The regulation of human granulocyte migration across endothelial cells has been studied extensively. Yet, little is known about the regulation of granulocyte migration across the epithelium of the lung. Bronchial epithelial cells not only form a passive barrier but also play an active role in the allergic immune response (8;9). Firstly, the epithelial cells can synthesize and release a wide range of proinflammatory mediators (8). Secondly, the epithelial cells express adhesion molecules that are involved in granulocyte migration, e.g. ICAM-1, CD47 and integrins (8;9). Thirdly, asthma-associated factors, such as platelet-activating factor (PAF), in vitro induce changes in lung epithelial monolayers resulting in augmented passage of eosinophils (10). The mechanism behind this phenomenon is as yet unclear. Taken together, these findings indicate that epithelial cells actively participate in the allergic immune response.

Whereas in vivo both eosinophils and neutrophils infiltrate the lungs of asthmatic patients during the late-phase response, to date the in vitro migratory behavior of these cell types has always been studied separately. In the present study, we investigated the influence of neutrophils on eosinophil migration in vitro across a confluent monolayer of lung H292 epithelial cell-line cells. Eosinophil transepithelial migration towards complement fragment 5a (C5a) proved to be strongly enhanced by the presence of neutrophils. The results suggest that human neutrophils are able to maintain the steepness of the C5a chemotactic gradient across an epithelial monolayer by inactivating diffused C5a, which results in favored eosinophil migration.
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Materials and Methods

Reagents

PAF, C5a and carboxypeptidase B were purchased from Sigma Chemical Co. (St. Louis, MO). Recombinant human (rh) RANTES was obtained from Gibco Life Technologies (Gaithersburg, MD). RhIL-5 were bought from Pepro Tech Inc. (Rocky Hill, NJ), and rhIL-8 from Boehringer Mannheim (Mannheim, Germany). Monoclonal antibody (mAb) 1B4 against the β2-integrin was a generous gift of Dr. S.C. Silverstein (Columbia University, Dept. of Phys. & Cell Biophys., New York, NY). C5a, PAF, RANTES, IL-5 and IL-8 were dissolved in phosphate-buffered saline, pH 7.4, (PBS) supplemented with 0.5 % (w/v) human serum albumin (HSA) (Central Laboratory of the Netherlands Blood Transfusion Service (CLB) Amsterdam, The Netherlands) and stored at -20°C. HEPES medium contained 132 mM NaCl, 6.0 mM KCl, 1.0 mM CaCl2, 1.0 mM MgSO4, 1.2 mM KH2PO4, 20 mM HEPES, 5.5 mM glucose and 0.5 % (w/v) HSA (pH 7.4). Lysis buffer consisted of PBS supplemented with 0.1 % (v/v) Tween-20 (Merck, Schuchardt, Germany), 0.2 % (w/v) N-cetyl-N,N,N-trimethyl-ammoniumbromide (Sigma), 0.2 % (w/v) bovine serum albumin (BSA) (Sigma) and 20 mM EDTA.

Cell culture

The human cell line H292 derived from lung adenocarcinoma (American Type Culture Collection CRL 1848, Rockville, MD) (11) was grown in RPMI-1640 (Gibco) supplemented with 10 % (v/v) heat-inactivated fetal calf serum (FCS) (Gibco), 100 U/ml penicillin (Gibco), 100 µg/ml streptomycin (Gibco) and 2 mM glutamine (Merck). Primary human bronchial epithelial cells (HBEC) were obtained from bronchial tissues with microscopically normal appearance from patients undergoing thoracotomy. The HBEC cells were cultured as described (10). The 4th to 30th passages of H292 cells and the 3rd to 4th passages of HBEC were used for the transmigration assay.

To study the eosinophil migration across epithelial monolayers in the physiological direction, epithelial cells were routinely cultured on the bottom side of Transwell polycarbonate membranes (3.0 μm pore size, 12 mm diameter) (Costar, Cambridge, MA), as described (12). Complete confluence of the epithelial cell monolayers was reached prior to their use in experiments, as determined by May-Grünwald/Giemsa staining and light microscopy. The confluence of the monolayers was confirmed by [3H]-inulin leakage experiments, as described (13).

Granulocyte isolation

Blood was obtained from healthy volunteers. Granulocytes were purified from a buffy coat of 500 ml of blood by density gradient centrifugation over isotonic Percoll, as described (14). After lysis of the erythrocytes in the pellet fraction with a cold lysis buffer containing 155 mM NH4Cl, 10 mM KHCO3 and 0.1 mM EDTA (pH 7.4), the granulocytes were washed twice in PBS. The granulocyte cell suspension consisted for more than 95 % of neutrophils. This cell suspension was used for the isolation of eosinophils.

Eosinophil purification

Human eosinophils were purified via the formyl-methionyl-leucyl-phenylalanine (fMLP) method (15). In brief, the granulocytes, resuspended in HEPES medium without CaCl2, were incubated for 30 min at 37°C. The cells were washed, resuspended in PBS supplemented with 0.5 % HSA (w/v) and 13 mM trisodium citrate, and incubated for 5 min at 37°C. The incubation was continued for precisely 10 min after the addition of 10 nM fMLP. Thereafter, the eosinophils were purified by centrifugation (15 min, 1000x g) over isotonic Percoll (1.082 g/ml, pH 7.4), washed and resuspended in HEPES medium. The purity and viability of the eosinophils were more than 95 %. This procedure leads to the isolation of relatively unprimed eosinophils compared to conventionally used isolation procedures with immunomagnetic beads (16).

Eosinophil transmigration assay

Fresh medium was added to the Transwells 4 hours prior to the start of the transmigration assay, and the Transwells were washed twice with HEPES medium just before starting the experiment. The lower compartment was filled with pre-warmed HEPES medium with or without individual chemoattractants.
The final concentrations of the chemoattractants were: C5a $10^{-8}$ M, PAF $10^{-6}$ M, RANTES 50 ng/ml and IL-8 $10^{-8}$ M. Eosinophils were labeled with Calcein-AM (Molecular Probes) before the onset of the transmigration assay (17). The eosinophils ($10^6$/ml) were labeled with 4 μg/ml Calcein-AM diluted in HEPES medium for 30 min at 37°C. After labeling, the eosinophils were washed 2 times with HEPES medium. Where indicated, purified eosinophils ($2\times10^6$ cells/ml) were primed with $10^{-10}$ M IL-5 in HEPES medium for 30 min at 37°C, and were washed in incubation medium. Where indicated, neutrophils were pre-treated with 10 μg/ml anti-$\beta_2$-integrin mAb for 30 min or pre-treated with 10 μM Jasplakinolide (Molecular Probes, Eugene, OR) for 15 min in HEPES medium, and the neutrophils were washed twice before addition to the eosinophils.

Eosinophils with or without neutrophils ($5\times10^5$ cells of either cell type were suspended separately or together in 0.5 ml of HEPES medium) were placed in the upper compartment. The Transwells were incubated for 1.5 h at 37°C with 5 % CO$_2$ and maximal humidity. After the incubation, the upper and lower compartments were washed with HEPES medium and lysis buffer, respectively, and the fluids were collected and the membranes were excised. The cells in the collected fluids and in the excised membranes were lysed in lysis buffer. The extent of eosinophil transmigration was quantified by means of fluorescence measurement, i.e. the concentrations of Calcein-AM in the upper compartment, lower compartment and membrane were measured with a spectrofluorometer (Model RF-540, Shimadzu Corporation, Kyoto, Japan). In this manner the migration of eosinophils is measured, and not the migration of added neutrophils, because the neutrophils were not labeled with Calcein-AM. The percentage of eosinophils that had transmigrated was calculated from the amount of fluorescence detected in the lower compartment in relation to the fluorescence of the originally added Calcein-AM-labeled eosinophils. The specificity of the calcein labeling for the detection of eosinophils was confirmed by specific quantification of eosinophils in the different compartments and the input fraction by means of an ELISA for eosinophil cationic protein (ECP).

Neutrophil transmigration assay
The assay is similar to the assay described for eosinophil transmigration except for the fact that the Transwells were incubated for 40 min in stead of 1.5 h. Where indicated, neutrophils were pre-treated with 10 μg/ml anti-$\beta_2$-integrin mAb for 30 min or pre-treated with 10 μM Jasplakinolide for 15 min in HEPES medium at 37°C and washed twice before placement in the upper compartment.

Measurement of the intracellular free Ca$^{2+}$ concentration ([Ca$^{2+}$])
For the [Ca$^{2+}$] measurement, human neutrophils ($6-10 \times 10^6$/ml in HEPES medium) were loaded with indo-1/AM (Molecular Probes) for 40 min at 37°C (18). The cells were washed, resuspended in HEPES medium to the previous concentration, and kept in the dark at room temperature. Before being transferred to a cuvette, the cells loaded with indo-1/AM were diluted 10 times in HEPES medium and were prewarmed for 5 min at 37°C. Fluorescence changes in the cells, magnetically stirred and kept at 37°C, were monitored with a spectrofluorometer (Model RF-540, Shimadzu Corporation), with 340 and 390 nm as excitation and emission wavelengths, respectively. To calibrate the indo-1 fluorescence as a function of [Ca$^{2+}$], all trappedindo-1 was saturated with Ca$^{2+}$ by addition of digitonin (10 mM), after which the indo-1 fluorescence was quenched with MnCl$_2$ (0.5 mM). A dissociation constant of 250 nM for the indo-1-Ca$^{2+}$ complex was used to calculate [Ca$^{2+}$] (19).

Statistical analysis
The results were expressed as the mean ± SEM of the number of different experiments mentioned in the legends and analyzed with the Student's t-test. Two-sided p values were calculated, and p values exceeding 0.05 were considered not to be significant.
Results

Human neutrophils enhance eosinophil transepithelial migration towards C5a

We investigated the influence of neutrophils on eosinophil transepithelial migration. The migration of human eosinophils across monolayers of lung H292 epithelial cells (Fig. 1a) towards chemoattractants, e.g. RANTES, IL-8, PAF or C5a, was relatively low (2% to 6%). Priming of the eosinophils resulted in a small increase in migration towards C5a (Fig. 1b). Surprisingly, the migration of both unprimed and IL-5-primed eosinophils towards C5a, but not towards RANTES, IL-8, or PAF was greatly enhanced when human neutrophils were added to the eosinophils (Fig. 1a). Moreover, preliminary results suggested that eotaxin-driven migration of eosinophils is not stimulated by neutrophils either. Thus, the neutrophils stimulate eosinophil migration selectively towards C5a.

![Figure 1](image_url)

**Figure 1.** Migration of eosinophils with or without neutrophils across H292 epithelial cell monolayers.

(a) Transmigration of IL-5-primed eosinophils with (solid bars) or without (open bars) neutrophils towards HEPES medium, RANTES (50 ng/ml), IL-8 (10^{-8} M), PAF (10^{-9} M) or C5a (10^{-9} M). The C5a-driven eosinophil migration with neutrophils was higher than without neutrophils (p<0.0001). Data are mean ± SEM of 4 different experiments. (b) Transmigration of unprimed and IL-5-primed eosinophils with (solid bars) or without (open bars) neutrophils towards C5a. The IL-5-primed eosinophil migration was higher than the migration of unprimed eosinophils in the presence of neutrophils (p<0.05). Data are mean ± SEM of 5 experiments.

The stimulatory effect of neutrophils was more pronounced with IL-5-primed eosinophils than with unprimed eosinophils (Fig. 1b). Furthermore, the effect was dependent on the number of neutrophils added (Fig. 2) and on the concentration of C5a, i.e. the effect was only observed when 10^8 M C5a was used. Eosinophil migration towards 10^{-7} M C5a was hardly and that towards 10^{-9} M C5a was not enhanced by neutrophils (data not shown). In addition, this effect was not only observed when lung H292 epithelial monolayers were used, but also when monolayers of primary cultured human bronchial epithelial cells (HBEC) were used. C5a-driven migration of IL-5-primed eosinophils across HBEC monolayers was higher with neutrophils than without neutrophils (33 % and 18 %, respectively). C5a-driven eosinophil migration across monolayers of primary human umbilical cord vascular endothelial cells (HUVECs) was also augmented by neutrophils, and was also found to be dependent of the concentration of C5a (optimum at 10^8 C5a) in the lower compartment (data not shown).
Migration of neutrophils is not required for enhancement of C5a-driven eosinophil transepithelial migration

Neutrophils migrate more rapidly than eosinophils across lung epithelial monolayers. Thus, when neutrophils and eosinophils are both present, neutrophils will migrate ahead of eosinophils. Possibly, the neutrophils induce changes in the epithelium or simply clear the way for the cells behind, resulting in enhancement of the subsequent migration of eosinophils. We therefore investigated whether or not previous neutrophil passage has an impact on eosinophil migration.

Two different approaches were taken. First, we assessed eosinophil migration through epithelial monolayers that had previously been crossed by neutrophils. When neutrophils were allowed to migrate towards C5a during 30 min before addition of IL-5-primed eosinophils, the eosinophil migration was not enhanced (data not shown). The migration was also unaffected when the neutrophils were removed, by means of washing of the Transwells, after a 30-min transmigration period and before addition of the eosinophils (Fig. 3). In addition, neutrophil passage did not seem to damage the monolayers, because both the morphology and the permeability of the monolayers were unaltered after neutrophil passage, as assessed by means of confocal microscopic analysis and FITC-conjugated Dextran leakage experiments (data not shown). Second, we investigated the influence of neutrophils with impaired migratory capacity on eosinophil transepithelial migration. For this purpose, neutrophils were pre-treated with anti-ß₂-integrin mAb, resulting in impaired C5a-driven transepithelial migration (Fig. 4a). These immobilized neutrophils were found to be equally capable of enhancing eosinophil transepithelial migration towards C5a as were untreated neutrophils (Fig. 4b). In parallel, pre-treatment of neutrophils with the actin-polymerizing agent Jaspelinolide (20) completely blocked migration towards C5a (Fig. 4a), whereas eosinophil migration was equally enhanced as with untreated neutrophils (Fig. 4c). Taken together, these results indicate that previous neutrophil passage does not facilitate subsequent eosinophil migration, and that the stimulatory effect of neutrophils is independent of neutrophil migration.

Figure 2. Migration of IL-5-primed eosinophils with increasing numbers of neutrophils across H292 epithelial cell monolayers. PAF and C5a were used as chemoattractants. The eosinophil migration was measured without neutrophils (open bars), with 0.2x10⁶ neutrophils/ml (hatched bars) and with 10⁶ neutrophils/ml (solid bars).

Figure 3. Migration of IL-5-primed eosinophils with neutrophils (solid bars) or after previous neutrophil transmigration (open bars) across H292 epithelial cell monolayers. In the latter situation, neutrophils were placed in the upper compartment and incubated for 30 min, followed by washing of both the upper and the lower compartments with HEPES medium, and addition of fresh HEPES medium and chemoattractants. PAF and C5a were used as chemoattractants. The eosinophil migration towards C5a was higher when neutrophils migrated simultaneously than when neutrophils had migrated previously (P<0.01). Data shown are mean ± SEM of 3 different experiments.
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**Figure 4.** Neutrophil and IL-5-primed eosinophil migration across H292 epithelial cell monolayers towards C5a after neutrophil pre-treatment with β2-integrin blocking mAb (CD18) or Jasplakinolide (JP). (a) Migration of neutrophils was measured without pre-treatment and after pre-treatment with CD18 or Jasplakinolide. (b) Migration of eosinophils was measured without neutrophils (PMN), with untreated neutrophils and with CD18-pre-treated neutrophils. Data are shown as a mean + SEM of 3 different experiments. (c) Migration of eosinophils was measured without neutrophils, with untreated neutrophils and with Jasplakinolide-pre-treated neutrophils. Data are shown as mean + SEM of 3 different experiments.

Neutrophils do not release mediators that stimulate C5a-driven eosinophil transepithelial migration

Another possible explanation is that neutrophils secrete mediators that enhance eosinophil transepithelial migration. For instance, neutrophils produce and release PAF after activation (21). Yet, addition of the specific PAF-receptor antagonist WEB 2086 did not diminish the C5a-driven transepithelial migration of IL-5-primed eosinophils with or without neutrophils, no matter whether neutrophils and eosinophils, epithelial cells, or all three cell types were incubated with WEB 2086 (data not shown). In contrast, this antagonist completely blocks PAF-driven eosinophil and neutrophil migration across epithelial cells (10, 22). Thus, PAF seems not be such a mediator.

To unravel whether other secreted mediators enhance eosinophil migration, the influence of neutrophil supernatant on eosinophil migration was assessed. Neutrophils were allowed to migrate towards C5a for 30 min, and the content of the upper compartment was collected, cleared from neutrophils by centrifugation, and used in the transmigration assay. It was found that suspending the IL-5-primed eosinophils in this supernatant did not result in augmentation of transepithelial migration towards C5a. Thus, the stimulatory effect of neutrophils on eosinophil transepithelial migration towards C5a seems not to be mediated via factors secreted by neutrophils, nor via factors secreted by epithelial cells in response to interaction with neutrophils.
Migration of IL-5-primed eosinophils across H292 epithelial monolayers towards HEPES medium (open bar) and towards C5a without (ctrl) or with 250 ng/ml carboxypeptidase B (CP), 5% (v/v) human AB serum (HS) or neutrophils (PMN) added to the upper compartment (solid bars). C5a-driven eosinophil migration was significantly enhanced by carboxypeptidase, human serum and neutrophils (p<0.005). Data are shown as mean ± SEM of 5 different experiments.

Inactivation of C5a by neutrophils may account for the efficient eosinophil transmigration towards C5a. Neutrophils enhance eosinophil migration when the neutrophils were placed in the upper compartment. However, when neutrophils were placed in the lower compartment, eosinophil migration was potently inhibited (94% inhibition). Likewise, addition of human serum to the upper compartment strongly enhanced migration of eosinophils towards C5a (Fig. 5), and serum in the lower compartment potently inhibited eosinophil transepithelial migration towards C5a (88% inhibition). Moreover, in the presence of neutrophils, addition of serum to the upper compartment did not further promote C5a-driven eosinophil migration, yet serum in the lower compartment still blocked eosinophil migration (88% inhibition).

A common feature of neutrophils and human serum is the capacity to degrade or neutralize C5a. When C5a was incubated with either neutrophils or serum, its biological activity was rapidly destroyed; i.e. the solution no longer induced an intracellular calcium response in neutrophils (Fig. 6a & 6b).

Eosinophils inactivate C5a not as efficiently as neutrophils, i.e. eosinophil incubation with C5a hardly decreased its biological activity (Fig. 6b). Thus, the inhibitory effect of neutrophils and serum when placed in the lower compartment may be explained by destruction of the C5a chemotactic gradient. Consequently, the stimulatory effect on eosinophil migration may be explained by maintenance of the C5a chemotactic gradient. In other words, neutrophils or serum degrade diffused C5a in the upper compartment and support in this manner the steepness of the C5a chemotactic gradient. This hypothesis is in line with the observation that neutrophils only enhance migration towards C5a, and not towards other chemoattractants. Furthermore, the C5a-degrading enzyme carboxypeptidase B (Fig. 6a) also promoted C5a-driven eosinophil transepithelial migration when added to the eosinophils (Fig. 5) and inhibited migration when added to the lower compartment (83% inhibition). The degrading effect of carboxypeptidase B was specific for C5a, because incubation with other chemotactic agents, e.g. PAF, IL-8 and fMLP did not affect the intracellular calcium response in neutrophils induced by these agents and had no effect on the chemotactic response of eosinophils towards these agents (data not shown).
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Figure 6. (a) Degradation of C5a by human serum or carboxypeptidase B. 10^{-8} M C5a was incubated for 30 min at 37°C with or without 5% human serum or 250 ng/ml carboxypeptidase B in HEPES medium. Thereafter, the solutions were added to Indo-AM-loaded neutrophils and the transient rise in [Ca^{2+}]_i was measured. Trace A, C5a incubated without agent; trace B, C5a incubated with human serum; trace C, C5a incubated with carboxypeptidase B. This figure is representative of 2 different experiments performed. (b) Degradation of C5a by IL-5-primed eosinophils or neutrophils. C5a (10^{-8} M) was incubated for 30 min at 37°C with or without eosinophils (10^5/ml) or neutrophils (10^6/ml) in HEPES medium. Thereafter, the granulocytes were spun down, the supernatant was added to Indo-AM-loaded neutrophils, and the transient rise in [Ca^{2+}]_i was measured. Trace A, C5a incubated without cells; trace B, C5a incubated with eosinophils; trace C, C5a incubated with neutrophils. This figure is representative of 3 different experiments performed.
Chapter III

Discussion

Mechanism of neutrophil enhancement of eosinophil transepithelial migration

From our studies, it appeared that neutrophils enhance transepithelial migration of eosinophils selectively towards C5a. The enhancement of eosinophil migration was not due to damage inflicted upon the epithelial cells by toxic agents released by neutrophils. The integrity of the monolayers after a 1.5-hour transmigration experiment was not diminished i.e. the monolayers remained microscopically confluent and both the morphology and the permeability were not altered.

The stimulatory effect of neutrophils on eosinophil migration was independent of transmigration of neutrophils, i.e. the enhancement also occurred when neutrophil migration was prevented. The effect was also found to be independent of production of migration-promoting mediators, although our failure to detect an effect of supernatant from C5a-treated neutrophils may be due to relative instability of such mediators.

The stimulatory effect of neutrophils seems to be due to maintenance of the C5a chemotactic gradient by inactivation of C5a that has diffused into the upper compartment. Eosinophil migration is rather slow, i.e. the plateau phase is reached after 1.5 h of incubation, whereas neutrophil migration already levels off after 30 min of incubation. This implies that eosinophil migration will be relatively sensitive to the steady decrease of the chemotactic gradient by diffusion of the chemoattractant into the upper compartment. When neutrophils are added to the upper compartment, the steepness of the gradient will be maintained, and the attraction of eosinophils towards C5a will be more potent during a longer period of time. This last conclusion is based on the following arguments: 1. We found that neutrophils inactivate C5a more efficiently than eosinophils do. 2. The stimulatory effect of neutrophils was mimicked by specific C5a-degrading agents, i.e. human serum (23;24) or carboxypeptidase B (25). 3. Addition of one of these C5a degraders to the lower compartment caused reduction of eosinophil migration towards C5a, presumably due to destruction of the chemotactic gradient. 4. The stimulatory effect was optimal when 10$^{-8}$ M C5a was used, indicating that the concentration of C5a is essential for the stimulatory effect.

Stimulation of eosinophil migration by neutrophils appears to be restricted to migration across confluent epithelial or endothelial cell monolayers. C5a-driven eosinophil migration across bare filters was not enhanced by neutrophils, i.e. the eosinophil migration was unaffected by the presence of neutrophils, also when different concentrations of C5a were added to the lower compartment (data not shown). The confluent cellular monolayer undoubtedly forms a barrier that reduces the diffusion of molecules from the lower to the upper compartment. The capacity of neutrophils to neutralize C5a may be sufficient to maintain a proper C5a gradient when little C5a diffuses into the upper compartment, e.g. when a cellular barrier prevents diffusion. However, the neutrophils may not be capable of effectively maintaining a proper C5a gradient when the diffusion is higher, e.g. when bare filters are used. Consequently, it can be expected that whether or not neutrophils enhance eosinophil migration may be dependent of the ability of C5a to cross the barrier and of the capacity of neutrophils to degrade C5a in the upper compartment.

It is as yet unclear how neutrophils neutralize C5a in this experimental set-up. The neutralization seems not to take place via secretion of soluble proteases because neutrophil supernatant did not enhance eosinophil migration. However, it is possible that the C5a degradation takes place at neutrophil membranes, or that C5a is ingested by neutrophils. In an attempt to clarify this notion, we have tried to inhibit the proteolytic activity of neutrophils with a specific carboxypeptidase inhibitor, but addition of this agent greatly enhanced the neutrophil adhesion to the monolayer in the absence of any chemoattractant and was therefore considered unsuitable for functional experiments.

In vivo, C5a is implicated in the inflammatory reaction in lungs of allergic asthmatic patients, i.e. C5a presumably acts as a chemoattractant and activator of both eosinophils and neutrophils, and C5a induces contraction of smooth muscle and enhances vascular permeability (26). Moreover, it has been shown that bronchial asthmatic lavage fluid contains C5a (4) and that complement fragments, such as C5a, are locally generated after exposure of the airway mucosa to mediators implicated in asthma (26). C5a is generated by means of cleavage of complement component C5. C5 can be cleaved by C5-
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convertase after activation of the classical or alternative pathway of complement, and by serine proteases contained in neutrophil granules (27).

During the late phase of an allergic asthmatic reaction, neutrophils migrate ahead of eosinophils (2) and thus arrive earlier in the lung lumen. Peripheral and invading neutrophils presumably neutralize C5a in the surrounding tissue and vasculature resulting in maintenance of the C5a chemotactic gradient. In this way, neutrophils may enhance attraction of eosinophils towards the site of inflammation. The stimulatory effect of neutrophils on eosinophil recruitment may be of physiological importance in situations of eosinophil influx into inflamed tissues, as in allergic inflammations of nose, gut or lung. Therapeutic approaches aimed at decreasing tissue damage during allergic inflammation must therefore take into account not only eosinophil influx but also neutrophil influx into the tissues.

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References


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