Migration of Primed Human Eosinophils Across Cytokine-Activated Endothelial Cell Monolayers

By René Moser, Jorg Fehr, Licia Olgiati, and Piet L.B. Bruijnzael

Eosinophils are known to adhere to cytokine-activated endothelium. Whereas transendothelial migration for neutrophils is an inevitable consequence of this endothelial-dependent adherence, this has not yet been shown for eosinophils. By means of human umbilical vein endothelial cells (HUVE) grown to confluence on microporous filters as an in vitro model of leukocytic migration across postcapillary venules, we have characterized the conditions leading to endothelium-driven transmigration of blood eosinophils from normals and from patients with allergic asthma. Freshly isolated eosinophils from nonallergic donors adhered to interleukin-1 (IL-1) and tumor necrosis factor-activated HUVE, but did not penetrate these monolayers. In contrast, eosinophils from allergic asthma patients showed an increased adherence and transmigration capacity. This increased functional competence was not caused by a difference in density phenotype, because the eosinophils from both groups showed a comparable density distribution over discontinuous Percoll gradients. Moreover, no difference existed within one group among eosinophils harvested from the Percoll density bands 1.080, 1.085, and 1.090 g/mL in terms of transendothelial migration. In vitro cultivation of freshly isolated eosinophils from nonallergic individuals in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-3 induced a stepwise decrease of the density distribution over such gradients. In contrast, eosinophils from patients with allergic asthma directly shifted to a final density of 1.075 g/mL within 24 hours of culture. Notwithstanding the kinetics of density changes, eosinophils from nonallergic donors already expressed the capacity to transmigrate IL-1-activated HUVE monolayers 20 hours after cultivation with different combinations of GM-CSF, IL-3, and IL-5. Inhibition studies with monoclonal antibodies showed that endothelium-driven transmigration of eosinophils predominantly implicates CD11/CD18 structures on the eosinophil surface, whereas no significant inhibition was found with the anti-VLA-4 monoclonal antibody HP2/1. From cytofluorometric studies, we conclude that spontaneous transmigration of eosinophils from allergic asthma patients is not accompanied by quantitative upregulation of these antigens. Taken together, these results allow the conclusion that blood eosinophils from allergic asthma patients have undergone in vivo priming, mimicked in vitro by cytokines such as GM-CSF, IL-3, and IL-5, leading to induction of the capacity to migrate across cytokine-activated HUVE monolayers.

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MATERIALS AND METHODS

Subjects. All allergic asthmatic subjects participating in this study met the American Thoracic Society definition of Asthma, including episodic airway obstruction and hyperresponsiveness to bronchoprovocation with histamine. At the time of the study all patients were asymptomatic. All allergic asthma patients had blood eosinophilia ranging between 7.9% and 18.5%, showed positive intracutaneous skin reactions to several allergens, and elevated specific IgE levels to the allergens used in the intracutaneous skin tests. During the study, the allergic asthma patients were under maintenance therapy. They had not received steroids (systemic or topical) for at least 2 weeks. They had stopped the intake of sodium cromoglycate, theophylline, and p-mimetics at least 12 hours before blood sampling. The normal healthy volunteers had no history of allergy or asthma and did not take any kind of medication before and at the time of blood sampling. All participating individuals gave their informed consent.

Preparation of cultured cells. Endothelial cells from human umbilical cord veins were harvested as reported elsewhere.8 Confluent primary cultures were trypsinized, seeded in a 1:3 split ratio on human fibronectin (4 μg/cm²; Collaborative Research, Inc, St Waltham, MA), and grown to confluence in Medium 199 enriched with sodium hepamin (90 μg/mL; Novo Industries, Copenhagen, Denmark), endothelial cell growth supplement (15 μg/mL; Collaborative Research), and 20% human serum as described.9 Final monolayers to study eosinophil-endothelial interactions were grown on 24-well plates (Falcon Labware, Becton Dickinson & Co, Oxnard, CA) and on our modified Boyden chamber system (Plastic Petica Inc, Chur, Switzerland), exactly as described.10 Confluent monolayers were used in their second to fourth passage and were routinely tested for exhibition of cytoplasmic factor VIII von Willebrand activity by indirect immunofluorescence with rabbit antihuman factor VIII antibody.

Purification of eosinophils. Heparinized (20 U/mL blood; Novo Industries) whole blood (50 to 400 mL) either from nonallergic individuals or from patients with allergic asthma was processed exactly as described for the separation of neutrophils.21 In brief, 20 mL of blood was layered onto 15 mL methocel-metrizoate (Methocel, MC 25C; Fluka AG, Buchs, Switzerland; Isopaque 440; Nyegaard & Co, Oslo, Norway), erythrocytes were sedimented at 1,000 g for 20 minutes at room temperature and counted by means of a counting chamber (Neubauer). As extensively described elsewhere,24 the eosinophils isolated in this way were not primed or activated.

As to exclude that activation of the eosinophils from allergic asthma patients would take place by FMLP pretreatment (under Ca2+-free conditions) during the isolation procedure, eosinophils were also isolated according to Yazdanbakhsh et al.25 This isolation uses two isotonic Percoll densities consecutively, i.e., 1.082 and 1.085 g/mL, in the same way as described above. In this isolation procedure, eosinophils of a purity greater than 90% may be harvested from the band under the Percoll layer with a density of 1.085 g/mL, although with a smaller recovery as with the method described above (20% to 40%). These eosinophils have been incubated with and without FMLP (10 nmol/L) for 30 minutes at 37°C, and the adherence and transendothelial migration has been measured (see Results).

Cultivation of eosinophils. Eosinophils (1 to 2 x 10⁶/mL) suspended in Iscove’s medium containing 20% of FCS were cultured up to 5 days at 37°C and 5% CO₂/saturated humidity as described20,21 by means of different combinations of recombinant human GM-CSF (11.5 x 10⁶ U/mg; Sandoz Lid, Basel, Switzerland), IL-3 (5 x 10⁵ U/mg; Sandoz Lid), and IL-5 (4 x 10⁵ U/mL; Dr C. Sanderson, London, UK) at a final concentration of 10 nmol/L. After cultivation, the eosinophils were layered onto an isotonic discontinuous Percoll gradient (layers, 1.070 and 1.075 g/mL). The eosinophils accumulating between these layers were collected, washed once with Iscove’s, and were resuspended in Iscove’s medium containing 20% of FCS. They always had a viability of greater than 95%.

Control for the possible loss of hypodense eosinophils due to the isolation procedure. Cultured eosinophils (10 x 10⁶ cells) with a density of 1.075 g/mL were added to 10 mL of heparinized whole blood from a nonallergic healthy individual. Ten milliliters of heparinized whole blood from the same volunteer was taken as control. Granulocytes were separated from the two specimens exactly as described above. One milliliter of the granulocyte suspension (30 x 10⁶/mL) was layered onto an isotonic discontinuous Percoll gradient consisting of the following densities: 1.100, 1.095, 1.090, 1.085, 1.080, 1.075, and 1.070 g/mL. The various bands in both gradients were collected, and the cell number and purity by means of cytocentrifuge preparation was determined.

Quantification of eosinophil adherence. HUVE grown to confluence on 24-well plates were washed twice with 1 mL of HBSS. Except where stated specifically, HUVE monolayers were preincubated with 1 mL of either normal culture medium or medium containing 5 U/mL of human recombinant IL-1α (Roche Inc, Nutley, NJ) and 10 ng/mL TNF (Cetus Corp, Emeryville, CA). Monolayers were then washed twice with 1 mL of HBSS. Thereafter, 500 μL/well of the eosinophil suspension (10⁶/mL) was layered onto the washed HUVE monolayer. After incubation for 30 minutes at 37°C and saturated humidity/5% CO₂, the monolayers were subjected to a high-shear stress wash procedure. The 24-well plate was three times repeatedly submerged in a bath of 300 mL PBS to discriminate between loosely and tightly attached eosinophils. The plate was then dried at 4°C and the number of adhering eosinophils was quantified by measuring extracted eosinophil peroxidase activity as described.23,27 Results of adhering eosinophils are expressed as the percentage of initially applied cells.

Eosinophil migration through endothelial cell monolayers. Nucleopore polycarbonate membrane filters (5 μm pore size; Nuclepore
Corp, Pleasanton, CA) mounted on modified Boyden chambers with an inner diameter of 9 mm were overgrown by HUVE. One week after seeding, the integrity of the monolayer was tested by toluidine blue staining followed by microscopic control. Transendothelial migration was performed as described, except that the cultures were preincubated with Iscove’s medium containing 20% of FCS alone (control) or together with IL-1 (5 U/mL) or TNF (10 ng/mL) for indicated periods of time at 37°C. Thereafter, the cultures were washed twice with Iscove’s and eosinophils 0.5 × 10⁶ suspended in 1 mL of Iscove’s 20% FCS were added to the upper compartment and were allowed to migrate for 90 minutes. Transendothelial migration was terminated by the addition of 25 μL of 1 mol/L EDTA to the upper and lower compartment followed by vibration, exactly as described. The number of migrated eosinophils, collected from the lower compartment, was counted manually in a blind fashion using a Neubauer counting chamber and was expressed as percent of migrated eosinophils in relation to the number of cells initially added to the upper compartment. In certain experiments, endothelial activation was performed together with actinomycin D (1 μg/mL; Sigma Chemical Co, St Louis, MO), which was solubilized and further diluted in PBS and added to the incubation medium at a final dilution of 1:100 (vol/vol). Experiments were also performed using the monoclonal antibodies (MoAbs) IB-4, HP2/1, and W6/32 at saturating concentrations of 20 μg/mL to show the dependence on CD11/CD18 and VLA-4 of the eosinophil transendothelial migration across cytokine-activated HUVE monolayers. In these experiments, the eosinophils were incubated at room temperature for 30 minutes before coincubation with HUVE monolayers.

Cytocentrifugation. Cytocentrifugation studies were performed essentially as described. Eosinophils (5 × 10⁶) were washed once and resuspended in 50 μL of ice-cold HBSS-A containing the primary antibody. Incubation was continued for 60 minutes at 4°C. After two washes with ice-cold HBSS-A, the cells were reincubated for 30 minutes in 50 μL of the same buffer containing fluorescein-conjugated goat antimouse-IgG antibody (Tago, Inc, Burlingame, CA) at a saturating concentration of the secondary antibody. The cells were washed twice again, controlled for vitality (trypan-blue exclusion: >91% unstained), and fixed in 1% paraformaldehyde and stored in HBSS-A at 4°C overnight. Cytocentrifugation was performed within 12 hours using a FACSscan HP310 (Becton Dickinson, San Jose, CA). Fluorescence intensity was calculated directly on a linear scale and is reported herein as the mean fluorescent channel of cells that had been reacted with a dilution of 1:100 units of MoAb with a known concentration. Specific fluorescence determination. Specific fluorescence was determined directly on a linear scale and is reported herein as the mean fluorescent channel of cells that had been reacted with a dilution of 1:100 units of MoAb with a known concentration. Statistical analysis. Where not specifically indicated, statistical analysis was performed using the Student’s two-tailed t-test for unpaired observations.

RESULTS

Density distribution of blood eosinophils over isotonic discontinuous Percoll gradients. Freshly isolated eosinophils from the blood of nonallergic individuals and allergic asthma patients were layered onto isotonic discontinuous Percoll gradients. The percentage of eosinophils recovered from the various bands is shown in Fig 1. Eosinophils from the circulation of nonallergic individuals (Fig 1A) and of patients with allergic asthma (Fig 1C) showed a similar density distribution. When the cells are cultured in Iscove’s medium supplemented with 20% FCS, 10 pmol/L of IL-3, and 10 pmol/L of GM-CSF for 5 days, their density distribution pattern over Percoll gradients changed (Fig 1B and D). Almost all eosinophils had turned into low-density eosinophils (d = 1.075 g/mL). The upper band of the gradient (<1.070 g/mL) contained mostly dead cells. Therefore, this band was not taken into account in calculating the percent of eosinophils recovered from the various density bands. Eosinophils with a density of 1.075 g/mL always contained over 90% viable cells and were used for adherence and transmigration experiments. The kinetics of the change in density during cultivation showed that eosinophils from patients with allergic asthma changed to a final density of 1.075 g/mL within 24 hours after culture, whereas eosinophils from nonallergic individuals changed gradually (Fig 2). Regarding vitality of the eosinophils after 5 days of cultivation, consistently more dead eosinophils accumulated in the density band less than 1.070 g/mL in cases of allergic asthma patients (~30%) than in cases of nonallergic individuals (~10%), although they were cultured under exactly the same conditions.

Adherence of blood eosinophils to nonactivated and IL-1-activated HUVE monolayers. We have compared the adherence of blood eosinophils from nonallergic and asthmatic subjects with IL-1-activated HUVE monolayers. In addition, the adherence capacity of eosinophils from those groups, cultured for 5 days in the presence of IL-3 and GM-CSF (both at 10 pmol/L), was investigated. Initially, the adhesive response of eosinophils to activation of the endothelial monolayer for 4 hours with different cytokines such as interferon-γ (IFN-γ; 1,000 U/mL), IL-3 (1,000 U/mL), IL-5 (100 pmol/mL), and GM-CSF (100 pmol/mL), IL-1 (5 U/mL), and TNF (10 ng/mL) was studied. Significant tight adhesion was found with IL-1 and TNF only (IL-1, 57.1 ± 4.8; TNF, 50.8 ± 2.3; control, 3.8 ± 1.3; results are means ± SD of triplicate determinations of one representative experiment of a series of three). The responses to other cytokines were similar to the control value. So far, these data confirm the findings of others. Compared with eosinophils from a group of nonallergic donors (n = 6), those from patients with allergic asthma (n = 6), despite similar density distribution (Fig 1A and C), showed a significantly enhanced capacity to adhere to IL-1-activated HUVE monolayers (Fig 3A; P < .0005). This enhanced adherence capacity did not change after in vitro cultivation together with IL-3 and GM-CSF for 5 days (Fig 3B).
Fig 1. Density distribution profiles over discontinuous isotonic Percoll gradients of eosinophils isolated from peripheral blood of nonallergic donors (A) and patients with allergic asthma (C). Density distribution pattern of the above eosinophils after cultivation for 5 days in the presence of GM-CSF and IL-3 (10 pmol/L) from nonallergic individuals (B) and from patients with allergic asthma (D). The percentage of eosinophils recovered from the various density bands is plotted versus Percoll density (g/mL). Each profile represents the distribution pattern of a different individual.

**Transmigration capacity of blood eosinophils across nonactivated and IL-1-activated HUVE monolayers.** By means of HUVE grown to confluence on the microporous membrane of a two-compartment migration chamber system (see Materials and Methods), the ultimate ability of adhering eosinophils to penetrate the monolayer was estimated. In this way, we have compared the transmigration capacity of eosinophils from nonallergic and asthmatic individuals either freshly isolated or after 5 days of cultivation with GM-CSF and IL-3. Surprisingly, transendothelial migration across nonactivated and IL-1-activated HUVE of eosinophils from nonallergic donors was low and not significantly different from controls (Fig 4A; Student’s paired t-test: $P = .06$). In contrast, freshly isolated eosinophils from patients with allergic asthma initially showed a significantly enhanced capacity to penetrate IL-1-activated monolayers (Fig 4A; $P < .0005$). When the eosinophils from nonallergic donors were cultured for 5 days in the presence of IL-3 and GM-CSF (10 pmol/L), there was a twofold increase in the capacity to migrate across IL-1-activated monolayers (Fig 4B; Student’s paired t-test: $P < .0005$). In case of allergic asthmatics, culture of the eosinophils did not further increase the originally enhanced capacity to transmigrate (Fig 4B; $P = .221$). Transmigration of eosinophils culminated between 4 to 6 hours of cytokine preincubation and persisted at a level of 70% after continuous stimulation with IL-1 or TNF (data not shown).

In a separate series of experiments, we showed that IL-3, IL-5, and GM-CSF are capable of inducing the ability of eosinophils from nonallergic individuals to migrate across IL-1-activated HUVE within 24 hours of cultivation. Com-
TRANSENDOTHELIAL MIGRATION OF EOSINOPHILS

pared with the response of noncultivated eosinophils, significant enhancement of transmigration was found after cultivation with the above cytokines alone or in combinations (Fig 5; \( P < .0005 \)). Time course experiments showed that the increased penetration of IL-1-activated HUVE monolayers could not be detected earlier than after 20 hours of cultivation (Fig 6). Again, it should be stressed that the density distribution of freshly isolated eosinophils from nonallergic and allergic asthmatic individuals were similar, although their transendothelial migration capacity was completely different. Because there always exists a certain density distribution of eosinophils over different Percoll density layers, a possible relation between decreased density and enhanced ability to cross the barrier of cytokine-activated endothelium was investigated. Eosinophils were isolated and layered onto a discontinuous Percoll gradient allowing the separation of eosinophils of the following densities: 1.090, 1.085, and 1.080 g/mL. Such eosinophils of defined density either from nonallergic or from allergic asthmatic individuals were compared for their transendothelial migration capacity (Table 1). These results confirm that eosinophils of various densities behave similarly with respect to transendothelial migration. The existence of functional heterogeneity certainly needs the exclusion of any modification of the eosinophils during separation. Thus, eosinophils from the circulation of allergic asthmatic individuals were isolated without the use of FMLP. The almost pure eosinophils were incubated as long as 30 minutes under calcium- and magnesium-free conditions at 37°C in the absence or presence of FMLP (10 nmol/L). After washing the cells, transendothelial migration across nonactivated HUVE (no eosinophil pretreatment: 5.0% ± 1.1%; FMLP pretreatment: 4.3% ± 0.6%) and IL-1-activated HUVE (no eosinophil pretreatment: 9.4% ± 2.5%; FMLP pretreatment: 10.2% ± 1.1%; results are means ± SD and are from one of three congruent experiments performed in triplicate) was determined. These results show that transendothelial migration of eosinophils is not affected by the use of FMLP during separation. As the density distribution of eosinophils from the blood of nonallergic donors and of patients with allergic asthma was identical, the question arose as to whether a possible
content of hypodense eosinophils was lost by the separation procedure. To exclude such a methodologic drawback, eosinophils were cultured for 5 days with IL-3 and GM-CSF and cells of a density of 1.075 g/mL were harvested from a discontinuous Percoll gradient and added to the whole blood of a nonallergic donor. Subsequent separation of the eosinophils on a Percoll discontinuous gradient showed a recovery of 85% (95% vitality) of the hypodense eosinophils of a density of 1.075 g/mL. Corroborated by the isolation of hypodense eosinophils from patients with hyper eosinophilic syndrome, the above data exclude a possible loss of hypodense eosinophils by the separation procedure.

Further experiments were performed to define the repertoire of adhesion molecules involved in IL-1- and TNF-provoked eosinophil transmigration. These experiments showed that MoAbs against CD18, the common β-structure of the β2-integrin family, strongly inhibited transmigration of eosinophils below the level of control (Table 2). However, no significant inhibition of transmigration across 4- and 18-hour preincubated HUVE was induced by the MoAb HP2/1 directed against the β1-integrin VLA-4 (Table 2). Keeping in mind that freshly isolated eosinophils from nonallergic donors, but not from allergic asthma patients, need in vitro priming to transmigrate, we asked the question whether such induction of transmigration would be reflected by quantitative upregulation of CD18 or VLA-4 on the eosinophil surface. Cytolymphometric determination of these antigens showed comparable expression of CD18 and VLA-4 in both groups (Table 3).

The eosinophil adherence to and transmigration across

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Table 1. Transendothelial Migration Across Nonactivated and IL-1–Activated HUVE Monolayers of Freshly Isolated Eosinophils With Different Densities Obtained From the Blood of Nonallergic Individuals and of Patients With Allergic Asthma

| Preincubation of HUVE Monolayer | Eosinophil Transmigration (%) | | | |
|---|---|---|---|
| | g/mL | g/mL | g/mL | |
| Nonallergic individuals | — | 5.7 ± 0.7 | 6.2 ± 0.3 | 4.0 ± 1.7 |
| | — | 6.5 ± 0.8 | 9.0 ± 1.1 | 5.3 ± 0.9 |
| Allergic asthma patients | — | 9.7 ± 2.0 | 8.8 ± 1.5 | 6.4 ± 0.7 |
| | — | 17.3 ± 2.6 | 17.8 ± 3.6 | 17.6 ± 4.6 |

Results are from one of three congruent experiments (means ± SD of triplicate determinations).

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Table 2. Effect of Anti-CD18 and Anti–VLA-4 MoAbs on Transendothelial Migration of Eosinophils From Nonallergic Donors After Cultivation for 3 Days With GM-CSF and IL-3

<table>
<thead>
<tr>
<th>Pretreatment of HUVE</th>
<th>MoAb</th>
<th>Eosinophil Transmigration (%)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>—</td>
<td>15.2 ± 1.7</td>
<td></td>
</tr>
<tr>
<td>IL-1 (4 h)</td>
<td>—</td>
<td>30.5 ± 2.1</td>
<td></td>
</tr>
<tr>
<td>IL-1 (4 h)</td>
<td>W6/32</td>
<td>28.9 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>IL-1 (4 h)</td>
<td>IB-4</td>
<td>7.1 ± 1.2*</td>
<td></td>
</tr>
<tr>
<td>IL-1 (18 h)</td>
<td>HP2/1</td>
<td>34.0 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>IL-1 (18 h)</td>
<td>—</td>
<td>30.2 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>IL-1 (18 h)</td>
<td>W6/32</td>
<td>30.9 ± 3.7</td>
<td></td>
</tr>
<tr>
<td>IL-1 (18 h)</td>
<td>IB-4</td>
<td>7.4 ± 1.6*</td>
<td></td>
</tr>
<tr>
<td>IL-1 (18 h)</td>
<td>HP2/1</td>
<td>29.8 ± 1.4</td>
<td></td>
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</tbody>
</table>

HUVE monolayers were pretreated with TNF (10 ng/mL) for 4 hours. Before the assay, the MoAb at 20 μg/mL was added to the eosinophil suspension and was incubated at room temperature for 30 minutes. Transendothelial migration was determined as described in Materials and Methods. Results are means ± SD from one of three congruent experiments using different batches of eosinophils and endothelial cells. Statistical analysis was performed by means of Student’s paired t-test within the 4- or 18-hour group of preincubation.

*Control experiments using MoAb W6/32 were compared with those using the MoAb IB-4 (P < .01).
cytokine-activated HUVE was of endothelial origin because addition of actinomycin D (0.1 μg/mL) during preincubation with IL-1 (5 U/mL) led to potent inhibition of the cytokine-provoked adherence (data not shown) and transmigration (HUVE: 10.6% ± 1.6%; HUVE + IL-1: 21.4% ± 1.4%; HUVE + IL-1 + actinomycin D: 9.5% ± 1.1%; one representative experiment of three; results depict means ± SD of triple determinations). In contrast, adherence and enhanced layer penetration of eosinophils was not inhibited by αa-methylprednisolone neither when added during the endothelial preincubation nor when it was together with the eosinophils. To answer the question if PAF might act as a final mediator of eosinophil transendothelial migration, the PAF antagonist WEB 2086 (1 μmol/L final concentration) was added to the eosinophil suspension before transendothelial migration across nonactivated and IL-1-activated HUVE (HUVE: 8.8% ± 1.2%; HUVE + IL-1: 18.9% ± 1.5%) and was compared with the effect of buffer alone instead of WEB 2086 (HUVE: 8.8% ± 0.8%; HUVE + IL-1: 18.9% ± 1.3%; results are means ± SD of triple determinations from one of two congruent experiments). The above data show that PAF is not the chemotactic agent presented by IL-1-activated HUVE to induce the ultimate layer penetration of adhering eosinophils.

**DISCUSSION**

Because neutrophil adherence implies transendothelial migration driven by the endothelial barrier exposed to IL-1 and TNF, the importance of this endothelial mechanism had to be demonstrated also for eosinophils. Surprisingly, freshly isolated eosinophils from nonallergic individuals had no spontaneous capacity to migrate across IL-1-activated monolayers. To induce the competence of such eosinophils to respond to cytokine-activated HUVE with transendothelial migration, in vitro maturation was introduced. Cultivation with GM-CSF, IL-3, and IL-5 alone or in combination has been reported to prolong the in vitro survival of eosinophils, was accompanied by a progressive decrease in density, and enhanced the capacity to kill larvae of *schistosoma mansoni*. Such low-density eosinophils, obtained after 5 days of in vitro cultivation together with GM-CSF and IL-3, adhered to IL-1-activated HUVE to the same extent as freshly isolated normodense eosinophils, but they became competent to penetrate cytokine-activated HUVE monolayers. Compared with the gradual density decrease of these eosinophils during cultivation, induction of the transmigratory response was already evident after 20 hours. Thus, in contrast to the instant transmigration of nonactivated neutrophils, which is an inevitable consequence of endothelial-dependent adherence, eosinophils need functional maturation by cytokines to penetrate the activated endothelium.

Table 3. Cytofluorometric Determination of CD18 and VLA-4 Expression on Freshly Isolated Blood Eosinophils From Nonallergic Individuals and Allergic Asthma Patients

<table>
<thead>
<tr>
<th>Net Arbitrary Fluorescence Units</th>
<th>CD18</th>
<th>VLA-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allergic asthma patients (5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VLA-4</td>
<td>28.8 ± 1.8</td>
<td>288.2 ± 8.3</td>
</tr>
<tr>
<td>HP2/1</td>
<td>28.3 ± 4.5</td>
<td>264.3 ± 21.9</td>
</tr>
<tr>
<td>Nonallergic donors (3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VLA-4</td>
<td>28.8 ± 1.8</td>
<td>288.2 ± 8.3</td>
</tr>
<tr>
<td>HP2/1</td>
<td>28.3 ± 4.5</td>
<td>264.3 ± 21.9</td>
</tr>
</tbody>
</table>

Binding of MoAbs was performed as described in Materials and Methods. Values for net arbitrary fluorescence units were calculated by subtracting values obtained with the control MoAb Mas144p, which is not expressed on eosinophils. Data represent means ± SE of the number of experiments given in parentheses.

As for neutrophils, transmigration of eosinophils was maximal after 4 to 6 hours of endothelial activation, persisted up to 24 hours, and needed active endothelial mRNA synthesis. With respect to the repertoire of adhesion molecules involved, the process of eosinophil extravasation was strongly inhibited by MoAbs against CD18. These findings correlate to what has recently been reported for eosinophil adherence that involves CD11/CD18, known to bind to intercellular adhesion molecule-1 (ICAM-1) expressed on the endothelium. ICAM-1 was shown to be crucial also for eosinophil infiltration in a primate model of asthma. Moreover, eosinophils can also bind to endothelial leukocyte adhesion molecule-1. Very recently, the involvement of vascular cell adhesion molecule-1 (VCAM-1) was shown for eosinophil adherence. VCAM-1 on the endothelial surface interacts with the β1-integrin VLA-4, which is expressed on eosinophils but not on neutrophils. We support the finding of others that eosinophil adherence to IL-1-activated HUVE is mediated by different endothelial ligands and is also partially inhibited by the MoAb HP2/1. Moreover, eosinophils can also bind to endothelial leukocyte adhesion molecule-1. However, transmigration across IL-1- and TNF-activated HUVE implied CD11/CD18 rather than VLA-4 structures on the eosinophil surface, despite the fact that our data do not finally exclude that other adhesion pathways might be contributory.

Compared with freshly isolated eosinophils from nonallergic individuals, adherence and transendothelial migration of eosinophils from allergic asthma patients was completely different. Freshly isolated, they showed an impressively enhanced adherence to cytokine-activated HUVE. More importantly, eosinophils from allergic asthma patients already transmigrated across IL-1-activated HUVE monolayers. Their density distribution over discontinuous isotonic Percoll gradients was comparable to that of eosinophils from nonallergic individuals, corroborating the data of Hodges et al. Moreover, isolation of eosinophils with defined density of 1.090, 1.085, or 1.080 g/mL either from nonallergic donors (after in vitro cultivation) or freshly isolated from patients with allergic asthma showed no density dependence in terms of transendothelial migration. From these results we conclude that the capacity to respond to cytokine-provoked transendothelial migration is not linked to the density phenotype of eosinophils. Nevertheless, when eosinophils from allergic asthmatics were cultivated in the presence of 10 pmol/L GM-CSF and IL-3, a decrease from the initial density of 1.085 to the final density of 1.075 g/mL occurred within 24 hours. Compared with the gradual response of eosinophils from nonallergic individuals (see above), this rapid shift of density might reflect in...
vivo priming during allergic inflammation. Such eosinophils showed no further enhancement of adherence and transendothelial migration after in vitro cultivation. Cytofluorometric determination of surface-marker expression showed that the ability to transmigrate is not dependent on quantitative upregulation of CD18 and VLA-4 on the eosinophil surface. VLA-4 has been shown to be constitutively expressed on eosinophils and it was not increased after stimulation with PAF.39 These facts do not exclude functional changes by stimulus-induced protein phosphorylation41 as it has been reported for CD11/CD18.42

Taken together, these results allow the following conclusions. (1) The density distribution of eosinophils over discontinuous isotonic Percoll gradients does not reflect their capacity to migrate across IL-1- and TNF-activated HUVE monolayers. (2) Eosinophils from patients with allergic asthma have the capacity to spontaneously penetrate cytokine-activated monolayers. (3) Cultivation with GM-CSF, IL-3, or IL-5 of eosinophils from nonallergic individuals simulates in vitro a condition that may lead in vivo to induction of the capacity of eosinophils to respond to infiltration-inducing mechanisms in allergic diseases. The recent demonstration of GM-CSF, IL-3, and IL-5 in the circulation of asthma patients and the spontaneous secretion of these cytokines into supernatants of T lymphocytes in culture points to the in vivo importance of these cytokines in allergic asthma.18,19

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