Freezing Adhesion Molecules in a State of High-avidity Binding Blocks Eosinophil Migration

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Summary

Leukocyte extravasation is mediated by multiple interactions of adhesive surface structures with ligands on endothelial cells and matrix components. The functional role of β_1 (CD29) integrins (or very late antigen [VLA] proteins) in eosinophil migration across polycarbonate filters was examined under several in vitro conditions. Eosinophil migration induced by the chemoattractant C5a or platelet-activating factor was fully inhibited by monoclonal antibody (mAb) 8A2, a recently characterized "activating" CD29 mAb. However, inhibition by mAb 8A2 was observed only under filter conditions that best reflected the in vivo situation, i.e., when the eosinophils migrated over filters preincubated with the extracellular matrix (ECM) protein fibronectin (FN), or when the filters were covered with confluent monolayers of cultured human umbilical vein endothelial cells (HUVEC). When bare untreated filters were used, mAb 8A2 had no effect, whereas the C5a-directed movement was prevented by CD18 mAb. Studies with α-subunit (CD49)–specific mAbs indicated that the integrins VLA-4 and -5 mediated migration across FN-preincubated filters, and VLA-2, -4, -5, and -6 were involved in eosinophil migration through filters covered with HUVEC. In contrast with the activating CD29 mAb 8A2, a combination of blocking CD49 mAbs or the nonactivating but blocking CD29 mAb AIIB2 failed to inhibit completely eosinophil migration over FN-preincubated or HUVEC-covered filters. mAb 8A2 stimulated binding to FN but not to HUVEC. Moreover, eosinophil migration over FN-preincubated or HUVEC-covered filters was significantly inhibited by anti-connecting segment 1 (CS-1) mAbs, as well as the soluble CS-1 peptide (unlike migration across bare untreated filters). Thus, inhibition of eosinophil migration by mAb 8A2 depended upon the presence of ECM proteins and not upon the presence of HUVEC per se. In conclusion, "freezing" adhesion receptors of the β_1 integrin family into their high-avidity binding state by the activating CD29 mAb 8A2 results in a complete inhibition of eosinophil migration under physiological conditions. Hence, activation of β_1 integrin–mediated cell adhesion may represent a new approach to prevent influx of inflammatory cells.

Although eosinophils constitute a minor fraction of the leukocytes in the circulation, they are a major component of the cellular infiltrate at extravascular sites of inflammation under certain pathological circumstances, such as allergic late phase responses in asthma and atopic skin reactions, parasitic infestation, and some delayed-type hypersensitivity reactions (1–5).

At the beginning of this process of localized eosinophilic infiltration, the eosinophils leave the vascular compartment. The extracellular matrix (ECM)[1] of the vascular endothelial cells comprises all kinds of proteins and heavily glycosylated structures (proteoglycans) to which the infiltrating immune cells can attach during this migration along gradients of chemotactic factors. Platelet-activating factor (PAF) and the

[1] *Abbreviations used in this paper:* CS-1, connecting segment 1; ECM, extracellular matrix; FN, fibronectin; HUVEC, human umbilical vein endothelial cell; PAF, platelet-activating factor; VLA, very late antigen.

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Volume 178 July 1993 279–284

active complement fragment C5a are all well-known chemoattractants for eosinophils (6, 7).

Of the integrin family of adhesion molecules, the β_2 subfamily of CD11/CD18 molecules as well as the very late antigen (VLA) 4 (CD49d/CD29) member of the β_1 subfamily have been reported to contribute to eosinophil adherence to endothelium through binding to endothelial ligands, such as intracellular and vascular cell adhesion molecule 1 (ICAM-1 and VCAM-1) (8–12). Blockage of integrins is believed to hamper not only adherence to endothelium, but also the subsequent transendothelial migration (diapedesis). Although the role of CD11/CD18 seems to be undisputed (12), the contribution of β_1 members (and VLA-4 in particular) is less clear.

From our present findings it is clear that several β_1 integrins contribute to eosinophil migration. Moreover, we demonstrate in the present study that a continuous modulation of integrins from low- to high-avidity state and vice versa is an essential requirement for cellular migratory responsiveness. A disturbance in this avidity-switch mechanism results in (an almost) complete inhibition of chemotaxis and diapedesis.

Materials and Methods

Eosinophil Purification. Eosinophils were purified from healthy blood donors (13). After isolation, the eosinophils (>95% pure) were washed and resuspended in incubation medium (132 mM NaCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 6 mM KCl, 1.2 mM KH_2PO_4, 20 mM Hepes, 10 U/ml heparin, 5.5 mM glucose, and 0.5% [wt/vol] human serum albumin, pH 7.4).

Eosinophil Migration. Chemotaxis was measured in a 48-well microchemotaxis chamber (NeuroProbe, Cabin John, MD) (14). Two cellulose nitrate filters were placed between the upper and lower compartments a lower stop filter (0.45-μm pore size; Cat No. HAWP01300; Millipore Corp., Bedford, MA) and a migration filter (150-μm thick, 8-μm pore size; Cat No. 11301.13.N; Sartorius GmbH, Göttingen, Germany). Human umbilical vein endothelial cells (HUVEC) were subcultured for 6 d on 10–20-μm thick nucleopore polycarbonate membrane filters precoated with fibronectin (FN) (8-μm pore size; Costar, Cambridge, MA) (15). These filters were placed on top of the migration filter, covering eight separate wells. The lack of E-selectin together with low levels of VCAM-1 expression was considered evidence for the resting state of the HUVEC. Chemoattractant or incubation medium was placed in the lower compartments. Recombinant human C5a (rC5a) and PAF (Sigma Chemical Co., St. Louis, MO) were used as chemoattractants at concentrations defined to be optimal in the assay: 10^{-8} and 10^{-6} M, respectively. Eosinophils were preincubated with saturating concentrations of mAb (15 min at 37°C) or incubation buffer, and subsequently added to the upper compartment (10^5 cells in 25 μl). The chambers were incubated for 2.5 h at 37°C. The number of transmigrated cells reached a maximum at 2 h, most likely because the chemotactic gradient is reduced at that time. The migration filter was removed, fixed with butanol–ethanol, and stained with Weigert's solution. Thee number of cells that had completely passed the 150-μm-thick filter was determined with light microscopy in 10 high power fields (at a magnification of 400) per filter. Spontaneous migration without rC5a (chemokinesis) consisted of one to three cells (mostly neutrophils) in 10 high power fields (at a magnification of 400) per filter.

Eosinophil Adhesion. Eosinophils were labeled with ^{51}Chromium as described for neutrophils (15). After labeling, eosinophils were resuspended in incubation medium. Cells (10^5 cells per well) were added to 48-well culture plates containing confluent HUVEC or fresh coatings of FN. After incubation for 30 min at 37°C, nonadherent cells were removed, and their radioactivity was counted together with two fixed volumes of washing buffer. Adherent cells were lysed with 1% Triton X-100 in water for 15 min at 37°C. The radioactivity of these cells was also counted.

Results and Discussion

We tested the migratory capacity of eosinophils in a microchemotaxis chamber assay through 150-μm thick filters toward the chemoattractants' recombinant complement fragment rC5a, or the lipid mediator PAF. When the migration filters were covered by confluent monolayers of HUVEC, diapedesis was inhibited by the CD18 mAb CLB-LFA1/1 by 60% and by the CD49d mAb HP1/2 by 30%, whereas eosinophil chemotaxis over untreated bare filters was inhibited only by the CD18 mAb (Fig. 1).

Freezing of cells onto the substrates of adhesion molecules by inducing high-avidity binding was considered as an alternative approach to interfere in eosinophil migration. The activating CD29 mAb 8A2 has recently been shown to increase adhesion of PBL and several hematopoietic clones and cell lines to the purified ECM proteins FN, or fragments thereof, and laminin (16, 17). Since bare polycarbonate filters lack appropriate ligands for VLA proteins, eosinophil chemotaxis across these bare uncoated filters was not affected by mAb 8A2. By contrast, migration across filters covered by confluent HUVEC was inhibited to a significantly greater extent than observed with either CD18 or CD49d mAb (Fig. 1).

Although the procedure to stain the filters did not allow surface markers (e.g., CD16) to be used to distinguish between eosinophils and neutrophils, the morphology of the nucleus as well as the granular staining indicated that the cells that

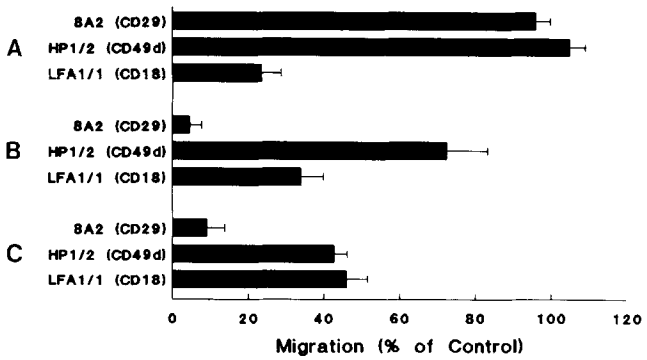

Figure 1. Eosinophil migration in response to recombinant C5a is inhibited by mAbs against the integrin receptor α_4, β_1, and β_2 subunits. The following conditions were tested: (*A*) bare untreated filters; (*B*) filters covered by resting endothelial cells derived and subcultured from HUVEC; and (*C*) filters preincubated with FN. Affinity-purified CD49d (HP1/2, IgG1), CD29 (8A2, IgG1), and CD18 (CLB-LFA1/1, IgG1) mAbs (10 μg/ml final concentration) differentially inhibited eosinophil migration. Migration in response to PAF gave identical results (data not shown).

had reached the stop filter consisted only of the contaminating neutrophils (about 5%). Microscopic examination revealed that the 8A2-treated eosinophils had not entered the migration filter at all. After elimination of these neutrophils by CD16-conjugated magnetic beads, the migration of the remaining eosinophils across HUVEC-covered filters was completely blocked by mAb 8A2 (data not shown).

Because the endothelial cells had been subcultured on FN-precoated filters, we also measured eosinophil chemotaxis over filters preincubated with FN. Under these conditions, mAb 8A2 markedly inhibited chemotaxis. CD49d mAb reduced migration by about 60%, i.e., to a similar extent as had the CD18 mAb (Fig. 1). We performed similar experiments with neutrophils, but never observed any effect of mAb 8A2 under the various experimental conditions (i.e., bare, FN-preincubated or HUVEC-covered filters). On the other hand, neutrophil migration was always fully inhibited by CD18 mAb (data not shown).

Endothelial cells are known to produce a complex ECM composed of several proteins, such as collagen, FN, and laminin (18–20). Many of these proteins are recognized by members of the β_1 subfamily previously described on leukocytes: VLA-1–6 (20–24). Surface expression of VLA-2α, -4α, -5α, and -6α on eosinophils was demonstrated by flow cytometry (Table 1). To explain the complete inhibition of eosinophil migration through HUVEC-covered filters by the activating CD29 mAb 8A2, various blocking CD49 mAbs were tested. Although a role for VLA-6 seemed limited, VLA-2, -4, and -5 were definitely involved, as indicated by the significant inhibition by the respective CD49 mAbs (Table 2). Eosinophil migration through FN-coated filters depended on VLA-4 and -5 (data not shown).

To determine the mechanism by which VLA-4 functioned in the eosinophil diapedesis, further experiments were performed to discriminate between eosinophil binding to endothelial VCAM-1 vs the ECM protein FN. The major recognition site for VLA-4 in FN has been localized to the connecting segment 1 (CS-1) region (25), comprising the first 25 amino acids of the alternatively spliced III-CS domain (IIICS) (26). The activating CD29 mAb 8A2 enhances binding of β_1 integrin VLA-4 to its extracellular ligand FN/CS-1, as well as to the cellular ligand VCAM-1 (16), and CD49d mAb HP1/2 inhibits both these VLA-4–mediated interactions (27). Large numbers of eosinophils were found to bind to FN-coated plates in the presence of mAb 8A2 (Fig. 2). mAb 8A2-stimulated adhesion occurred in a rapid (optimal within 15 min) and prolonged (more than 3 h) fashion. Binding of the mAb 8A2-activated eosinophils to FN was markedly inhibited by the CD49d mAb HP1/2 (Fig. 2). On the other hand, adherence of eosinophils to resting HUVEC was neither significantly induced by mAb 8A2, nor inhibited by mAb HP1/2. Thus, the mAb 8A2-dependent inhibition of eosinophil migration through endothelium-covered filters

Table 2. *Inhibition of Eosinophil Diapedesis across HUVEC-covered Filters by mAbs against Various VLA-α Subunits*

Addition	Number of cells			Inhibition
	Expt. 1	Expt. 2	Expt. 3	
				%
Control mAb	122	91	104	–
CD49b	21	19	39	75 ± 7.4
CD49d	64	57	70	40 ± 5.3
CD49e	73	62	74	36 ± 3.7
CD49f	68	74	89	28 ± 9.6

Number of cells represents the mean number of eosinophils migrated along a chemotactic gradient of rC5a through the 150-μm filter, counted in 10 high power fields (400 ×) per filter. Percent inhibition is expressed as mean ± SEM. Migration was significantly inhibited by all CD49 mAbs listed ($p < 0.05$). CD49a as well as CD49c mAb did not show any inhibition (data not shown).

Table 1. *Expression of VLA Proteins on Eosinophils*

mAb	Specificity	Fluorescence
Control mAb	anti-TNP	14.8 ± 2.1
CD49a TS2/7	VLA-1α	14.9 ± 2.0
CD49b Thromb/4	VLA-2α	24.5 ± 3.1
CD49c J143	VLA-3α	15.0 ± 1.8
CD49d HP1/2	VLA-4α	55.8 ± 4.9
CD49e SAM-1	VLA-5α	23.8 ± 4.7
CD49f GoH3	VLA-6α	30.0 ± 2.0

Flow cytometric determination expressed as mean channel of fluorescence ± SEM of four different experiments. Control antibody was directed against TNP.

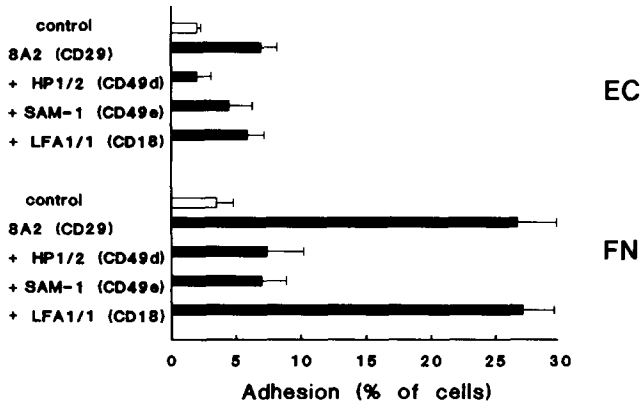

Figure 2. CD29 mAb 8A2 stimulates eosinophil adherence to FN. The CD29 mAb 8A2 did not induce eosinophil adhesion to unstimulated endothelium (*EC*), but VLA-4 and VLA-5–dependent adhesion to fibronectin (*FN*) was strongly induced.

was not due to enhanced binding to the untreated endothelial monolayer. The anti-VCAM-1 mAb 4B9 (28) did not significantly inhibit eosinophil binding to or migration across resting HUVEC, which is in keeping with VCAM-1 expression being predominantly restricted to cytokine- (or endotoxin-) activated endothelium (29–32; data not shown). On the other hand, two out of three anti-CS-1 mAbs which will be more extensively described elsewhere (Gaeta, F. C. A., V. Tollefson, and M. J. Elices, manuscript in preparation), as well as soluble CS-1 peptide, significantly reduced eosinophil diapedesis across HUVEC-covered filters (Fig. 3) because of an inhibition of cell binding to FN (Fig. 4). All three anti-CS-1 mAbs recognized our plasma FN preparation from plasma as well as the ECM of subcultured HUVEC equally well, as determined by ELISA (data not shown). Further control experiments showed that the addition of mAb HP1/2 to the blocking anti-CS-1 mAbs (90.45 and 116/32) or the CS-1 peptide did not result in any additional inhibition of eosinophil migration and/or attachment. Moreover, neutrophil migration was not affected by either the anti-CS-1 mAbs or the soluble CS-1 peptide (data not shown). In conclusion, mAb 8A2 most likely inhibited eosinophil diapedesis because of enhanced binding to the subendothelial matrix formed by HUVEC during culture on the filters.

To investigate the relative importance of β_2 vs β_1 integrins, we further delineated the process of eosinophil migration by using a blocking but nonactivating CD29 mAb AIIB2 (33). mAb CD29 mAb AIIB2 (or the combination of CD49 mAbs [CD49b, CD49d, CD49e, and CD49f]), failed to completely inhibit eosinophil migration (Table 3). The same was true for CD18, as was shown before (Fig. 1). Only the combination of CD29 mAb AIIB2 with CD18 mAb prevented eosinophil migration as efficiently as the activating CD29 mAb 8A2 (Table 3). Microscopic examination revealed that the mechanism by which the activating CD29 mAb 8A2 inhibited migration differed from the mechanism by which the blocking CD29 mAb AIIB2 and CD18 acted. Eosinophils preincubated with mAb 8A2 were completely prevented from entering the migration filter and adhered to the top filter covered by HUVEC or preincubated with FN, whereas with the combination of mAb AIIB2 and the CD18 mAb, very few cells were found on top of the filter indicating that eosinophil adherence was dramatically affected.

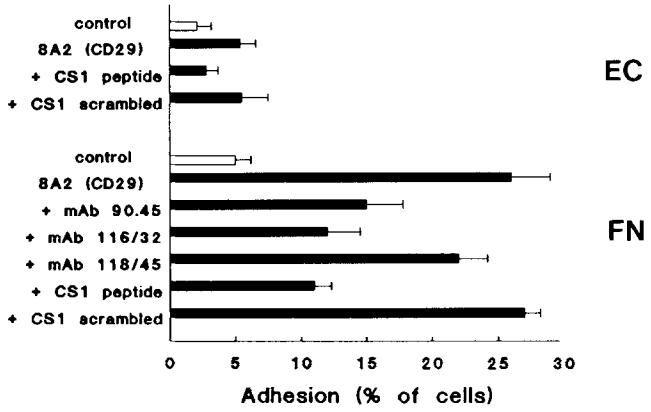

Figure 4. Inhibition of 8A2-stimulated eosinophil adherence by anti-CS-1 mAbs or soluble CS-1 peptide. Experimental approach was as described essentially in the legend of Fig. 3. The anti-CS-1 mAbs were preincubated with the HUVEC or FN coatings, whereas the eosinophils were preincubated with peptides before use. Binding to FN was significantly inhibited by mAb 90.45, mAb 116/32, or the CS-1 peptide ($p < 0.05$).

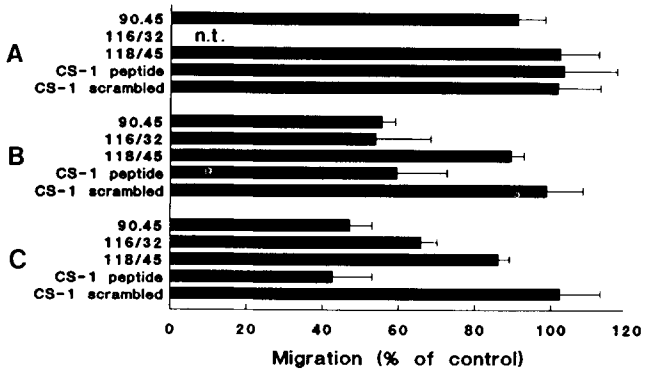

Figure 3. Eosinophil migration in response to recombinant C5a is inhibited by mAbs against CS-1 or the soluble CS-1 peptide. The same conditions as in Fig. 1 were tested. Filters were preincubated for 30 min with an optimal dilution (1:2–4) of hybridoma culture supernatant in case of the anti-CS-1 mAbs 90.45 (IgM), 116/32 (IgM), and 118/45 (IgM) before the cells were added. In contrast, eosinophils were preincubated with the CS-1 peptide (comprising the 25-amino acid sequence) or a scrambled version of the CS-1 amino acid sequence (up to 250 μg/ml) for 30 min at 37°C before addition to the migration chambers. (*B* and *C*) Eosinophil migration was significantly inhibited by mAb 90.45, mAb 116/32, and the CS-1 peptide ($p < 0.05$). (*A*) mAb 116/32 was not tested (*n.t.*).

Table 3. *Inhibition of Eosinophil Diapedesis across HUVEC-covered Filters by mAbs against the β_1 (CD29) and β_2 (CD18) Subunits*

Addition	Number of cells Expt. 1	Expt. 2	Expt. 3	Inhibition
				%
Control mAb	97	116	112	–
CD49 (combi)*	23	28	15	78 ± 4.6
CD29 (AllB2)	25	21	19	80 ± 2.2
CD18	30	52	44	61 ± 7.9
CD18 and CD29 (AllB2)	3	2	4	97 ± 0.8
CD29 (8A2)	4	5	7	95 ± 1.2

Number of cells represents the mean number of eosinophils migrated along a chemotactic gradient of rC5a through the 150-μm filter, counted in 10 high power fields (400×) per filter. Percent inhibition is expressed as mean ± SEM. Migration was significantly inhibited under the conditions tested ($p < 0.05$).

* The CD49 combination comprises Thromb/4 (CD49b), HP1/2 (CD49d), SAM-1 (CD49e), and GoH3 (CD49f).

In summary, eosinophil migration through bare filters is clearly defined by the CD11/CD18 molecules. However, the eosinophilic response became less dependent on CD18 as soon as substrates for VLA integrin receptors were made available (i.e., FN coated upon filters, or ECM protein deposited by HUVEC cultures) (Fig. 1). Concomitantly, there was a significant increase in inhibition of eosinophil migration by CD49d mAb HP1/2 from about 5% on bare, untreated filters, to 30% on filters with HUVEC, or 60% on FN-preincubated filters. The lesser inhibition of migration across HUVEC versus FN produced by the CD49d mAb (Fig. 1) is explained by the involvement of alternative VLA integrin receptors recognizing matrix proteins other than FN. HUVEC are able to generate a complex subendothelial matrix in which collagen and laminin are also important constituents (18–20). These proteins apparently function as the respective ECM ligands for VLA-2 and -6 expressed by eosinophils (Tables 1 and 2). For the first time, the VLA-4–CS-1 interaction is shown to be functionally involved in in vitro transendothelial migration of a leukocyte population (Fig. 3). The relative distribution and functional importance of FN splice variants in vivo are currently unknown. The control of mRNA splicing in the IIICS region of FN during inflammation or wound healing, for example, may thus contribute to the specificity of VLA-4 binding to its ligands (Elices, M. J., A. Goel, J. Fikes, and D. Strahl, manuscript submitted for publication).

Even when the eosinophils were primed by cytokines (e.g., GM-CSF, IL-3, and IL-5) to enhance their chemotactic properties (34), as may occur with eosinophils from allergic patients (12, 35), mAb 8A2 completely blocked migration of these primed eosinophils into HUVEC-covered or FN-coated filters (data not shown).

Activation of the β_1 integrin receptors by mAb 8A2 dramatically interfered with eosinophil migration under conditions that mimic migration in vivo, i.e., chemotaxis across matrix-coated filters or diapedesis across HUVEC monolayers. These findings show that functional responses such as chemotaxis or nondirected movement require that integrin receptors modulate binding avidity for ligand. Paralysis in low avidity state by low temperature or cellular ATP depletion (36, and data not shown) will severely hamper binding. Conversely, an induced, persistent state of high-avidity binding will result in loss of proper function as well. Freezing integrins in a high-avidity state may represent a novel approach to prevent a rapid tissue invasion by VLA-positive cells during inflammation. Such an approach may not be limited to leukocytes. Since adhesion and invasion of tumor cells also involve β_1 integrins (29, 37), activating β_1 mAbs might reduce metastasis as well.

We thank Dr. F. Sanchez-Madrid for anti-VLA-4α HP1/2 (CD49d). We are very grateful to Drs. D. Roos, A. Sonnenberg, A. J. Verhoeven, and C. G. Figdor for discussions and helpful comments.

This study we supported by the Dutch Kidney Foundation (C89.909), the Dutch Asthma Foundation (14.89.25), and the U.S. Public Health Service (HL-18645).

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Received for publication 6 November 1992 and in revised form 10 January 1993.

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