Mechanisms of eosinophil adhesion to endothelial cells under flow conditions

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Mechanisms of eosinophil adhesion to endothelial cells under flow conditions

Mechanismen van hechting van eosinofiele granulocyten aan endotheelcellen onder stromingscondities

(met een samenvatting in het Nederlands)

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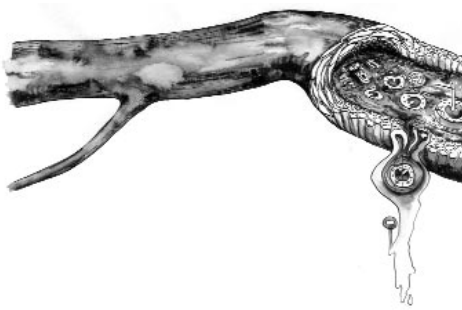
From the moment I picked your book up
until I laid it down I was convulsed with laughter.
Someday I intend reading it.

Groucho Marx

Voor Opa van Lokin



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Abbreviations

$[Ca^{2+}]_i$	intracellular Ca^{2+} concentration
ADP	adenosine 5' -diphosphate
APC	antigen presenting cell
BAL	broncho-alveolar lavage
BHR	broncho hyperresponsiveness
CR3	complement receptor 3
DAG	diacylglycerol
EAR	early asthmatic response
ECM	extracellular matrix
ECP	eosinophil cationic protein
EDN	eosinophil-derived neurotoxin
EGF	epidermal growth factor
EPO	eosinophil peroxidase
Erk	extracellular regulated kinase
ESL-1	endothelial selectin ligand-1
FACS	fluorescence activated cell scan
FEV1	forced expired volume in one second
fMLP	N formylmethionine leucyl phenylalanine
FN	fibronectin
FucT	fucosyl transferase
GAP	GTP-ase activating proteins
GDI	guanine nucleotide dissociation inhibitors
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factors
GlyCAM-1	glycosylation-dependent cell adhesion molecule-1
GM-CSF	granulocyte/macrophage colony stimulating factor
G-protein	guanosine phosphate binding protein
GRO-α	growth related oncogene-α
GTP	guanosine triphosphate
HIV	human immunodeficiency virus
HSA	human serum albumin
HUVEC	human umbilical vein endothelial cells
ICAM-1	intercellular adhesion molecule-1
IL-..	interleukin-..
IP_3	inositol 1,4,5-trisphosphate
LAD	leukocyte adhesion deficiency

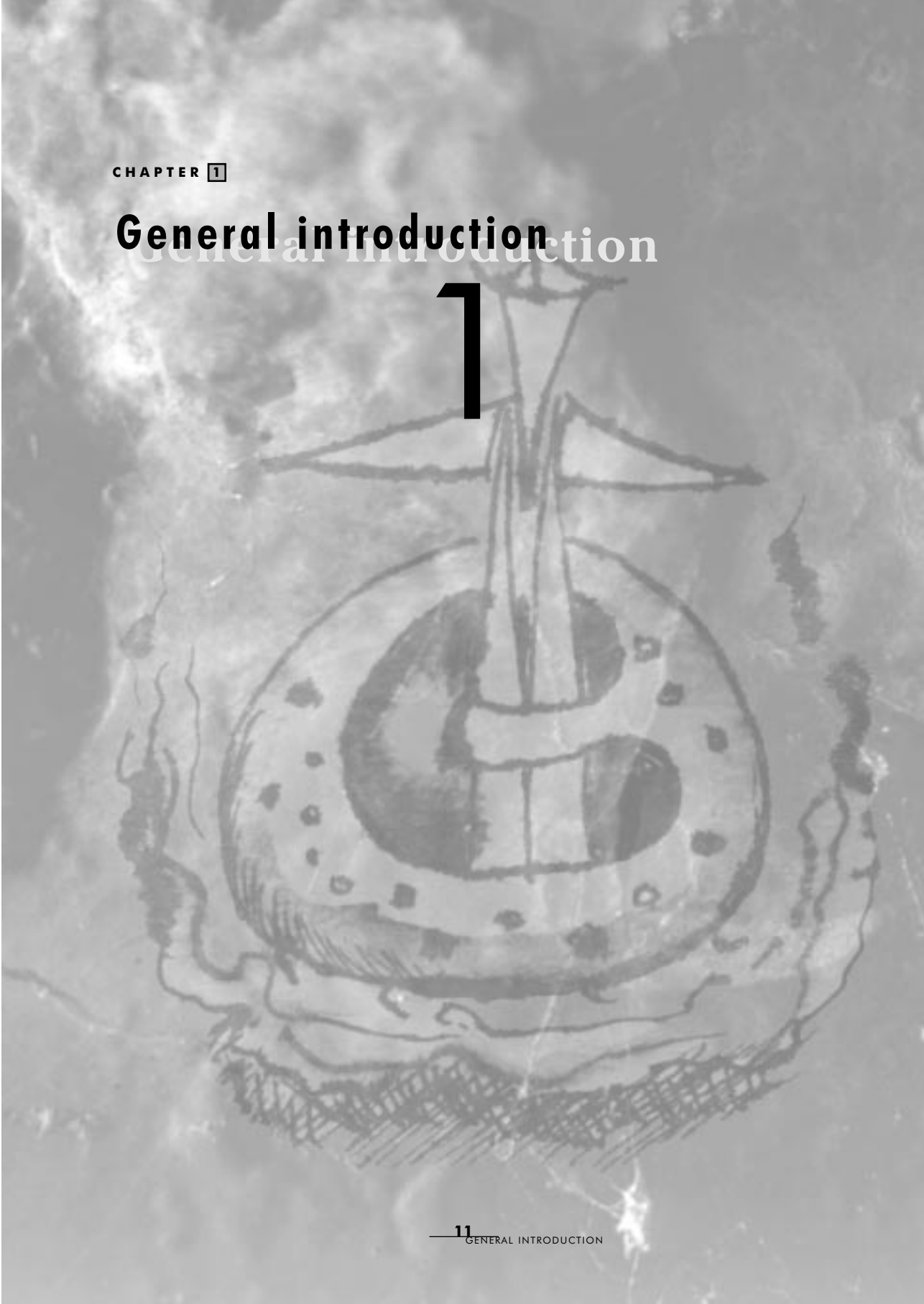
LAR	late asthmatic response
LPA	lysophophatidic acid
LPS	lipopoly saccharide
MadCAM-1	mucosal addressin cell adhesion molecule-1
MBP	major basic protein
MCP	monocyte chemoattractant protein
MLC	myosin light chain
MoAb	monoclonal antibody
PAF	platelet activating factor
PAK	p21-activated kinase
PECAM-1	platelet endothelial cellular adhesion molecule-1
PIP_2	phosphatidylinositol (4,5) bisphosphate
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PLC	phospholipase C
PMA	phorbol myristate acetate
PMN	polymorphonuclear cells
PNAd	peripheral node addressin
PSGL-1	P-selectin glycoprotein ligand-1
PTX	pertussis toxin
RACK	receptor for activated C-kinase
RANTES	regulated on activation normal T-cell expressed and secreted
ROCK	Rho-associated coiled-coil forming protein kinase
SCR	short consensus repeat
SLC	secondary lymphoid-tissue chemokine
TNF	tumor necrosis factor
VCAM-1	vascular cell adhesion molecule-1
VLA-4	very late antigen-4
Zap70	zeta chain-associated protein 70

CHAPTER 1

General introduction

General introduction

1



Multistep paradigm of leukocyte extravasation

For a leukocyte to leave the blood stream and to enter an organ a general model has been described, often referred to as the multistep model (figure 1) (1;3). The first step involves tethering and rolling of leukocytes along the endothelial cell layer. The second step is the exposure of chemoattractants in soluble form and/or presented by endothelial cells to the leukocytes. The third step is the activation of the leukocytes resulting in firm adhesion and spreading. And the fourth step implies the migration of the leukocytes through the endothelial layer to the inflammatory site. These four steps are described in detail in the following paragraphs.

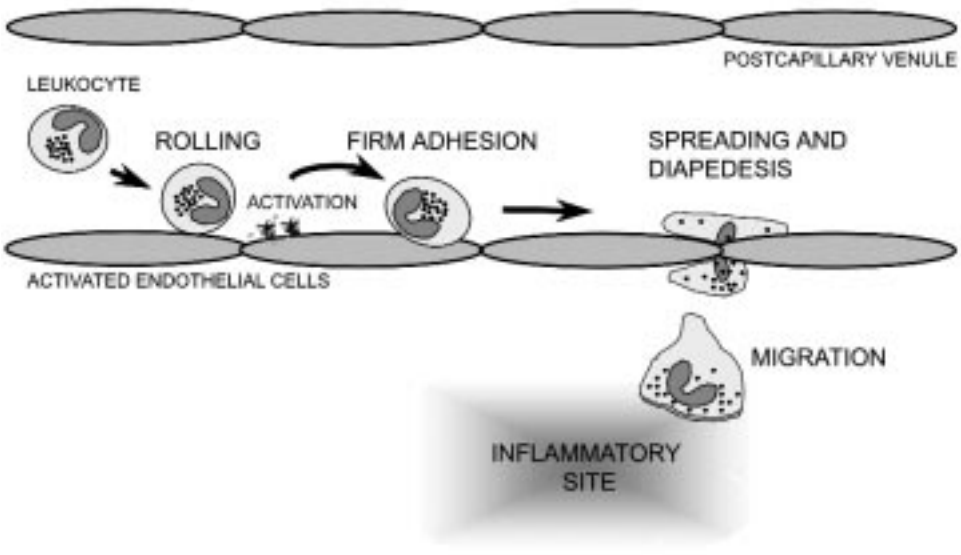


Figure 1. Multi step model for leukocyte extravasation. Cells roll on the activated endothelial cells. Leukocyte activation by encountered chemoattractants results in firm adhesive interactions to the endothelium. Finally, the cells migrate through the endothelial lining to the site of inflammation.

Tethering and Rolling

Leukocyte migration to inflammatory sites occurs mainly in the postcapillary venules. The blood flow at these sites roughly varies between 3-6 cm/sec. Cells flowing with these velocities can tether (=a short transient interaction) on the endothelium that lines the vessel walls. Subsequently, they roll with a speed that is around a thousand times lower than the speed of free flowing leukocytes in the venule. The moment a cell is rolling it endures a force of the blood stream called “shear stress”. A certain minimal shear stress is required for rolling interactions to occur (4;5). As the first interaction between leukocytes and endothelial cells preceding the rolling phase is called “primary tethering”, additional recruitment of cells to the surface involves the tethering of fast flowing leukocyte to an already bound leukocyte. These homotypic interactions or “secondary tethering” facilitate the subsequent formation of clusters (6) or strings (7) of cells on the vessel wall. The molecules involved in tether and rolling interactions are the selectins and their ligands (figure 2), which are mucin-like carbohydrate structures that are sialylated, fucosylated and/or sulfated.

Selectins

The selectins consist of an N-terminal Ca^{2+} dependent lectin-like domain (8), a single epidermal growth factor (EGF)-like domain, from two to nine short consensus repeats (SCR), a single membrane-spanning region and a cytoplasmic tail. Three types of selectins are known, L(eukocyte)-, E(ndothelium)- and P(latelet)- selectin. L-selectin (CD62L) is present on almost all leukocytes and is located on the tips of the microvilli (9). This extended cellular distribution is ideal for capturing and rolling of cells on the endothelium (10;11). Upon leukocyte activation L-selectin is shed from the surface (12). Although healthy individuals have already soluble L-selectin in their blood, increases in plasma levels of soluble L-selectin is associated with disease status and might be used for monitoring diseases (13-15). E-selectin (CD62E) is expressed on activated endothelial cells. Inflammatory mediators such as TNFα, IL-1 and bacterial lipopolysaccharide (LPS), control the expression at the level of transcription. Typically, E-selectin is expressed 2-3 hours after stimulation, peaks around 4-6 hours and returns to basal levels after 10 to 12 hours on human umbilical endothelial cells (HUVEC), that are commonly used as an *in vitro* model for endothelial cell monolayers. Besides its upregulation in inflammation it is known that E-selectin is constitutively expressed on skin venules mediating rolling of leukocytes, thereby increasing the immune-surveillance (16). P-selectin (CD62P) is pre-stored in Weibel-Palade bodies and α-granules present in endothelial cells and platelets, respectively. Upon activation by inflammatory mediators such as

histamine, thrombin or complement factors (17) the granules fuse with the plasma membrane ensuring a fast expression of P-selectin on the cell surface. Besides this direct expression, P-selectin can also be induced at the transcriptional level by inflammatory mediators. E- and P-selectin have overlapping functions *in vivo*. Knock-out models have shown that E-selectin and P-selectin double knock-out mice are susceptible for spontaneous cutaneous infections whereas their single deficient counterparts are not (18).

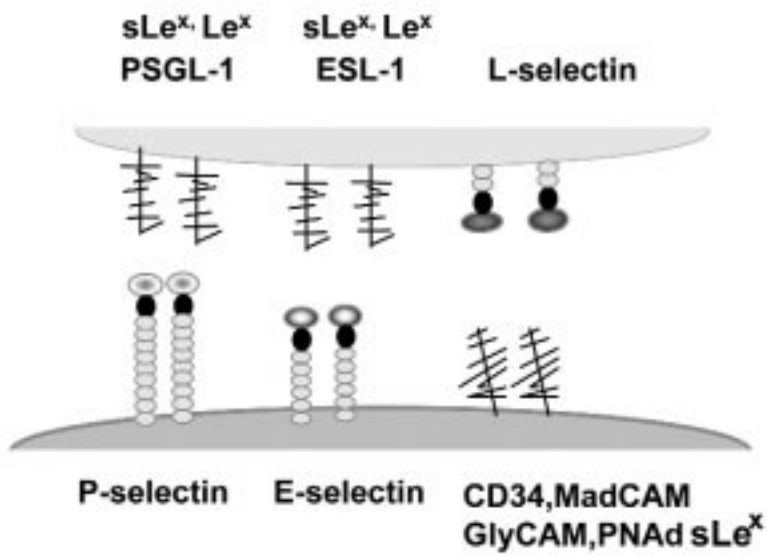


Figure 2. Selectins and selectin ligands are presented by eosinophils and endothelial cells. PSGL-1 binds to P-selectin but can also bind to E- and L-selectin. ESL-1 is a specific ligand for E-selectin. L-selectin has many specific ligands: CD34, MadCAM, GlyCAM and PNAD. Further ligands for E-,P- and L-selectin are discussed in the text.

Selectin ligands

The counter ligands of the selectins form a diverse group of carbohydrate structures attached to a core of specific glycoproteins (figure 2). Fucose-, sialic acid- and sulfate residues on the carbohydrates largely determine the functionality of the ligand (19). This is illustrated by the fact that removal of sialic acid from leukocytes or cell lines by neuraminidase treatment results in reduced binding to selectins (20;21). Furthermore, sulfotransferases have been shown to add a sulfate to a glycan chain, leading to specific

structures (22). Also, much research has been done on the enzymes that decorate glycoproteins with fucose, of which fucosyltransferases (FucT) IV and VII are the most important in leukocytes (23;24). A study on skin venules in FucT-VII and -IV knock-out mice has established that FucT-VII is most important for decoration of P-selectin ligands and subsequent tethering to the endothelium, whereas both FucT- VII and -IV are needed for efficient E-selectin ligands and subsequent slow rolling (16). The need for fucose residues is further illustrated by the rare genetic human disease leukocyte adhesion deficiency type II (LADII) (25). The deficient fucose metabolism in these patients results in afunctional selectin epitopes, leading to decreased leukocyte adhesion to the endothelium and therefore recurrent episodes of bacterial infection. Thus far, oral fucose therapy has been shown to be a succesful therapy in one patient because it induced the expression of fucosylated selectin ligands and as a consequence the infections disappeared (26).

All three selectins can recognize the sialyl Lewis^x and sialyl Lewis^a tetrasaccharides (27;28) and their sulfated equivalents, as suggested from adhesion studies. However, Sle^x has a very low affinity for selectins ranging from micro- to milli-molars (29;30). Therefore, the recognition of these structures *in vitro* does not automatically imply that these interactions occur *in vivo*. The context in which the residues are presented seems more important for the functionality of the ligand. In this respect high affinity ligands have been described for each selectin. L-selectin has four specific high affinity ligands, PNAd (Peripheral Node Addressin) (31), GlyCAM-1 (Glycosylation-Dependent Cell Adhesion Molecule-1) (32), CD34 (33) and MadCAM-1 (Mucosal Addressin Cell Adhesion Molecule-1) (34). E-selectin binds with high affinity to ESL-1 (E-selectin ligand-1) (35). PSGL-1 was originally described as a specific ligand for P-selectin (36) but it can also bind E-selectin (37) and L-selectin (38). The presence of Sle^x moieties in P-selectin glycoprotein ligand-1 (PSGL-1) is not enough for the high affinity binding to selectins. Binding of PSGL-1 to P-selectin requires α1,3 fucosylation by FucT-IV and/or -VII and α2,3 sialylation. Also, the location of Sle^x on O-branched glycans and sulfation of tyrosine residues within the N-terminal of the polypeptide makes PSGL-1 a specific ligand for the selectins (39). A recent paper demonstrated an additional complexity; the presence of the O-linked branching enzyme core 2 β1,6-N-glucosaminyltransferase (C2GlcNacT-1) adds an additional O-linked glycan chain on an existing one and creates an epitope for high affinity binding to P-selectin, which is not required for binding to E-selectin (40). It is thought that what a selectin actually recognizes is a clustered saccharide patch (reviewed in (27)). Treatment of cells with the enzyme O-sialoglycoprotease (41) that recognizes and cleaves large numbers of closely spaced O-linked oligosaccharides abrogates the binding to selectins (42). Overall, the presence of specific enzymes in a cell determines the decoration of the selectin ligands, thereby controlling the interactions with the different selectins.

Chemoattractants and their receptors

Binding of chemoattractant to receptors on the rolling leukocyte induces firm adhesion of the cell to the endothelial lining and subsequent migration. It is thought that an important part of the chemoattractants are presented by glycoproteins on the endothelium and this has been shown for IL-8 (43). Chemoattractants can be divided in classical chemoattractants that have been described decades ago and chemokines that have been described more recently. Both groups bind to GTP-binding protein (G-protein)-coupled seven spanning transmembrane receptors (or serpentine receptors). These receptors are characterized by the presence of a G-protein that consists of an α and a $\beta\gamma$ subunit. Upon receptor activation the G-protein exchanges GDP for GTP resulting in dissociation of the α from the $\beta\gamma$ subunit. This process leads to activation of phospholipase C (PLC) and this enzyme hydrolyses its substrate phosphatidylinositol (4,5)- bisphosphate (PIP_2) into inositol (1,4,5) trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 binds to its receptor on intracellular Ca^{2+} storage organelles with subsequent release of Ca^{2+} in the cytosol, whereas DAG activates protein kinase C (PKC) (44). Ca^{2+}, PKC and other intracellular messengers that are activated control a broad array of cellular processes such as chemotaxis, differentiation, proliferation and survival (45).

Classical chemoattractants

The classical chemoattractants activate most leukocyte subsets. These chemoattractants can be divided in at least 3 different groups; bacterial peptides such as fMLP, serum factors such as C5a and C3a, and bioactive lipids such as LTB4 and PAF.

Chemokines

The chemokine family can be divided in four groups, the CCL, CXCL, CX_3CL and CL chemokines that bind to their receptors CCR, CXCR, CX_3CR and CR, respectively (46). All chemokines share structural elements including conserved cysteine residues that form disulfide bonds in the tertiary structure. The CCL, CXCL and CX_3CL have four cysteines that form disulfide bonds with no, one or three amininoacids (X) in between the cysteines, respectively. The C group only has one disulfide bond. Of each of the subgroups CL and CXC3L only one member is known today, lymphotactin (47) and fractalkine/neurotactin (48), respectively. The number of chemokines in the CCL (28 members) and CXCL (16 members) groups has grown extensively in the last decade,

and they have overlapping functions, although some specificity is present (49). Besides the structural classification of chemokines a classification according to function can also be made. Part of the chemokines are continuously expressed in the body for direct homing of leukocytes, thereby securing immuno-surveillance. An example is the stromal cell derived factor (SDF-1, CXC chemokine), which plays a major role in homing of stem cells to the bone marrow (50). Furthermore, secondary lymphoid-tissue chemokine (SLC) is expressed by lymph nodes and spleen and attracts lymphocytes to these sites (51). Other chemokines are expressed upon an inflammatory reaction, and might therefore be called inflammatory chemokines. Generally, it is thought that CCL chemoattractants are more chemotactic for monocytes, eosinophils, lymphocytes and basophils whereas the CXCL chemoattractants are probably more specific for neutrophils and to a lesser extent for basophils and T-cells (52). One reason why chemokines have overlapping functions is that different chemoattractants can bind to the same receptor and that one chemokine can bind to distinct chemokine receptors. In this respect RANTES, eotaxin and MCP2 and MCP3 can all bind to CCR3 (53;54) and RANTES binds to CCR1, CCR3 and CCR5 (55).

Firm adhesion and spreading

Activation of the rolling cell by chemoattractants induces firm adhesion and subsequent spreading of the cell. Firm adhesion is mediated by integrins on the leukocytes that bind to their counter ligands on the endothelium belonging to the immunoglobulin superfamily (figure 3).

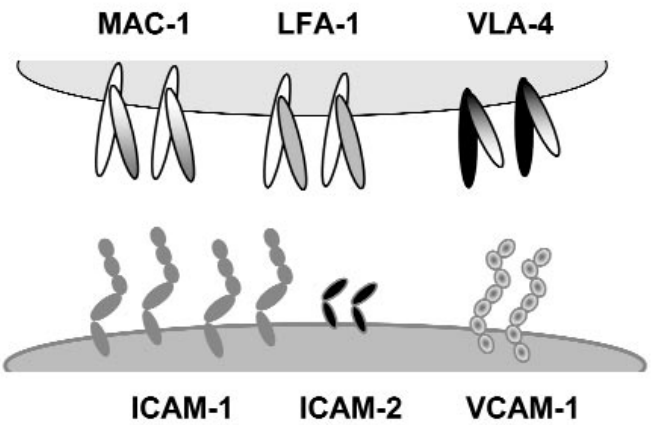


Figure 3. Integrins are expressed by leukocytes and their ligands of the IgS are expressed on endothelial cells. For eosinophils the integrins VLA-4 and MAC-1 are important for firm adhesion to VCAM-1 and ICAM-1, respectively. LFA-1 is less abundant on eosinophils.

Integrins

Integrins are heterodimeric proteins that consist of an α subunit that is non-covalently bound to a β subunit. At least 8 different β subunits ($\beta_{1\text{-}8}$) and 18 different α subunits ($\alpha_{L,M,X,D}$, $\alpha_{1\text{-}11}$, α_{E}, α_{IIb}, α_{v}) have been described on human cells that combine to form 24 known adhesion receptors (56) (Hemler Keystone 2001) (Table 1). The integrins that are important for leukocyte adhesion to endothelium are all four members of the β_2 integrin family; $\alpha_L\beta_2$ (CD11a/CD18, LFA-1), $\alpha_M\beta_2$ (CD11b/CD18, Mac-1, CR3), $\alpha_X\beta_2$ (CD11c/CD18, p150,95) and $\alpha_D\beta_2$(CD11d/CD18) and two members of the β_1 and β_7 integrin family, $\alpha_4\beta_1$ (VLA-4) and $\alpha_4\beta_7$, respectively. $\alpha_L\beta_2$ is expressed on all leukocytes although the highest expression is found on lymphocytes. $\alpha_M\beta_2$ and $\alpha_X\beta_2$ are predominantly expressed on granulocytes, monocytes and macrophages. $\alpha_D\beta_2$ was characterized as an integrin present on foam cells (57) but also appears to be present on a subset of leukocyte populations (58). $\alpha_L\beta_2$ binds to ICAM-1,-2 and -3, whereas $\alpha_M\beta_2$ binds to ICAM-1 (59), C3bi (60), fibrinogen (61), heparin (62), LPS (63), factor X (64) and an unknown molecule (65). $\alpha_D\beta_2$ and $\alpha_4\beta_1$ integrins both bind to VCAM-1 (66;67). The importance of functional β_2 integrins in host defense mechanisms is illustrated by the rare genetic disease LAD I (Leukocyte adhesion deficiency I) (68;69). These patients have no expression of β_2 integrins on their leukocytes and suffer from recurrent bacterial infections. The only permanent therapy for this disorder is allogeneic bone marrow transplantation of which graft verus host disease is the major drawback (70).

On non-activated, resting cells integrins maintain a conformationally inactive state. An exception of this characteristic accounts for the $\alpha_4\beta_1$ integrin that is present on resting cells and is able to bind to its ligand VCAM-1 (71-73). Upon stimulation by chemoattractants or other stimuli integrins change their conformation leading to increased affinity and/or avidity. This process is called “inside-out” signaling (74-76). The binding, however, of integrins to their ligands in itself may lead to activation of intracellular signaling pathways; this process is called “outside-in” signaling.

Table I The integrin receptor family. Table is a modified form of Hynes (56) with additives from (77, 78, 74).

Subunits		Alternative names	Ligands counter receptors	Binding sites
β_1	α_1	VLA-1	collagens, laminin	
	α_2	VLA-2, gpIa/Iia	collagens, laminin	DGEA
	α_3	VLA-3, VCA	fibronectin, laminin, collagens	RGD
	α_4	VLA-4	fibronectin, VCAM-1	EILDV
	α_5	VLA-5,FNR	fibronectin	RGD
	α_6	VLA-6, gpIc/Iia	laminin	
	α_7	VLA-7	laminin	
	α_8	VLA-8	tenascin,fimbronectin,vitronectin	RGD
	α_9	VLA-9	tenascin	
	α_{10}	VLA-10	collagen II	
	α_{11}	VLA-11	collagen	
	α_v		vitronectin, fibronectin	
β_2	α_L	CD11a/CD18, LFA-1	ICAM-1,-2,-3	
	α_M	CD11b/CD18,Mac-1 CR3	ICAM-1,-2, C3bi, fibrinogen, factor X, HMWK, LPS, β-glucan, heparin, NIF, elastase, gp1b	
	α_X	CD11c/CD18, p150/95	fibrinogen, C3bi, LPS	GPRP
	α_D	CD11d/CD18	ICAM-3, VCAM-1	
β_3	α_{IIb}	gpIIb/IIIa	fibrinogen,von Willebrand Factor fibronectin, vitronectin thrombospondin	RGD KQAGDV
	α_v	VNR	vitronectin, fibrinogen, vWF thrombospondin,FN,osteopontin collagen,tenascin,PECAM-1	
β_4	α_6	gpIc/IcBP	laminin-5	
β_5	α_v		vitronectin	RGD
β_6	α_v		fibronectin	RGD
β_7	α_4		fibronectin,VCAM-1,MadCAM-1	EILDV
	α_{IEL}	$\alpha_E\beta_7$	E-cadherin	
β_8	α_v		vitronectin	

Adhesion counter-receptors belonging to the Ig superfamily members

Immunoglobulins are plasma proteins including all antibody molecules and are characterized by an immunoglobulin domain. Members of the immunoglobulin superfamily share structural and genetic features with immunoglobulin molecules and contain at least one immunoglobulin domain. The vascular endothelium expresses molecules of the immunoglobulin superfamily, which act as counter-receptors for leukocyte integrins. Three immunoglobulin type ligands are present on the endothelium, of which ICAM-2 is constitutively present and ICAM-1 and VCAM-1 are induced by cytokine stimulation. ICAM-1 is also expressed on activated epithelial cells, mediating eosinophil adhesion and activation in patients with allergic asthma (79;80). VCAM-1 is constitutively expressed on bone marrow endothelium and is thought to play a role in homing to this site (81).

Migration

Chemoattractant-induced activation of leukocytes leads to firm adhesion and spreading on the endothelium. Subsequently, cells crawl to the site where endothelial cells connect to each other and migrate in between them to the inflammatory site. It was shown that outside-in signaling by adhesion of neutrophils to endothelial receptors increased the endothelial permeability by disorganizing endothelial cell-to-cell adherent junctions and thus facilitating migration (82). Another mechanism for passing the endothelial layer is the observation that neutrophils do not crawl in between endothelial cells but migrate through an endothelial cell (83). When cells have passed the endothelium they interact with the extracellular matrix (ECM) (84). For neutrophils β_1- and β_3- but to a lesser extent β_2-integrins are essential for adhesion to ECM proteins such as fibronectin, collagen I and IV, laminin, vitronectin and tenascin (85). Finally, cells enter the site of inflammation.

The process of migration

The spread cell has a typical tear-drop resembling shape (figure 4). The broad and flat edge is the leading front of the cell also called the lamellipodium, while the narrow end at the rear of the cell is called uropod (86). Signals both from chemoattractants and from integrin crosslinking lead to extension of the lamellipodium membrane, a process called protrusion. Filopodia are other protrusions and resemble thin cylindrical

needle-like projections. Protrusion is associated with an increased polarization of filamentous actin (F-actin), and in fibroblasts it has been shown that in the protruding lamellipodium focal adhesion formation takes place. Focal adhesions are structures of clustered integrins associated intracellularly with structural and signaling molecules (87). In fibroblasts, focal adhesions were shown to increase in size as the cell moves over them, and when they reach the rear of the cell, they are left behind (88;89) and/or are recycled (90). In leukocytes smaller structures than focal adhesions are present that are called focal complexes or adhesisomes, and these appear to have the same characteristics (91). In neutrophils, $\alpha_5\beta_1$ integrins are translocated from the uropod to the leading edge via endosomes (92). This might be a Ca^{2+} dependent process since buffering of Ca^{2+} transients was shown to inhibit the recycling of integrins. Moreover, eosinophils showed low Ca^{2+} concentration in the leading edge and a high Ca^{2+} concentration in the uropod (93). However, in neutrophils this could not be shown (94). Such Ca^{2+} distribution might enable release of cell-substratum attachments in the uropod without also contracting the leading edge, since the myosin based contraction is a Ca^{2+} dependent process.

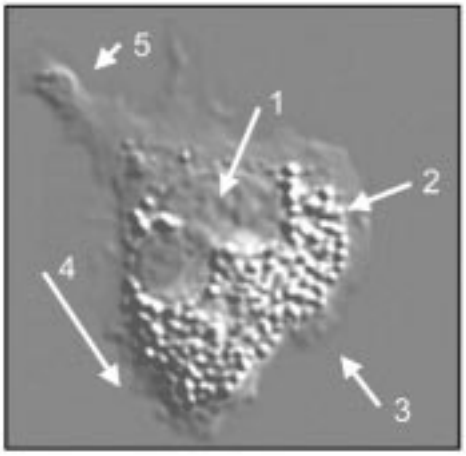


Figure 4. Specific characteristics of a migrating eosinophil. Clearly visible are the bilobed nucleus (1) and the granules (2). The leading lamellipodium (3) protrudes on the albumin coated surface in the direction of migration (4). The rear of the cell is called the uropod (5) and release of this part allows movement of the cell.

Role of Rho GTP-ases in migrating cells

The most important molecules regulating cell migration are the members of the Rho GTP-ase family including Rac, Cdc42 and RhoA (95). Rho GTP-ases are low molecular weight molecules that switch between an inactive GDP bound state and an active GTP bound state (96). Rac1 is implicated in lamellipodium formation (97), Cdc42 mediates filopodia formation (98) and RhoA controls actomyosin mediated contraction and focal adhesion formation (99). In general, the adhesion sites in fibroblasts induced by Rac activation are smaller than those induced by RhoA and are called focal

complexes. Adhesion sites induced by RhoA are large arrow-shaped complexes and are called the focal adhesions. The latter are connected to the RhoA-induced stress fibers in the cell which in this way can generate traction force in the cell leading to migration. As discussed before, focal adhesions and stress fibers are not present in leukocytes; however, RhoA is implicated in generating traction force and therefore could be important for retraction of the uropod.

The role of the multistep model in chronic inflammatory diseases

The multistep paradigm is a valid model for leukocyte homing to different organs. For immunological homeostasis it is important that leukocytes travel back and forth between the blood, the lymphoid tissue and the peripheral tissues. The adhesion molecules and cytokines needed for this leukocyte extravasation are continuously present. This is in clear contrast to inflammatory situations in the tissues. Pathogens present at a specific site of the body should be eliminated. At that site an inflammatory response is evoked and characterized by *de novo* cytokine production and adhesion molecule upregulation. This leads to the extravasation of leukocytes that clear the pathogens from the body. However, when the immune system cannot control this response properly, a chronic inflammatory response may be evoked. A typical example of this situation is the disease allergic asthma that is characterized by a sustained leukocyte infiltration and inflammation in the bronchial tissue. However, one important difference with a pathogen-driven inflammatory reaction is that in asthma the response is directed to non-hazardous allergens (see below).

Facts and clinical aspects of asthma

Over the last decades the prevalence of asthma has dramatically increased (100-102). The incidence of asthma is up to 30% of certain populations in the Western civilization (103, 104). The clinical symptoms of this disease, like wheezing, dyspnoea and cough, are due to reversible airway obstruction and hyperresponsiveness of the airways (105). Airway obstruction is caused by contraction of smooth muscle cells, secretion of mucus, oedema due to enhanced vascular permeability and cellular infiltration of the airway wall. Hyperresponsiveness is an exaggerated bronchoconstrictive response to a variety of non-specific stimuli, which under healthy conditions do not evoke an airway obstruction. Pharmacological agents like histamine and methacholine can also induce this hyperresponsiveness and are used in the clinic to determine the

severity of the disease. An asthmatic reaction can be divided in an early and a late asthmatic response (EAR and LAR respectively) (106). In general, the onset of the EAR is immediately after allergen challenge; it peaks around 15 minutes and resolves within 1 to 3 hours. In approximately 50% of the patients the EAR is followed by a LAR that starts around 4-6 hours after allergen challenge and can last for 12 hours or even longer. Both EAR and LAR are associated with decreased but reversible changes in lung function, as expressed by forced expired volume in one second (FEV1), but the underlying mechanisms causing the shortness of breath are different. The main event evoking the early asthmatic response is the IgE-dependent release of histamine and other mediators from the mast cell. Histamine is a potent bronchoconstrictor and can also induce mucus secretion and microvascular leakage. Besides mast cells, macrophages and epithelial cells are also activated and release acute-phase and chemotactic factors like leukotrienes, thromboxanes and platelet activating factor (PAF) and cytokines. The development of the LAR is characterized by a mobilization of inflammatory cells. Several leukocyte subtypes, of which eosinophils are the most dominant, migrate into the mucosa of the bronchi upon locally produced chemoattractants (107). The number of eosinophils present in bronchial biopsies and bronchoalveolar lavage (BAL) fluid are associated with the severity of the disease (108).

Immunological processes in asthma

It is well accepted that the clinical features of allergic asthma are caused by a chronic inflammation of the airways (109-110) (figure 5). However, the exact mechanism of the onset of the disease is not known. Genetic and environmental factors are thought to play a role in creating the conditions in which the immune system is triggered to react on non-hazardous antigens (allergens). Allergens can be divided in two catagories: indoor allergens (like house-dust mite, cat and cockroach) and outdoor allergens (like birch pollen and hay). Indoor allergens are believed to account for the increase in asthmatic patients seen in the last decades (112). Antigen-presenting cells (APC) take up allergens from the environment on a daily basis in any individual. The immune system of part of the population sees these allergens as hazardous. In these people the induction of atopy starts. Atopic individuals have allergen-specific IgE in their serum and show positive reactions to extracts of common allergens on skin prick tests. However, the presence of atopy does not necessarily mean that a person develops an allergic disease like asthma. Also, the moment of production of allergen-specific IgE and the moment of an asthmatic response can be years apart. The allergens are presented by APCs in a MHC class II restricted way to $CD4^+$ T-cells that become activated and subsequently start secreting cytokines like IL-4, IL-13, IL-5, GM-CSF and IL-3 (113). IL-4 induces isotype

switching of B cells that start to produce allergen-specific IgE (114). This IgE binds to high-affinity IgE receptors on mast cells.

An early asthmatic response can be initiated when an allergen crosslinks the IgE molecules on a mast cell. This induces a direct release of histamine, cytokines and other mediators from pre-stored granules. Subsequently, these compounds mediate the recruitment and survival of leukocytes and induce a shift of T cells to a Th_2 like subtype (113;115). At this point the late response leads to chronic inflammation. Additionally, on top of the chronically inflamed bronchial tissue in allergic asthmatics, an additional allergen challenge can induce an early and a late response and an exacerbation of the disease.

Eosinophils are one of the main leukocyte subtypes present in allergic asthmatic airway tissue. They are thought to contribute to the severity of the disease. Therefore, this thesis focuses on eosinophils and how they extravasate to bronchial tissue.

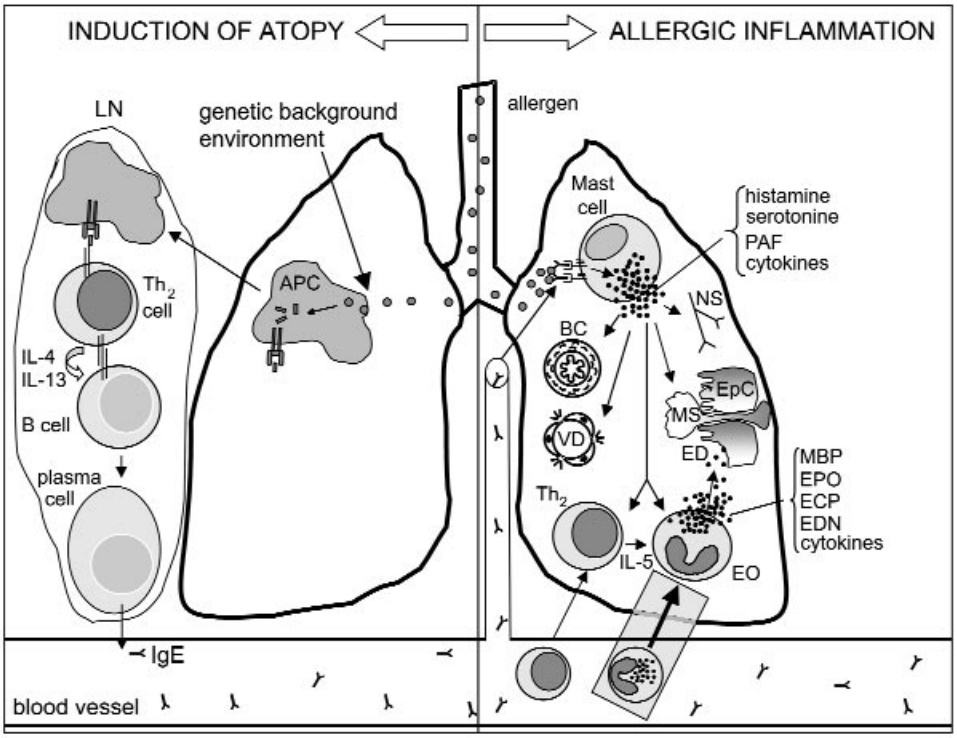


Figure 5. The onset of an immune response against allergens involves the uptake of the allergen by the antigen presenting cell (APC). Environmental and genetic factors are thought to contribute to the decision of the immune system to respond to "non-hazardous" allergens. The allergen is processed by the APC and presented in a MHC class II restricted way to a T-cell in a draining lymph node (LN). The Th_2 cell produces IL-4 and IL-13 and activates a B-cell, and the latter starts producing allergen-specific IgE. At this stage atopy is induced. This stage can be maintained for years without developing allergic symptoms. The IgE present in the blood and tissues can bind to IgE receptors on mast cells. The early reaction starts when an allergen crosslinks IgE bound to the mast cell, which leads to activation and degranulation of the mast cell. Secretion of histamine, PAF and other cytokines leads to several effects: bronchoconstriction (BC), vasodilatation (VD), nerve stimulation (NS), mucus secretion (MS). The late response starts when inflammatory cells, such as eosinophils, enter the lung. The cascade triggered by mast cells eventually leads to activation of endothelial cells and subsequently, T cells and eosinophils (eos) enter the lung. IL-5, secreted by Th_2-cells, is a potent priming agent for eosinophils. The grey rectangle depicts the working field of this thesis.

Eosinophils

Eosinophil structure and function

Eosinophils were already recognized in 1880 (116;117). They contain a bilobed nucleus and many granules. These granules, that could be stained by the acidic dye eosin, gave eosinophils their name. The granules contain many cationic proteins, of which eosinophil peroxidase (EPO), eosinophil-derived neurotoxin (EDN), eosinophil cationic protein (ECP) and major basic protein (MBP) are most abundant (118). These basic proteins are thought to play an important role in eosinophil associated diseases such as parasitic infections and allergic asthma. In parasitic infections, eosinophils are thought to play a positive role. Indeed, it has been shown that the basic proteins can kill helminths (119). In allergic asthma eosinophils are thought to play a negative role. The basic proteins secreted by eosinophils might be responsible for remodeling the bronchial epithelial layer, resulting in an increase in bronchial hyperreactivity (120). Eosinophils of asthmatic patients have been found in close contact with the epithelium and filled the holes of desquamated epithelial cells (109). Also, bronchoalveolar lavage fluid of hyperreactive compared to normoreactive mild atopic asthma patients has been shown to contain more eosinophils, MBP and epithelial cells (121). In addition, IL-5 activated eosinophils have been shown to degranulate and secrete eosinophil cationic protein when bound to activated epithelial cells in a β_2 integrin-dependent way (122). Thus, eosinophils that appear in the lung during the late asthmatic response contribute to the damage of functional structures, leading to impairment of the disease.

Priming of eosinophils

A characteristic of allergic asthma is not only the elevated number of eosinophils in the bronchial mucosa, but also in the peripheral blood. Normally 1-3% of the white blood cell population are eosinophils, whereas in allergic asthmatics this is generally between 3-6% and it can even be higher. Many data suggest that the presence of the eosinophilotrophic factor IL-5 causes elevated levels of eosinophils. A correlation between elevated eosinophil counts in peripheral blood and tissues and an increase in IL-5 was found in asthmatic patients (123;124) but also in other diseases such as a parasite infection (125) and some forms of hypereosinophilia (126). In accordance, it is well established that IL-5 is important for the differentiation of a pluripotent stem cell towards the eosinophilic lineage (127;128) and the survival (129) of mature eosinophils.

Since eosinophils are thought to be the major effector cells in chronic asthma, many studies have addressed the role of IL-5 in the development of this disease. It has been shown that IL-5 inhalation by asthmatics induces an increase in eosinophils in the peripheral blood and an increase in BHR (130;131). Also, many animal models of asthma show correlations between IL-5, pulmonary eosinophilia and airway hyperresponsiveness (132-135). Furthermore, anti-IL-5 treatment abolished pulmonary eosinophilia and bronchohyperresponsiveness in animal models (132;133). Together, these studies suggest that antagonism of IL-5 might be an attractive anti-asthma therapy. Leckie *et al.* (136) studied the effect of a humanized anti-IL-5 antibody in allergic asthmatics and showed a reduction in blood eosinophils, but not a change in airway hyperresponsiveness and LAR. These first results seem to be disappointing but some critical points can be raised regarding this study: 1) the investigated group was very small and 2) the mild asthmatics did not have a clear induction of BHR upon allergen provocation at the start of the study, making it difficult to see an effect of therapy (137). Although the role of IL-5 in allergic asthma has not crystalized yet, the role of IL-5 on eosinophil function is much more clear.

An important effect of IL-5 and also of the related cytokines IL-3 and GM-CSF on the eosinophil is preactivation or priming of the cell (138). Priming by itself does not induce effector responses of leukocytes but instead elevates the (pre)activation status of the cell. Upon addition of a second stimulus the cellular responses are much stronger than those induced by the stimulus alone. For example, eosinophils of healthy subjects when primed with IL-5, IL-3 or GM-CSF have an enhanced respiratory burst in response to opsonized particles (139) and migration in response to PAF (140) compared to the addition of these activators alone. Eosinophils from allergic asthmatic patients show similar responses in the absence of a priming cytokine, indicating that a primed phenotype is already induced *in vivo* in these patients (140;141). Priming of the cells is a potent mechanism that is likely to contribute to the severity of the disease. Especially adhesion processes of eosinophils are increased by priming of the cells (140;142-149).

Eosinophil rolling, adhesion and migration to allergic inflammatory sites

The observation that eosinophils are specifically recruited to the bronchial mucosa in allergic asthmatic patients suggests the existence of specific adhesion pathways. However, common pathways are used by different cells and probably only the combination of molecules used in each step might give a specificity in recruitment (146;150). Eosinophils roll on activated endothelial cells, using all three selectins, E-, P- and L-selectin. L-selectin has been shown to mediate eosinophil adhesion to activated endothelium in a rotation assay (151) and also *in vivo* (152). The observation that a specific antibody against L-selectin (LAM1-11) can block eosinophil-, but not neutrophil-dependent L-selectin interactions with the endothelium suggests that the recognition site on L-selectin differs between neutrophils and eosinophils (151). This might contribute to specificity. Many studies suggest that P-selectin/PSGL-1 interaction contributes to specific eosinophil recruitment. A role for P-selectin was found in binding of eosinophils to nasal polyp endothelium (153), under flow conditions (154-156) and in animal models for allergy (157-159). Eosinophils bind with greater affinity to P-selectin than neutrophils. However, it is not known whether this is due to the increased expression of PSGL-1 on eosinophils compared to neutrophils (153), a difference in structure of PSGL-1, or the presence of other P-selectin ligands on eosinophils. Although P-selectin seems important in eosinophil recruitment to allergic inflammatory sites, E-selectin is likely to play a role too. E-selectin, ICAM-1 and VCAM-1 expressions are increased on venules in bronchial mucosa of allergic asthmatics compared to controls (160-163), whereas no immunohistochemical studies have reported the presence of P-selectin. Although eosinophils cannot roll as efficiently as neutrophils on E-selectin (164;165), eosinophil recruitment in a murine model of allergic skin was dependent both on P-selectin as well as on E-selectin (166). Also, in P-selectin-deficient mice the eosinophil recruitment to the lung after ovalbumin challenge was only reduced by 50% compared to wild-type mice, suggesting that additional pathways such as E-selectin and VCAM-1/VLA-4 can occur (159). The versatile $\alpha_4\beta_1$ integrin behaves not only as an integrin but can also mediate tethering and rolling (167;168). Eosinophils, like lymphocytes, use α_4 integrins for initial attachment to VCAM-1-expressing endothelial cells, and subsequently it is used for firm adhesion (154). Although VLA-4 has been shown to be present on neutrophils (169;170), many studies have shown that eosinophils but not neutrophils adhere to VCAM-1-expressing endothelium in an $\alpha_4\beta_1$ integrin dependent way (71-73;171). Many *in vivo* (172-176) studies suggest that the α_4 integrin/VCAM-1 interaction is important for eosinophil recruitment to allergic inflammatory sites. Of the β_2 integrin members the $\alpha_M\beta_2$ (Mac-1) integrin is most highly

expressed on granulocytes whereas $\alpha_L\beta_2$ integrin is most prominent on lymphocytes. Upon stimulation, the $\alpha_M\beta_2$ integrin on eosinophils is activated (177) and binds to its diverse ligands, including ICAM-1. This interaction is a prerequisite for firm adhesion and migration of eosinophils, as determined by *in vitro* (178;179) and *in vivo* data (180-183). Another level of controlling specific recruitment is the presence of cytokines and chemoattractants. The cytokines IL-13 and IL-4 specifically upregulate VCAM-1 and P-selectin on endothelial cells (184), whereas IL-1β and TNFα induce E-selectin, VCAM-1 and ICAM-1. Adhesion and migration studies have shown that C5a (185), PAF (186;187), RANTES (188) and eotaxin-1, -2 and -3 (181;189;190) are potent chemoattractants for eosinophils, of which the eotaxins are most specific. However, the eotaxins also induce migration of basophils (191) and under specific conditions of T-cells (192), since the eotaxin receptor (CCR3) is present on these cells. The mechanism by which eosinophils pass the endothelial layer is not known. PECAM-1 has been shown to be involved in migration for different leukocyte subtypes (193-195) however not for eosinophils (196;197). Finally, eosinophils enter the bronchial tissue by directed migration through the ECM proteins. Since asthma is characterized by airway remodeling (198), the composition of the ECM in asthmatic patients (199) might finally also give specificity to the migration of the eosinophils.

Aim of the study

This study focuses on the different mechanisms by which eosinophils interact with the vasculature. A prerequisite for studying these interactions is that experiments are performed under flow conditions. Only under flow conditions the different steps described in the multistep model (tethering, secondary tethering, rolling and firm adhesion) can be studied. Figure 6 depicts the model system used to answer the research questions 1-3, as described below. Migration processes have been studied with a time-lapse microscopy technique on 2D substrates, and this was used to answer the fourth research question.

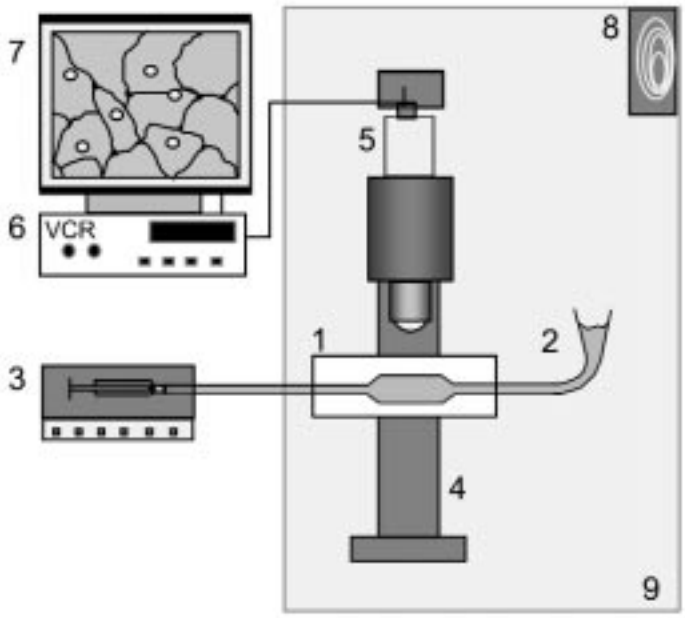


Figure 6. *In vitro* flow chamber model. Coverslips with a layer of confluent TNFα-activated endothelial cells are placed in the *in vitro* flow chamber (1). Eosinophils are put in a reservoir (2) and a pump (3) pulls the eosinophils through the flow chamber over the endothelial cells. On the microscope (4) a video camera (5) is mounted and with the video recorder (6) video sequences of rolling and adhering cells (7) are recorded. Experiments are performed at 37°C. A heater (8) regulates the temperature in the incubation box (9).

Research questions:

1) *Which adhesion molecules mediate the recruitment of eosinophils on activated endothelium?*
In chapter 2, the adhesion molecules involved in the rolling and adhesion events of eosinophils to TNFα-activated HUVEC are characterized under flow conditions.

2) *Do eosinophils of allergic asthmatic patients differ from eosinophils of healthy controls in adhesive behaviour on activated endothelial cells under flow conditions?*
In chapter 3, the adhesive behaviour of eosinophils of healthy individuals and allergic asthmatic patients on activated HUVEC is compared.

3) *Which effects do different chemoattractants have on the transition of rolling to firm adhesion of eosinophils and how is this regulated?*
In chapter 4 and 5, the effects of IL-8, eotaxin and C5a are examined on the transition from a rolling to a (firm) adhesive state. Furthermore, the effects of IL-8, eotaxin and C5a on integrin-mediated inside-out control are investigated.

4) *Which mechanisms are used by granulocytes to migrate in response to chemoattractants?*
In chapter 6, the role of the GTP-ase RhoA in the detachment process of granulocytes on 2D surfaces is investigated.

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CHAPTER 2

Characterization of eosinophil adhesion to TNFα-activated endothelium under flow conditions: α_4 integrins mediate initial attachment and E-selectin mediates rolling

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Abstract

The multistep model of leukocyte adhesion reveals that selectins mediate rolling interactions and integrins mediate firm adhesion processes. In this study the interaction between eosinophils and TNFα-activated HUVEC (second or third passage) was studied under flow conditions (0.8 and 3.2 dyn/cm^2). Especially the role of α_4 integrins on eosinophils and E-selectin on HUVEC was studied. Inhibition of the integrin α_4 chain on eosinophils reduced the number of firmly adhered resting eosinophils to TNFα-stimulated endothelium by 43 %, whereas the percentage of rolling cells increased 2.2-fold compared to untreated control eosinophils. Blocking of E-selectin on the endothelium reduced the number of adherent eosinophils only by 23 % and 16 %. In this situation however, hardly any rolling adhesion was observed, and the few rolling cells showed a low rolling velocity. Blocking both α_4 integrin on eosinophils and E-selectin on HUVEC reduced the number of adhered eosinophils by 95 %. P-selectin did not significantly participate in eosinophil adhesion to TNFα-activated HUVEC. Inhibition of both α_4 integrins and β_2 integrins on eosinophils resulted in a reduction of adhered cells by 65 % and a 3-fold increase in percentage of rolling cells. Taken together, these results clearly show that resting eosinophils preferentially use constitutively active α_4 integrins ($\alpha_4\beta_1$, $\alpha_4\beta_7$) for the first attachment to TNFα-activated HUVEC. In addition, α_4 integrins and E-selectin work synergistically in eosinophil adherence to TNFα-activated HUVEC. While E-selectin is important for eosinophil rolling under these conditions, P-selectin plays only a minor role.

Introduction

Eosinophils play an important role in allergic inflammatory diseases such as allergic asthma. Infiltrates of these cells are present in the interstitium and the lumen of the bronchi of asthmatic patients (1). Eosinophils must pass the endothelium to enter this site of inflammation. A widely accepted paradigm for leukocyte extravasation is the multistep model. In this model selectins mediate rolling interactions between leukocytes and endothelium and subsequently, activated integrins facilitate firm adhesion and extravasation of the cells (2). For eosinophils these specific interactions with the endothelium have not been fully elucidated and are subject of this study.

In marked contrast to neutrophils, eosinophils constitutively express the β_1 integrin $\alpha_4\beta_1$ (very late antigen 4, VLA-4, CD49dCD29) (3-6). VLA-4 is also present on monocytes, lymphocytes and basophils, while its counter structure VCAM-1 is present on activated endothelium (7;8). Although selectins primarily mediate rolling interactions, it has

been suggested that VLA-4 on lymphocytes (9;10) and cell lines (11) can play a role in this process as well. For eosinophils, discrepancies exist on the role of α_4 integrin in mediating initial attachment to the endothelium. A study in postcapillary venules of IL-1β-activated rabbit mesentery showed that eosinophils utilize VLA-4 and L-selectin for rolling interactions. However, still 50 % of the rolling interactions persisted even though VLA-4 and L-selectin were blocked (12). Very recently, Patel *et al.* (13) showed that eosinophils use α_4 integrins in tethering to IL-4-stimulated endothelial cells under flow conditions. In contrast, Kitayama *et al.* showed in a flow chamber model that α_4 integrins did not mediate initial attachment nor constitutive rolling interactions of eosinophils on TNFα-activated HUVEC but only mediated immediate arrest after P-selectin-dependent initial attachment (14).

In contrast to α_4 integrins, E-, P- and L-selectin are generally agreed upon as very important molecules in initial tethering and rolling adhesion of leukocytes to endothelium, including eosinophils. Under static conditions eosinophils can adhere both to P- and E-selectin (4;15). Under flow conditions eosinophils have been reported to accumulate more avidly on P-selectin compared to neutrophils (16). On the other hand, neutrophils adhere more efficiently to E-selectin-coated surfaces under flow conditions (17). In concordance with these findings, Kitayama *et al.* showed a role for P-selectin in the first attachment of eosinophils to TNFα-stimulated first passage HUVEC and did not see an effect on primary tethering when E-selectin was blocked (14). Taken together, eosinophils seem to be less E-selectin-dependent for rolling interactions with activated endothelium when compared to neutrophils. The role of L-selectin in rolling of eosinophils is thought to be of less importance than E- and P-selectin. However, L-selectin has been shown to mediate adhesion of eosinophils to HUVEC under conditions of shear, although experiments were performed at 4°C and no controlled shear stress was used (18).

Increased expression of VCAM-1, E-selectin and ICAM-1 is associated with pulmonary allergic inflammation (19-22). Therefore, these molecules and their ligands might be adhesion receptors for eosinophils to adhere to postcapillary venules present in the bronchial mucosa. However, many animal models do not lead to a consensus regarding this issue. An interesting primate model for asthma showed that anti-ICAM-1 therapy reduces lung eosinophilia and hyperreactivity (23). In addition, the latter study showed that after single antigen exposure anti-E-selectin treatment reduces lung neutrophilia in primates. In rats, anti-VLA-4 treatment did not have an effect on neutrophilia (24) nor on eosinophilia (25). In contrast, the combination treatment of anti-α_4 integrins and anti-VCAM-1 showed a reduction in eosinophil and lymphocyte infiltration in the lung of mice (26). *in vivo* studies in mice showed that recruitment of eosinophils (27;28) and other

leukocytes (29) at inflammatory sites is mainly mediated by P-selectin. However, these studies also suggest a role for E-selectin in this process (28;29).

In the current study the interactions between eosinophils and TNFα-activated HUVEC were investigated in an *in vitro* flow chamber model. Special emphasis was given to α_4 integrins on the eosinophils and E-selectin on the endothelium.

Materials and Methods

Reagents

Percoll was obtained from Pharmacia (Uppsala, Sweden). N-formyl-methionyl-leucyl-phenylalanine (fMLP) was purchased from Sigma Chemicals (St. Louis, MO). Human serum albumin (HSA) was purchased from the Central Laboratory of The Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands). Recombinant human TNFα was purchased from Boehringer Mannheim (Germany). HEPES incubation buffer contained 20 mM HEPES, 132 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.2 mM KH_2PO_4, supplemented with 5 mM glucose, 1.0 mM $CaCl_2$, and 0.5 % (w/v) HSA. All other materials were reagent grade.

Antibodies

The MoAb HP2/1 (anti VLA-4, CD49d) was purchased from Immunotech (Marseille, France). Two blocking anti E-selectin (CD62e) MoAbs were used; ENA_2 was kindly provided by Dr. W.A. Buurman (University Hospital, Maastricht) (30) and BBIG-E4 (5D11) was purchased from R&D systems (Abingdon, UK). MoAb IB4 and MoAb DREG56 were isolated from the supernatant of a hybridoma obtained from the American Type Culture Collection (Rockville, MD, USA). The MoAb WASP12.2 (anti P-selectin, CD62p) (31) was purchased from Endogen (Boston, MA, USA). The MoAb PL-1 (anti PSGL-1, anti CD 162) was purchased from Immunotech (Marseille, France). All MoAbs mentioned above are functionally blocking antibodies. Control antibody W6/32 (anti HLA-A,B,C) was isolated from the supernatant of a hybridoma obtained from the American Type Culture Collection (Rockville, MD, USA). MoAbs were incubated with eosinophils ($4x10^6$ cells/ml) or with confluent HUVEC layers on cover slips at 10 μg/ml during 15 minutes before the experiments. The cell suspensions were diluted once with incubation buffer (final concentration of 5 μg/ml MoAb at $2x10^6$ cells/ml in HEPES incubation buffer), and the coverslips were placed directly in the system.

Isolation of eosinophils

Blood was obtained from healthy volunteers from the Red Cross Blood Bank, Utrecht, The Netherlands. Mixed granulocytes were isolated from the buffy-coat of 500 ml blood anti-coagulated with 0.4 % (w/v) trisodium citrate (pH 7.4) as previously described (32). Mononuclear cells were removed by centrifugation over isotonic Ficoll (1.077 g/ml). After lysis of the erythrocytes with an isotonic ice-cold NH_4Cl solution, the granulocytes were washed and resuspended in RPMI 1640 (Gibco, Paisley, UK) with 0.5 % (w/v) human serum albumin (HSA).
Granulocytes were incubated for 30 min at 37°C to restore the initial density of the cells. Thereafter, the cells were washed and resuspended in phosphate-buffered saline (PBS) supplemented with 0.5 % HSA and 13 mM trisodium citrate, and incubated with fMLP (10 nM) for 10 min at 37°C, to decrease the specific gravity of the neutrophils, but not that of the eosinophils. Subsequently, eosinophils were obtained by centrifugation (20 min, 1000 g) over isotonic Percoll (density 1.082 g/ml, layered on Percoll with a density of 1.1 g/ml), washed and resuspended in HEPES incubation buffer. Purity of eosinophils was >95 %, (and recovery was usually 80-90 %). This procedure leads to the isolation of relatively unprimed eosinophils compared to conventionally used isolation procedures with immunomagnetic beads (33).

Endothelial cells

Human umbilical vein endothelial cells (HUVEC) were isolated from human umbilical cord veins according to Jaffe *et al.* (34), with some minor modifications (35). The cells were cultured in RPMI 1640 containing 20 % (vol./vol.) heat-inactivated human serum, 200 µg/ml penicilin/streptomycin (GIBCO, Life Technologies, Breda, The Netherlands) and fungizone (GIBCO, Life Technologies, Breda, The Netherlands). Cell monolayers were grown to confluence in 5-7 days. Endothelial cells of the second passage or third passage were used in perfusion assays. HUVEC was activated by TNFα (100 U/ml, 7 hours, 37°C) prior to the perfusion experiments.

Perfusion chamber

Perfusions under steady flow were performed in a modified form of transparent parallel plate perfusion chamber (36) as previously described by Van Zanten *et al.* (35). This micro-chamber has a slit height of 0.2 mm and width of 2 mm. The chamber contains a circular plug on which a coverslip (18 mm x 18 mm) with confluent HUVEC was mounted.

Eosinophil perfusion and evaluation

Eosinophils in suspension ($2x10^6$ cells/ml in HEPES incubation buffer) were aspirated from a reservoir through plastic tubing and the perfusion chamber with a Harvard syringe pump (Harvard Apparatus, South Natic, MA). In this way, the flow rate through the chamber could be precisely controlled. The wall shear stress (t) was calculated according to the Navier Stokes equation: $t = (6Q \bullet \eta)/(w \bullet h^2)$. In this equation Q is the volumetric flow rate, η is the suspending medium coefficient of viscosity (assumed to be equal to water at 0.01 poise), w the slit width and h is the slit height. The shear stress is proportional to the rate of flow of the cells and can be calculated as dyn/cm^2.

Eosinophil perfusions were performed as individual runs under specific shear conditions all in a 37°C temperature box. During the perfusion, the flow chamber was mounted on a microscope stage (DM RXE, Leica, Weitzlar, Germany) which was equipped with a B/W CCD-video-camera (Sanyo, Osaka, Japan), coupled to a VHS video recorder. Perfusion experiments were recorded on video tape. Video images were evaluated for the number of adhered cells, the rolling velocity per cell and the cluster index, using dedicated routines made in the image-analysis software Optimas 6.1 (Media Cybernetics systems, Silverspring, MD, USA). The eosinophils which were in contact with the surface, appeared as bright white-centered cells after proper adjustment of the microscope during recording. The adhering cells on the HUVEC were detected by the image-analyzer. The number of surface-adhered eosinophils was measured after 5 minutes perfusion at a minimum of 25 randomized high power fields (total surface of at least 1 mm^2). Then buffer was perfused and shear rates were increased from 0.8 to 2, 3.2 and 6.4 dyn/cm^2 each for 1 minute during which high power fields were recorded to determine rolling velocities at these shear rates. To automatically determine the velocity of rolling cells custom-made software was developed in Optimas 6.1. A sequence of 50 frames representing an adjustable time interval (δt, with a minimal interval of 80 milliseconds) was digitally captured. At each frame, the position of every cell was detected and for all subsequent frames the distance travelled by each cell and the number of images in which a cell appears in focus was measured. The velocity of a cell (v) in micrometers per sec was calculated from the equation: $v = L/\delta t(x-1)$ in which L is the covered distance (μm), δt is the time interval between images (seconds), and x the number of images in which a cell appears. The cut-off value to distinguish between rolling and static adherent cells was set at 1 μm/sec. Rolling velocity classes were depicted which is defined as the fraction of the total number of rolling cells in each group exhibiting a rolling velocity corresponding to the velocity classes as indicated (figure 3). With this method, static adherent, rolling and freely flowing cells (which were not in focus) could be clearly distinguished. Cluster index was measured as previously

described (37). The number of surface adhered eosinophils per mm^2 was measured after 5 minutes of perfusion at a minimum of 20 randomized fields. For each adherent cell the number of cells in the surrounding area of approximately 1750 μm^2 was measured. In the case of a random distribution, the expected number of cells inside this area was calculated based upon the mean number of surface-adherent cells per mm^2. The cluster index was set to be the difference between the measured and the expected number of cells inside an arbitrary area around the cell. In equation: Cluster index per cell = |m-(X*a/A-1)|, in which m is the measured number of cells in the rectangle area, X is the total number of cells in the image, A is the size of the total image and a is the size of a rectangular cell-surrounding area. For each experiment the mean cluster index of a minimum of 500 cells was calculated.

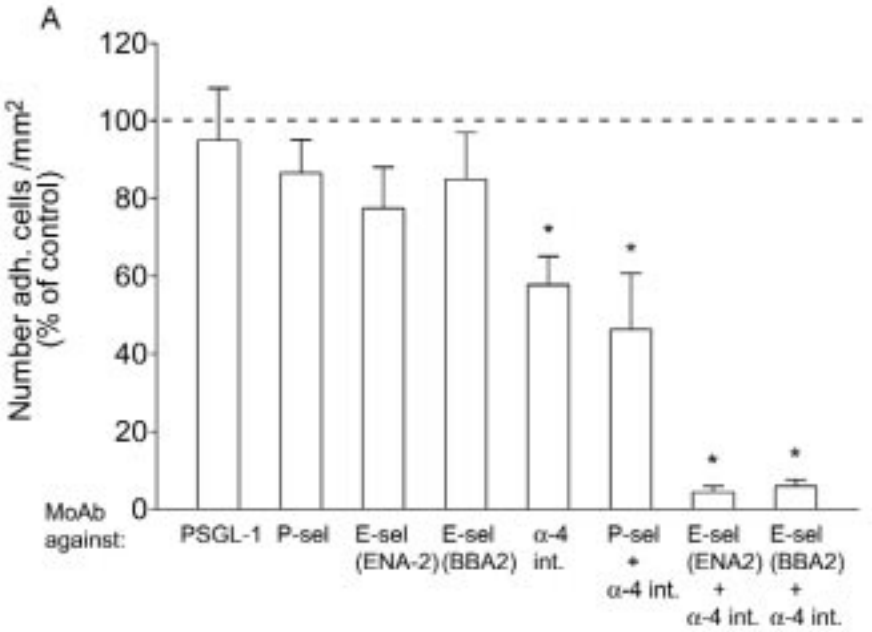
Statistical Analysis. Results are expressed as mean ± standard error of the mean (SEM). Statistical analysis of the data was performed using a one-way ANOVA against fixed value with Bonferroni correction, one-way ANOVA with Bonferroni correction or paired Student T test as indicated in the figures. P values < 0.05 were considered to be significant.

Results

E-selectin and α_4 integrins are important adhesion molecules in initial attachment to TNFα-stimulated HUVEC

The role of different adhesion molecules in eosinophil-endothelium interactions was examined using blocking antibodies. Figure 1 shows the number of adherent cells per mm^2 (= total number of firmly adhered and rolling cells) to activated HUVEC (TNFα, 100 U/ml, 7h) under flow conditions (0.8 dyn/cm^2) as a percentage of control (untreated) eosinophils. Panel A focuses on the adhesion molecules mediating rolling and panel B focuses on those mediating adhesion interactions. Treatment of eosinophils with the anti-PSGL-1 antibody PL-1 or treatment of HUVEC with anti-P-selectin WASP12.2 did not significantly decrease the number of adherent cells, showing the minor importance of these adhesion molecules under these conditions. HUVEC incubated with anti-E-selectin antibodies (ENA-2 or 5D11) showed a slight decrease in adherent cells. However, this did not reach significance. When eosinophils were incubated with the anti-integrin α_4 chain antibody HP2/1, only 58 ± 7 % of the eosinophils were able to adhere. Interestingly, a combination of anti-α_4 MoAb with anti-E-selectin MoAbs ENA-2 or BBA2 almost completely abolished adhesion. The percentage of binding eosinophils was

only 4.3 ± 1.6 % and 5.9 ± 1.7 %, respectively. This additive effect was not observed for anti-P-selectin treatment in combination with HP2/1 (46 ± 16 % adhesion, figure 1A). In panel 1B the treatment of eosinophils with the anti-integrin α_4 chain antibody HP2/1 is shown again. Furthermore, the combination of blocking α_4 integrins and β_2 integrins resulted in an additive effect whereby 35 ± 9 % of the cells remained adherent. This effect was significant (paired Student T test; $p=0.002$). The combination of blocking E-selectin and α_4 integrins is significantly different from blocking α_4 integrins alone (ANOVA with Bonferroni correction: $p<0.005$). Treatment of β_2 integrins by MoAb IB4 resulted in 89 ± 7 % of adherent eosinophils compared to control. Also, blocking both β_2 integrins on the eosinophils and E-selectin on the endothelium did not result in a change in percentage adherent cells (89 ± 12 %). Treatment of eosinophils with the antibody W6/32 (anti HLA-A,B,C) compared to control eosinophils (no MoAb) showed no difference in adhesion, 1169 ± 233 and 1292 ± 134, respectively ($p=0.362$ paired Student T test).



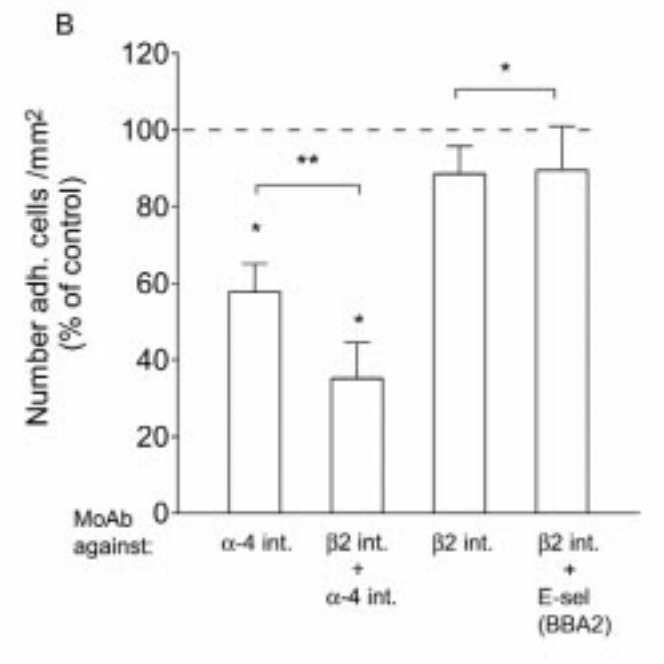
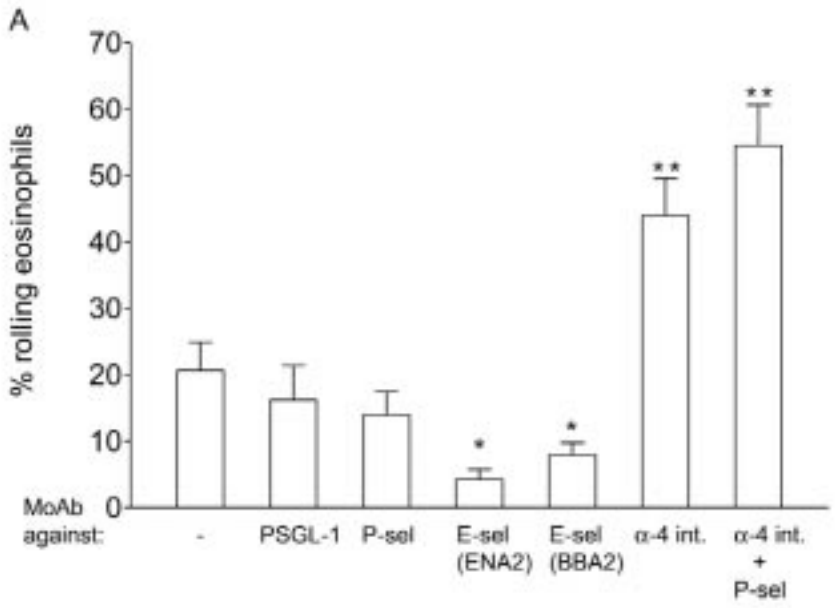


Figure 1. Effect of inhibition of selectins and integrins (int.) with blocking antibodies on the interaction of eosinophils to TNFα-activated endothelium. Panel A shows the effect of blocking rolling receptors and panel B shows the effect of blocking firm adhesion receptors (except the combination in which E-selectin is blocked). Cells ($2x10^6$/ml) preincubated in the presence or absence of blocking monoclonal antibodies (10 μg/ml, 15 minutes, 37°C) were perfused at a shear stress of 0.8 dyn/cm^2 during 5 minutes, and images were recorded on video. At least 30 images were counted off line by image analysis (see Materials and Methods section). Number of adherent eosinophils (rolling and firm adherent cells) was determined as a percentage of control values (resting, non treated eosinophils on 7h TNFα-stimulated HUVEC). The absolute number of adherent cells in the control situation was 1142 ± 167 eosinophils/mm^2. Percentages are shown ± SEM of 3 to 5 different experiments. The statistically significant effects of the blocking antibodies were determined by ANOVA against fixed value with Bonferroni correction for non-paired data (* $p<0.001$) or by paired Student T test for paired data (** $p=0.002$).

E-selectin, α4 integrins and β_2 integrins function differently in the rolling behaviour of eosinophils

The role of different adhesion molecules in rolling processes of eosinophils on activated endothelium was examined using blocking antibodies. Percentage of rolling cells was determined by image analysis of video images (see Materials and Methods section, figure 2). Panel 2a focuses on adhesion molecules mediating rolling interactions. On activated HUVEC (TNFα, 7h, 37°C), 21 ± 4.4 % of the surface-interacting eosinophils were rolling. Blocking PSGL-1 or P-selectin did not affect percentage rolling compared to the control situation. By blocking E-selectin the percentage rolling cells decreased by more then 50 % compared to control (ENA-2: 4.2 ± 1.8 %, BBA2: 8.0 ± 2.0). A significant increase in the percentage rolling cells was observed when α_4 integrins were blocked (44 ± 5.9 %). However, blocking both α_4 integrins and P-selectin did not significantly differ from blocking α_4 integrins alone (54 ± 7.5 %). Panel 2b focuses on adhesion molecules mediating firm adhesion. The control situation and treatment of eosinophils by anti α_4 integrins are depicted as in figure 1a. Blocking β_2 integrins did not have a significant effect on percentage of rolling cells. By blocking both β_2 and α_4, again a significant increase in the percentage of rolling cells was observed (61 ± 8.1 %). Blocking α_4 integrins and E-selectin simultaneously resulted in an amount of cells too low to evaluate percentage of rolling cells.



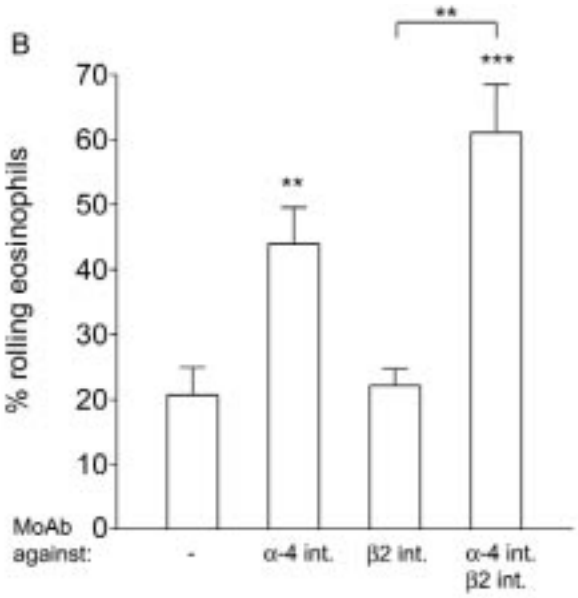


Figure 2. Effect of inhibition of selectins and integrins (int.) with blocking antibodies on the percentage of rolling eosinophils on TNFα-activated endothelium. A) shows the effect of blocking rolling receptors and B) shows the effect of blocking firm adhesion receptors. Cells ($2x10^6$/ml) preincubated in the presence or absence of blocking monoclonal antibodies (10 μg/ml, 15 minutes, 37°C) were perfused at shear stress 0.8 dyn/cm^2 and during the second and third minute of perfusion at least three areas were recorded on video for a period of at least 5 seconds. At least 100 cells were studied per perfusion and data were analyzed off line by image analysis (see Materials and Methods section). Percentage of rolling cells (>1 μm/sec) of the total amount of adhering cells was calculated. Means are plotted for 3 to 4 experiments ± SEM. The statistically significant effects of the blocking antibodies against control situation or between different treatments (as indicated in figure) were determined by paired Student T-test (*: $p<0.05$) or One Way ANOVA with Bonferroni correction (**: $p< 0.005$, ***: $p<0.001$).

Rolling velocities are shown in figure 3. The percentage cells in each velocity class is plotted against the different velocity classes. Rolling velocity classes are defined as the fraction of the total number of rolling cells in each group exhibiting a rolling velocity corresponding to the velocity classes as indicated. The median and mean rolling velocities are shown in table I. The mean rolling velocity of control eosinophils to activated endothelium was 4.8 μm/sec (median; 2.6 μm/sec). Eosinophils treated with anti PSGL-1 (PL-1, median; 2.8 μm/sec), anti β_2 (IB4, median; 3.1 μm/sec) and HUVEC treated with WASP12.2 (anti-P-selectin, median; 3.6 μm/sec) showed the same profile in frequency distribution as control cells (data not shown). However, when E-selectin was

blocked, the mean rolling velocity decreased to 4.3 μm/sec and the median rolling velocity was shifted to 1.7 μm/sec, although this was not significantly different from the control situation. By blocking α_4 integrins it was shown that besides the significant increase in percentage of rolling cells, the mean rolling velocity of the cells increased to 12.2 μm/sec (median; 9.4 μm/sec). Blocking both α_4 integrins and P-selectin or both α_4 integrins and β_2 integrins resulted in similar velocity profiles compared to blocking of α_4 integrins alone (data not shown, medians; 12.2 μm/sec and 10.0 μm/sec, respectively).

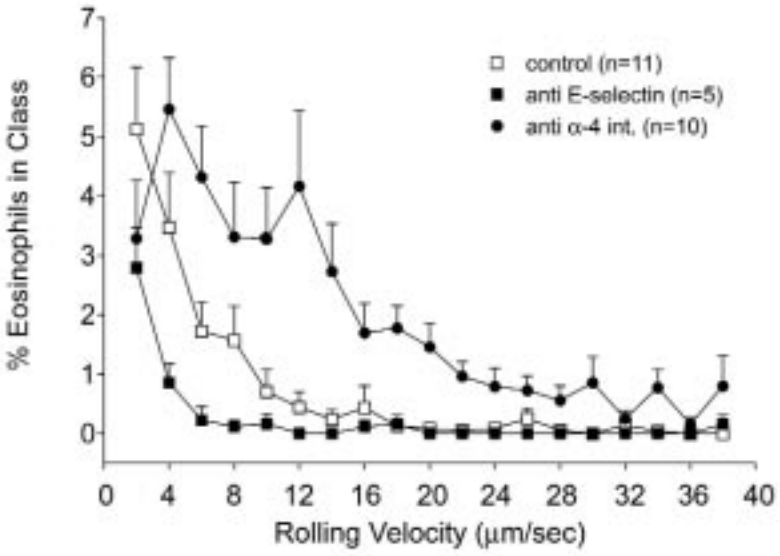


Figure 3. Effect of inhibition of selectins and integrins with blocking antibodies on the rolling velocity of eosinophils on TNFα-activated endothelium. Frequency distribution of rolling cells at shear stress 0.8 dyn/cm^2. Percentage of rolling cells of the total number of cells (= rolling and firmly adhering to the endothelium) in each velocity class is plotted. The sum of the percentages per group equals the percentages shown in figure 2. First point represents velocity class of 1 to 2 microns/sec, following points represent velocity class of 2 to 4, 4 to 6 microns/sec etc. Cells rolling faster than 1 μm/sec were considered as rolling cells. Data represent mean percentages ± SEM of indicated number of experiments.

Table I. Effect of inhibition of selectins and integrins with blocking antibodies on the median rolling velocity of eosinophils on TNFα-activated endothelium.

Blocking:	Median rolling velocity (μm/sec)	Mean rolling velocity (μm/sec)	p value
- (control)	2.6	4.8	-
P-selectin	3.6	5.2	n.s.
PSGL-1	2.8	6.1	n.s.
E-selectin	1.7	4.3	n.s.
VLA-4 (α4 integrin)	9.4	12.2	< 0.001
VLA-4 and P-selectin	12.2	14.4	< 0.001
VLA-4 and β2 integrins	10.0	12.7	< 0.001
β2 integrins	3.1	5.5	n.s.

Eosinophils were incubated with indicated MoAbs (10 μg/ml) for 15 minutes and flow experiments on 7h TNFα-activated HUVEC at 0.8 dyn/cm^2 were performed. The rolling velocities of at least 100 cells per experiment out of at least 3 experiments were determined (see Materials and Methods) and the median and mean rolling velocities (μm/sec) were calculated. The statistically significant effects (p) of the blocking antibodies against control situation were determined by one-way ANOVA with Bonferroni correction.

Effect of different shear rates on rolling velocities and behaviour of eosinophils

The effects of different shear rates on median rolling velocities of resting untreated eosinophils and HP2/1-treated eosinophils were examined. Eosinophils were allowed to interact with the endothelial surface for 5 minutes (0.8 dyn/cm^2). Then buffer was perfused with increasing shear stresses of 2, 3.2 and 6.4 dyn/cm^2 each for 1 minute. Rolling velocities of at least 100 cells were measured at these time intervals and the median rolling velocity of the total amount of rolling cells is shown in table II. Increasing shear forces of 0.8, 2, 3.2 and 6.4 dyn/cm^2 had a slight increasing effect on median rolling velocity of resting eosinophils on TNFα-activated HUVEC. The mean rolling velocity of control eosinophils at a shear rate of 3.2 and 6.4 dyn/cm^2 was significantly different ($p<0.05$) from the rolling velocity at shear rate 0.8 dyn/cm^2. The median rolling velocity of eosinophils treated with HP2/1 increased from 8.6 μm/sec at 0.8 dyn/cm^2 to 19.5 μm/sec at 6.4 dyn/cm^2. These cells increased their mean rolling velocity with

increasing shear stresses of 2, 3.2 and 6.4 dyn/cm^2 significantly ($p<0.001$) compared to a shear stress of 0.8 dyn/cm^2. The mean rolling velocity of control eosinophils versus HP2/1-treated eosinophils was significantly lower at all four different shear rates ($p<0.001$, table II).

Table II. Median rolling velocities and mean rolling velocities (± SEM) of total amount of rolling cells at different shear rates for resting control eosinophils and anti-α_4 integrin-treated eosinophils on 7h TNFα-stimulated HUVEC.

	Median (μm/s)		Mean (μm/s ± SD)		p value
dynes/cm^2	-	HP2/1	-	HP2/1	
0.8	3.2	8.6	4.7 ± 0.34	10.9 ± 0.54	< 0.001
2.0	3.8	12.8$^{\#}$	5.5 ± 0.24	13.6 ± 0.41*$^{\#}$	< 0.001
3.2	4.3	15.0	5.8 ± 0.27*	16.3 ± 0.41*	< 0.001
6.4	5.8	19.5	8.0 ± 0.30*	21.0 ± 0.43*	< 0.001

Effect of different shear stresses on the rolling velocity of eosinophils on TNFα-activated HUVEC in the presence or absence of an anti-α_4 integrin MoAb (HP2/1). After 5 minutes of perfusion at a shear stress of 0.8 dyn/cm^2 the shear was increased to 2.0, 3.2 and 6.4 dyn/cm^2 during 1 minute, respectively. Median and mean rolling velocities (μm/sec) ± SEM were calculated from at least 100 cells (see Materials and Methods section) out of three experiments (except $^{\#}$: n=2). The statistically significant effects (p) of anti-α_4 integrin treatment (HP2/1) versus control situation were determined for each velocity class by one way ANOVA. The asterisk (*) indicates the significant effects ($p<0.05$) of the mean rolling velocity in the shear class 2, 3.2 and 6.4 dyn/cm^2 versus the 0.8 dyn/cm^2 value in each group (control- and HP2/1-treatment) determined by one-way ANOVA.

Effect of high shear stress on adhesion and rolling of eosinophils

When resting, untreated eosinophils were perfused at a shear stress of 3.2 dyn/cm^2 during 5 minutes, the total number of adherent cells was 1089 ± 167, which was not significantly different from experiments performed at a shear stress of 0.8 dyn/cm^2 ($p=0.72$ paired Student T test). At a shear stress of 3.2 dyn/cm^2, strings (cell clusters in the direction of the flow) were visible. This suggests a role for L-selectin (19;38). To address the role of L-selectin in this situation, blocking studies were performed. In the control situation strings were observed (figure 4a) and the cluster index was

1.5 ± 0.2. Inhibition of L-selectin by blocking MoAb DREG-56 (10 μg/ml) resulted in a cluster index of 0.85 ± 0.2 (p=0.05 Paired Student T-test) and less strings were observed compared to the control situation as shown in figure 4b. Incubation of eosinophils with control antibody W6/32 (anti HLA-A,B,C) did not affect percentage of adherent cells (103 ± 14 %). Also, blocking E-selectin did not significantly influence the number of adherent cells (102 ± 14 %) while blocking α_4-integrins did (39 ± 8 %). When both L-selectin and E-selectin were blocked still 30 ± 8 % of the eosinophils remained adherent (table III). No cells were attached to the surface when both E-selectin and α_4 integrins were blocked, which is similar to the situation at a shear stress of 0.8 dyn/cm^2 (n=1). The percentage rolling cells at a shear stress of 3.2 dyn/cm^2 increased 1.5-fold compared to 0.8 dyn/cm^2. When α_4 integrins were blocked at a shear stress of 3.2 dyn/cm^2, 67.5 ± 14 % of the cells were rolling. In contrast, when E-selectin was blocked only 13.6 ± 6 % of the cells showed a rolling interaction, which did not decrease further when L-selectin was blocked in addition to E-selectin. The mean rolling velocity in the control situation was 7 ± 0.5 μm/sec, which is 1.5 times higher than at a shear stress of 0.8 dyn/cm^2. Inhibition of α_4-integrin increased the rolling velocity to 18 ± 0.6 μm/sec which is 1.6 times as high as at a shear stress of 0.8 dyn/cm^2 (table II). However, blocking E-selectin did not significantly reduce the rolling velocity compared to the control situation. Also, inhibition of both L-selectin and E-selectin did not significantly differ from the control situation.

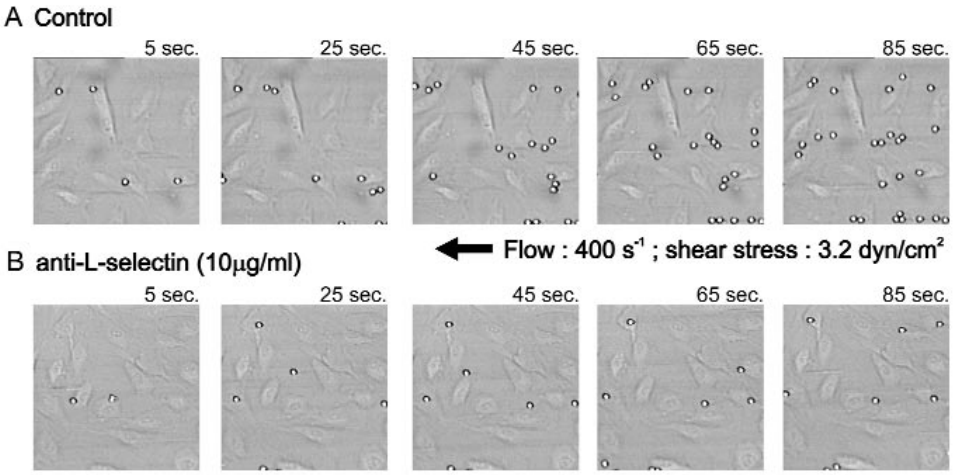


Figure 4. Effect of inhibition of L selectin with blocking antibody DREG56 (10 μg/ml) on the distribution pattern of eosinophils on TNFα-activated endothelium. Cells were perfused in the presence or absence of anti-L-selectin over 7h TNFα-activated HUVEC at a shear stress of 3.2 dyn/cm^2. Images were recorded on video during 5 minutes, and cluster indexes were determined subsequently (see Materials and Methods): A;1.8 and B;1.1. The images depicted above were taken after 30 seconds of cell perfusion in a time frame as indicated. The experiment shown is representative of 2 experiments.

Table III. Effect of inhibition adhesion molecules with blocking antibodies on adhesion, percentage rolling cells and the mean rolling velocity of eosinophils on TNFα-activated endothelium at a shear rate of 3.2 dyn/cm^2.

Blocking:	Adherent cells (% of control)	% rolling cells	Mean rolling velocity (μm/s)
-	100	32.6 ± 8.5	7.1 ± 0.48
VLA-4 (α4 integrin)	38.9 ± 7.7*	67.5 ± 14.2**	18.2 ± 0.64***
E-selectin	101.6 ± 13.6	13.6 ± 6.0**	7.3 ± 0.84
E-selectin and L-selectin	29.8 ± 7.6*	14.2 ± 3.4**	4.6 ± 0.69

Eosinophils were incubated in the presence or absence of indicated MoAbs (10 μg/ml) for 15 minutes, and flow experiments on 7h TNFα-activated HUVEC at 3.2 dyn/cm^2 were performed. Images were recorded on video. Data are plotted for 3 to 7 experiments ± SEM. At least 30 images were counted off line by image analysis and number of adherent eosinophils (rolling and firm adherent cells) was determined as a percentage of control values (resting, non treated eosinophils on 7h TNFα-stimulated HUVEC). The statistically significant effects of the blocking antibodies were determined by ANOVA against fixed value with Bonferroni correction (*: $p<0.005$). Percentage rolling cells (>1μm/sec) of the total amount of adherent cells was calculated. The statistically significant effects of antibody-treated eosinophils in contrast to control cells on percentage rolling cells were determined by a paired Student T-test (**: $p<0.05$). The rolling velocities of at least 40 cells per experiment were determined (see Materials and Methods). The statistically significant effects of antibody treated eosinophils in contrast to control cells on mean rolling velocity were determined by one-way ANOVA with Bonferroni correction (***: $p<0.001$).

Discussion

In this study, the interactions between resting human eosinophils and TNFα-stimulated HUVEC were evaluated using a flow chamber model. TNFα-stimulated HUVEC (7 hours) was used to induce increased VCAM-1, E-selectin and ICAM-1 expression, simulating the vasculature in allergic inflammation. The influence of inhibition of different adhesion molecules with blocking monoclonal antibodies was evaluated with real-time video-assisted image analysis. By determination of the number of adherent cells, percentage rolling cells and rolling velocity distributions, we showed that eosinophils preferentially use constitutively active α_4 integrins for mediating the first attachment to activated HUVEC. Also, E-selectin on the activated endothelium is used for initial attachment, and it mediates rolling interactions even at high shear stress. Most strikingly, blocking α_4 integrins and E-selectin together totally abolished adhesion, indicating the strong cooperation of these two molecules in eosinophil adhesion, to activated HUVEC and the minor importance of β_2 integrins under these conditions.

The well-established multistep paradigm for leukocyte-endothelium interactions describes rolling adhesion mediated by selectins and firm adhesion mediated by integrins. These processes are divided in time and place. However, the situation is more complex, since several recent studies described a situation in which both tethering and firm adhesion are mediated by the same adhesion receptors. A clear example for this overlapping behaviour are the α_4 integrins ($\alpha_4\beta_1$, $\alpha_4\beta_7$), which have been described as rolling receptors (9-12) besides their role in firm adhesion (5;14;39). The α_4 integrins are present on lymphocytes, monocytes, basophils and eosinophils but are not expressed on neutrophils. Berlin *et al.* (9) were the first to show α_4-dependent rolling of mouse lymph node cells on VCAM-1- and MADCAM-1-coated surfaces. Later studies also showed initial tethering and rolling interactions by α_4 integrins of human T lymphocytes (11) and B lymphocytes (40) in a VCAM-1-dependent way. For monocytes some studies showed that α_4 integrins play a role in stabilizing initial tethering on activated endothelium (39;41) and another study showed that monocytes can tether on VCAM-1 (42). For eosinophils little is known about the role of α_4 integrins in attachment to inflamed endothelium. Alpha 4 integrins on resting, non activated eosinophils are functionally active for adherence to VCAM-1-coated substrates or activated HUVEC under static conditions (3;4;5;6;43). We confirmed this by performing experiments in which resting eosinophils bound to VCAM-1-coated magnetic beads or VCAM-1-coated 96-well plates. This interaction could be specifically blocked by MoAb HP2/1 (data not shown). However, the question remained if α_4 integrins on human eosinophils are functionally active in mediating initial attachment to activated HUVEC under flow conditions. Thus far, a few groups have investigated this in an *in vitro* flow system (13;14). We used

an *in vitro* flow chamber assay to study the interactions of the α_4 integrins on eosinophils with activated endothelium under different shear forces. Also, the importance of the rolling receptor E- and P-selectin were studied, under the same conditions.

We found that inhibition of α_4 integrins (CD49d) by blocking anti-α_4 chain MoAb HP2/1 resulted in more than 40 % decrease in the total number of adherent eosinophils to TNFα-activated HUVEC (7h, 100 U/ml), while blocking E-selectin or P-selectin did not have a significant effect on adhesion. Remarkably, the percentage adherent eosinophils decreased by more than 95 % by blocking both α_4 integrin and E-selectin (figure 1). This was found for each of the two anti-E-selectin antibodies (ENA-2, 5D11) that were tested. In contrast, no synergistic effect was observed when α_4 integrin and P-selectin were both blocked. We conclude that in our system α_4 integrin and E-selectin are both important for the first attachment to activated HUVEC at a shear stress of 0.8 dyn/cm^2. In contrast with another report (14), P-selectin did not play a role under these conditions (see below). Thus, α_4 integrins and E-selectin together are responsible for initial attachment and work synergistically in our model.

These results are in disagreement with the study of Kitayama *et al.* (14). These authors concluded that α_4 integrins play a minor role in initial attachment, while we did find a major role for α_4 in this process. They also showed that P-selectin and not E-selectin is important in eosinophil rolling on TNFα-stimulated HUVEC (6h, 100 U/ml). These differences are difficult to explain. However, two considerations must be taken in account. (I) The (pre)activation status of eosinophils is of major importance in this type of studies. Eosinophils from mild eosinophilic donors (44) or cells isolated via different isolation procedures (33;44) might have been preactivated and have upregulated their β_2 integrins and thereby have altered adhesion characteristics to endothelial cells (see below). (II) We used second passage HUVEC, which did not express an appreciable amount of P-selectin (data not shown), whereas Kitayama *et al.* used first passage HUVEC possibly expressing sufficient amounts of P-selectin (39;42;45). P-selectin can be expressed significantly on HUVEC in response to stimulation with histamine (46) or thrombin (31;47). However, another important source for P-selectin are platelets. In this respect it was shown that activated and P-selectin-expressing platelets can bind leukocytes, thereby facilitating P-selectin-dependent secondary tethering to the surface (48). Interestingly, platelets have been shown to adhere to eosinophils of allergic donors and might contribute to P-selectin-mediated interactions between eosinophils and endothelial cells (49). In our studies, eosinophils were carefully studied and no attached platelets were present on the cell surface.

Blocking the β_2 integrins did not result in a significant decrease in adhesion, whereas the combination of anti-α_4 integrin antibodies and anti-β_2 integrin antibodies resulted in a percentage adherent cells of 35 ± 9 % compared to the control situation (figure 1). This 65 % reduction was significantly different from anti-α_4 treatment alone, suggesting that β_2 integrins only play a minor role for resting eosinophil adherence to activated HUVEC. In contrast, Kitayama *et al.* (14) and Patel (13) showed that the combination of anti-β_2 integrins and anti α_4 integrins totally abolished accumulation of eosinophils on 6h TNFα-activated HUVEC and 24 h IL-4-activated HUVEC, respectively. This suggests differences in activating state of the β_2 integrins of eosinophils isolated by different methods (see above). The combination of blocking β_2 integrins and E-selectin did not have a significant effect on adhesion compared to control. This further suggests that α_4 integrins are perfectly capable in mediating initial attachment to the endothelium under these conditions.

Although eosinophils express less sialylated Lewis^x (Sle^x) than neutrophils, causing these cells to bind E-selectin less avidly (50), neutrophils as well as eosinophils are able to roll on E-selectin (51). Figure 2 shows that eosinophils roll on E-selectin, because inhibition of E-selectin resulted in an immediate arrest of more than 90 % of surface-interacting cells. This α_4 integrin-dependent immediate arrest of eosinophils was also shown by others (13;14) and it has also been shown for monoblastoid and lymphoblastoid cells (52). When α_4 integrins were blocked however, eosinophils rolled on the surface using E-selectin. The anti α_4-treated cells rolled faster compared to untreated control eosinophils. This suggests that the α_4 integrin/VCAM-1 interaction decreases cell rolling velocity. Simultaneous inhibition of α_4 and β_2 integrins did not result in higher rolling velocities (figure 3, table I), thereby confirming the unprimed phenotype of eosinophils (53;54). Blocking E-selectin reduced the rolling velocity of the eosinophils, whereas blocking PSGL-1 did not. This indicates that PSGL-1 is not the ligand for E-selectin on eosinophils. Alternatively, one (or more) of the known E-selectin ligands (cutaneous lymphocyte antigen (CLA) (55), E-selectin ligand 1 (ESL-1) (56) and/or L-selectin (57)) might be involved.

Next, we wanted to test the resistance of the rolling interactions of eosinophils on activated HUVEC. Therefore, the shear stress was increased at the end of 5 minutes cell perfusion from 0.8 to 2, 3.2 and 6.4 dyn/cm^2 each for 1 minute (table II). Rolling velocities of resting eosinophils increased with increasing shear stresses: 0.8 to 2, 3.2 and 6.4 dyn/cm^2 and a significant difference between mean rolling velocities was found between 0.8 and 6.4 dyn/cm^2. When these shear forces were applied to anti-α_4 integrin treated eosinophils, cells rolled with velocities which were at least two times higher

at all four different shear stresses. These results again show that α_4 integrins are also active in reducing the rolling velocity at high shear stress.

At a shear stress of 0.8 dyn/cm^2, L-selectin played a minor role in eosinophil recruitment to the activated endothelium (data not shown). It is known that L-selectin is functionally important above a certain shear rate threshold (39;42). To address the importance of L-selectin on eosinophils, which had been shown to mediate adhesion to activated endothelium under shear (18), we applied 3.2 dyn/cm^2 to the system during 5 minutes (figure 4). L-selectin-dependent tethering with formation of cell clusters became important in the eosinophil recruitment to TNFα-activated HUVEC at this shear stress. Inhibition of both α_4 integrin and E-selectin totally abolished adhesion of eosinophils as was also observed at a shear stress of 0.8 dyn/cm^2, suggesting that L-selectin only mediates secondary tethering processes at high shear stresses. Remarkably, inhibition of both L-selectin and E-selectin resulted in a residual binding of 30 ± 8 % of the eosinophils compared to the control situation (table III). Thus, even at high shear stress α_4 integrins are functional in mediating initial attachment to activated endothelium.

In conclusion, we showed that resting eosinophils use constitutively active α_4 integrins and to a lesser extent E-selectin on TNFα-activated HUVEC for initial attachment under flow conditions. Also, E-selectin can mediate stable rolling interactions even at high shear stresses. These data provide new insights in the way eosinophils can get recruited to inflamed endothelium, and emphasize the importance of E-selectin as rolling receptor for eosinophils on TNFα-activated endothelium. The importance of identification of the mechanisms involved in eosinophil extravasation is that it might lead to development of specific antagonists of this process, which might be used as targets for specific anti-allergic treatment.

Acknowledgements

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CHAPTER 3

Adhesion of eosinophils of allergic asthmatic patients to activated endothelial cells under flow conditions: role of platelets

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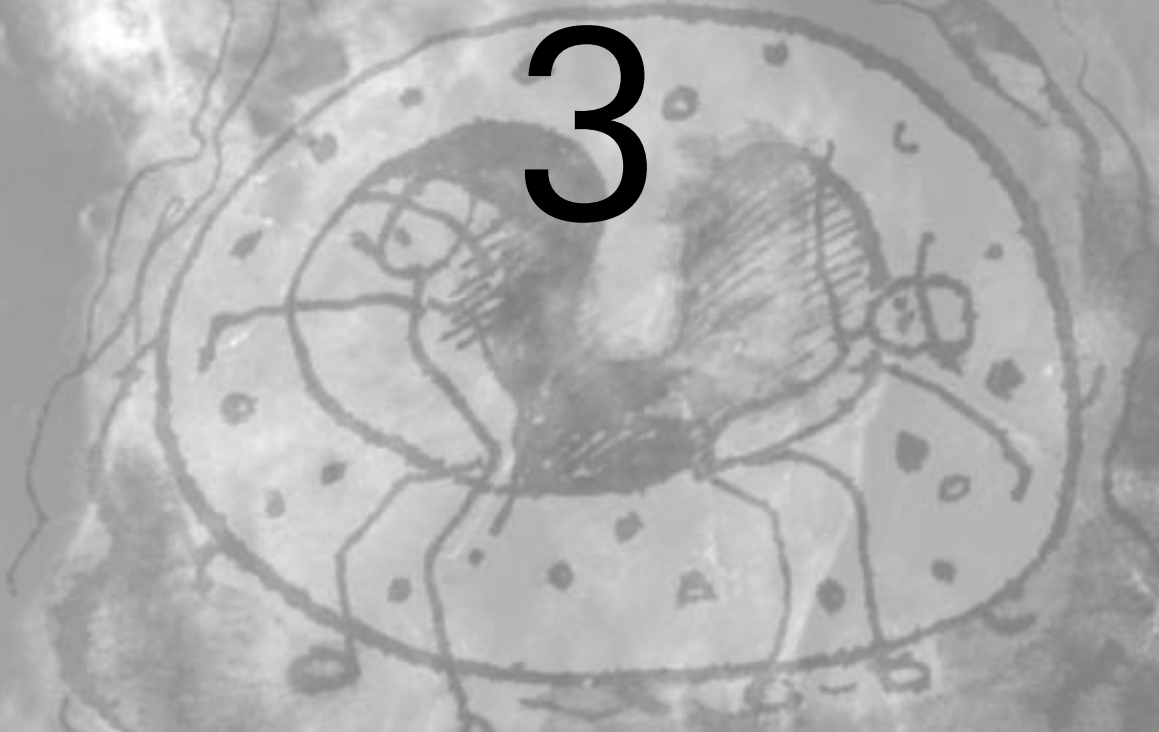

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Abstract

During the late-phase asthmatic response eosinophils migrate to the bronchial tissue and cause severe damage. In this study we compared *in vivo* primed eosinophils from allergic asthmatics with eosinophils from healthy controls in their adhesion behaviour to TNF-α-activated endothelium under flow conditions (0.8 dyn/cm^2). More eosinophils from asthmatic patients compared to cells from healthy controls adhered to activated endothelium (1240 ± 130 vs. 890 ± 90 cells/cm^2 respectively). In the presence of blocking antibodies directed against VLA-4 and E-selectin the residual binding of the cells of allergic asthmatic individuals was significantly higher than that of the healthy controls (28 ± 4 vs. 12 ± 3 %, $p<0.02$, of control situation, respectively). In addition, secondary tethering of the eosinophils of allergic asthmatic patients was significantly increased compared to the healthy controls (cluster indices 1.8 ± 0.3 vs.0.8 ± 0.2, respectively, $p<0.05$). Since patient cells showed an enhanced interaction with platelets during the perfusions, the role of P-selectin on platelets was investigated. Blocking antibodies directed against P-selectin reduced the enhanced binding and clustering of eosinophils of allergic asthmatic patients. We conclude that P-selectin-bearing platelets contribute to secondary tethering processes of eosinophils to activated endothelium. Therefore, platelets might play an important role in chronic inflammatory processes.

Introduction

Eosinophil accumulation is a hallmark of allergic inflammation, as found in the airway mucosa of asthmatic patients. Adhesion of eosinophils to endothelial cells of the blood vessel is one of the initial and crucial steps in the accumulation of eosinophils to these allergic inflammatory sites. As all leukocytes, eosinophils use adhesion molecules in a multistep mechanism to adhere to endothelial cells and migrate to the inflammatory site (reviewed in (1;2)). First, specific carbohydrate structures interact with selectins expressed by activated endothelium and allow them to slow down from the circulation in a rolling interaction with the substrate. *In vitro* flow chamber studies have addressed the relative importance for all three selectins, E-, P- and L-selectin (3-6) in the rolling process of eosinophils to TNFα-activated endothelium. For eosinophils and monocytes also the $\alpha_4\beta_1$ integrin (VLA-4) / VCAM-1 interaction plays a role in the initial adhesion (3;7). Firm adhesion is subsequently mediated by chemokine-induced activation of β_1 and β_2-integrins (8) that bind to their Ig-superfamily member counter structures, VCAM-1 and ICAM-1, respectively, on the endothelium. Finally, the cells spread and migrate to the site of inflammation.

Studies with animal models have addressed the importance of different adhesion molecules used by eosinophil to migrate to allergic inflammatory sites (reviewed in 9). A role for P-selectin was found by using P-selectin knock-out mice in a ragweed allergen-induced peritonitis. The percentage of rolling eosinophils decreased drastically compared to control mice in the mesentery as determined by intravital microscopy (10). In this study, eosinophil migration in the peritoneum was not only dependent on P-selectin but also on ICAM-1 and VCAM-1. Remarkably, in a delayed-onset allergic reaction in skin, both E-selectin and P-selectin were needed for eosinophils recruitment (11). Furthermore, α_4 and β_2 integrins were found to play a role in eosinophil migration (12;13). Also, a role for ICAM-1 was found: I) using ICAM-1 knockout mice in mice models of allergen challenge (14;15) and II) using blocking antibodies in a monkey model of asthma (16). Overall, the *in vivo* animal studies address the importance of the selectins (E- and P-selectin), the integrins (α_4 and β_2 integrins), and the Ig superfamily members (VCAM-1 and ICAM-1) in homing of eosinophils to allergic inflammatory sites. Studies showing that endothelial cells in biopsies of allergic asthmatic patients after allergen challenge have increased expression of E-selectin, ICAM-1 (17) and VCAM-1 (18;19) confirm the importance of these molecules in eosinophils adhesion and migration in humans. Static adhesion experiments showed that eosinophil from allergic asthmatic patients have increased adhesion to and transmigration through IL-1-stimulated HUVEC (20). Another study showed that eosinophils from asthma patients had an increased adhesion to VCAM-1 and ICAM-1 compared to eosinophils from healthy controls (21). Enhanced physiological functions of eosinophils of asthmatic patients compared to healthy controls are known to be a result of priming of the cells *in vivo* (22). Eosinophil priming *in vivo* can be mimicked *in vitro* by adding the cytokines IL-3, GM-CSF and particularly IL-5 to eosinophils of healthy controls. In an *in vitro* Boyden chamber assay it was shown that eosinophils from allergic asthmatic patients had an enhanced migration towards a PAF gradient compared to healthy controls. Subsequently, priming of eosinophils from healthy controls with IL-3, IL-5 or GM-CSF induced comparable enhanced migration as was observed for the eosinophils from the patients (23).

Although it is well established how eosinophils interact with inflamed endothelium under static conditions, only few studies address the role of eosinophils from allergic asthmatic patients under flow conditions. Sriramarao *et al.* (6) showed that eosinophils of mild allergic asthmatic patients use L-selectin and VLA-4 for rolling interactions with inflamed endothelium in a rabbit model. However, eosinophils from a non-asthmatic control group were not included in the study.

Thus far, it is unclear whether the primed phenotype of eosinophils in the peripheral blood from allergic asthmatic patients will influence adhesion to endothelial cells under physiological flow conditions. Therefore, we investigated whether eosinophils from

allergic asthmatic patients differ in rolling and adhesive behaviour on TNFα-activated endothelial cells in comparison with eosinophils from healthy individuals in an *in vitro* flow chamber model. We found an unexpected role for platelets contributing to the adhesion of eosinophils of allergic asthmatics to inflamed endothelium.

Materials and Methods

Reagents

CD16, CD14 and CD3 beads and isolation tools were purchased from Miltenyi, Biotec (Germany). Human serum albumin (HSA) was obtained from the Central Laboratory of The Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands). Recombinant human TNFα was purchased from Boehringer Mannheim (Germany). Incubation buffer contained 20 mM HEPES, 132 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.2 mM KH_2PO_4, supplemented with 5 mM glucose, 1.0 mM $CaCl_2$, and 0.5 % (w/v) HSA. All other materials were reagent grade.

Antibodies

The MoAb HP2/1 (anti VLA-4, CD49d) was purchased from Immunotech (Marseille, France). MoAb BBIG-E4 (5D11, anti E-selectin, anti CD62e) was purchased from R&D systems (Abingdon, UK). MoAb IB4 was isolated from the supernatant of a hybridoma obtained from the American Type Culture Collection (Rockville, MD, USA). The MoAbs as mentioned above are functionally blocking antibodies. Control antibody W6/32 (anti HLA-A,B,C) was isolated from the supernatant of a hybridoma obtained from the American Type Culture Collection (Rockville, MD, USA). MoAbs were incubated with eosinophils ($4x10^6$ cells/ml) at 10 μg/ml during 15 minutes before the experiments. The cell suspensions were diluted once with incubation buffer (final concentration of 5 μg/ml MoAb at $2x10^6$ cells/ml in incubation buffer), and the coverslips were placed directly in the system.

Patients had documented allergy as shown by positive skin-test reactions to several allergens (including inhalation allergens such as house dust mite, pollen and cat allergens), correlated with raised levels of specific IgE antibodies (Rast>2). Patients had respiratory complaints and were examined for asthmatic symptoms. For this study, patients were included according to the criteria of the American Thoracic Society (ATS, 1986). Briefly, patients were included who showed increased bronchial hyperresponsiveness to histamine. These patients used inhaled bronchodilators when needed (and some were receiving daily therapy with inhaled corticosteroids). The study was approved by the hospital ethics committee, and all patients gave informed consent before entering the study.

Isolation of eosinophils

Blood was obtained from healthy volunteers from the Red Cross Blood Bank (Utrecht, The Netherlands) anti-coagulated with 0.4 % (w/v) trisodium citrate (pH 7.4) or by sodium heparine and from allergic asthmatic patients anti-coagulated by sodium heparine. Mixed granulocytes were isolated as described previously (24). Mononuclear cells were removed by centrifugation over isotonic Ficoll (1.077 g/ml). After lysis of the erythrocytes with an isotonic ice-cold NH_4Cl solution, the granulocytes were washed and resuspended in isolation buffer. Eosinophils were purified from granulocytes by negative immunomagnetic selection using anti-CD16-conjugated microbeads (MACS; Miltenyi Biotec) (25). To avoid mononuclear cell contamination also anti-CD3 and anti-CD14- conjugated microbeads were added to the granulocyte suspension. Purity of eosinophils was always >95 %.

Endothelial cells

Human umbilical vein endothelial cells (HUVEC) were isolated from human umbilical cord veins according to Jaffe *et al.* (26), with some minor modifications (27). The cells were cultured in RPMI 1640 containing 20 % (vol./vol.) heat-inactivated human serum, 200 µg/ml penicilin/streptomycin (GIBCO, Life Technologies, Breda, The Netherlands) and fungizone (GIBCO, Life Technologies, Breda, The Netherlands). Cell monolayers were grown to confluence in 5-7 days. Endothelial cells of the second passage or third passage were used in perfusion assays. HUVEC was activated by TNFα (100 U/ml, 5-7 hours, 37°C) prior to the perfusion experiments.

Perfusion chamber

Perfusions under steady flow were performed in a modified form of transparent parallel plate perfusion chamber as described previously by Van Zanten *et al.* (28). This micro-chamber has a slit height of 0.2 mm and a width of 2 mm. The chamber contains a circular plug on which a coverslip (18 mm x 18 mm) with confluent HUVEC was mounted.

Eosinophil perfusion and evaluation

Eosinophil adhesion under flow conditions was performed as described before (3;8). In short, eosinophils were aspirated from a reservoir through the perfusion chamber. Eosinophil perfusions were performed as individual runs under shear conditions at 37°C. During the perfusion, the flow chamber was mounted on a microscope stage (DM RXE, Leica, Weitzlar, Germany) which was equipped with a B/W CCD-video-camera (Sanyo, Osaka, Japan), coupled to a VHS video recorder. Perfusion experiments were recorded on video tape. Video images were evaluated for the number of adherent cells, cluster index and velocity of rolling cells using dedicated routines made in the image-analysis software Optimas 6.1 (Media Cybernetics systems, Silverspring, MD, USA). The eosinophils which were in contact with the surface appeared as bright white-centered cells after proper adjustment of the microscope during recording. The adherent cells on the HUVEC were detected by the image-analyzer. The eosinophil suspension was perfused during 5 minutes at a shear stress of 0.8 dyn/cm^2. The number of surface-adherent eosinophils and the cluster index (29) was measured after 5 minutes perfusion at a minimum of 25 randomized high-power fields (total surface of at least 1 mm^2).

Statistical Analysis. Results are expressed as dot plots with mean values. Statistical analysis of the data was performed using the Student's T test and p values < 0.05 were considered to be significant.

Results

Increased adhesion of eosinophils of allergic asthma patients compared to healthy controls

To investigate whether eosinophils from allergic asthma patients showed increased adhesion to activated HUVEC compared to cells from healthy individuals, cells were perfused over activated HUVEC at a shear stress of 1 dyn/cm^2 for 5 minutes in an *in vitro* flow chamber.

Figure 1a shows that 1240 ± 130 patient cells adhered to the surface compared to 890 ± 90 control cells, which did not reach significance ($p=0.059$). The adhesion molecules responsible for capturing eosinophils from healthy individuals from the flow stream are α_4-integrins on the eosinophils and E-selectin on TNF-α-activated endothelium, as determined by a study using blocking MoAbs (3). To investigate whether these adhesion molecules mediated the increased adhesion of patient cells, blocking

MoAbs against α_4 integrins and E-selectin were used, and 28 ± 4 % of the control W6/32 treated patient cells remained adhered. Blocking both α_4 integrins on the healthy control cells and E-selectin on the endothelium 12 ± 3 % of the control W6/32 MoAb treated healthy control cells remained adhered ($p<0.02$), which is comparable to previous data (3). These results show that in addition to α_4 integrins and E-selectin, additional adhesive interactions mediate the increased adhesion of eosinophils of allergic asthmatic patients to activated endothelial cells.

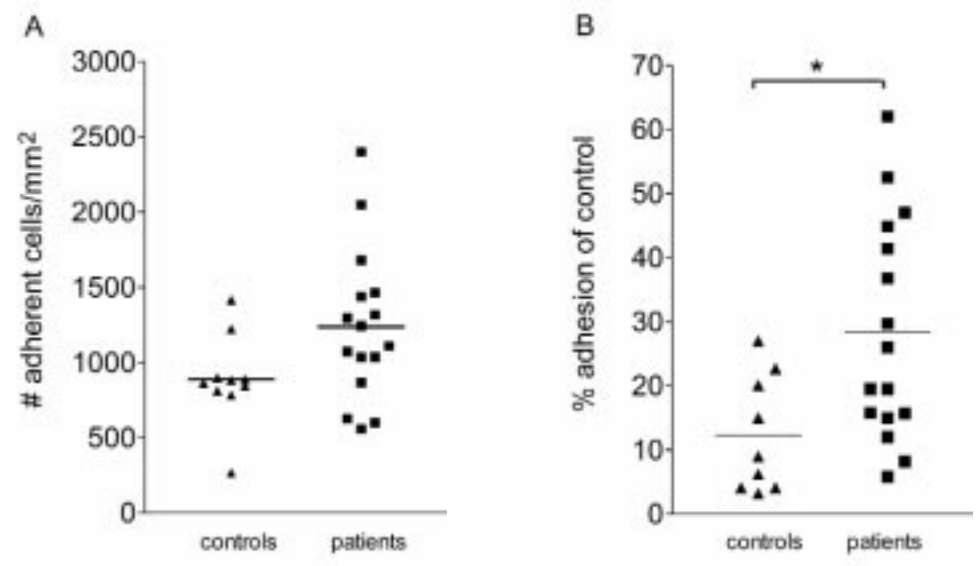
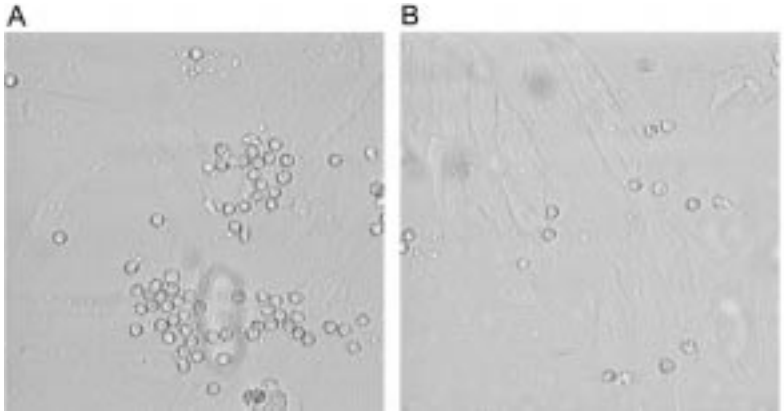


Figure 1. Adhesion of eosinophils of control donors vs. allergic asthmatic patients on TNFα-activated HUVEC under flow conditions. Cells were perfused during 5 minutes at a shear rate of 0.8 dyn/cm^2 and then the amount of cells and the cluster index were determined. A) The amount of adherent cells of controls (887 ± 94) vs. patients (1237 ± 126). B) Eosinophils incubated with anti-α_4 integrin MoAb and HUVEC was incubated with anti-E-selectin MoAb and % adhesion of control situation (control W6/32 MoAb-treated cells) is depicted. Asterisk (*) indicates $p<0.05$.



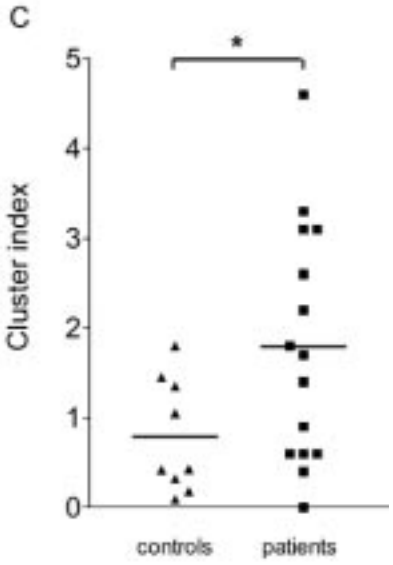


Figure 2. Cluster index of eosinophils of control donors vs. allergic asthmatic patients on TNFα-activated HUVEC under flow conditions. When the adhesion molecules α_4 integrins on eosinophils and E-selectin on the endothelium were blocked, cells of patients but not of healthy controls clustered on the endothelium. In A) a non random, clustered, distribution of eosinophils of allergic asthmatic patients is depicted, and in B) a random distribution of eosinophils of control subjects. C) quantified cluster index of control vs. asthmatic subjects. Unpaired Students T-test, asterisk (*) indicates $p<0.05$.

Platelet-derived P-selectin and activated β_2 integrins mediate residual binding of eosinophils from allergic asthmatics

Remarkably, the residual adhesion of patient cells in the presence of blocking MoAbs against α_4 integrins and E-selectin was characterized by clusters, in marked contrast to healthy control cells (figure 2a and b respectively). Indeed, when this clustering was expressed as an index for non-random adhesion (as described in (29)), patient cells showed a significant increase in cluster index as compared to controls (1.8 ± 0.3 vs. 0.8 ± 0.2 resp, $p<0.05$, figure 2c). Furthermore, it was clear that patient cells interacted with platelets, forming clear eosinophil/platelet rosettes (figure 3a). The eosinophil/platelet interactions participated in the formation of flow-orientated strings of clustered cells, also known as secondary tethering. This was not observed with control cells (figure 3b).

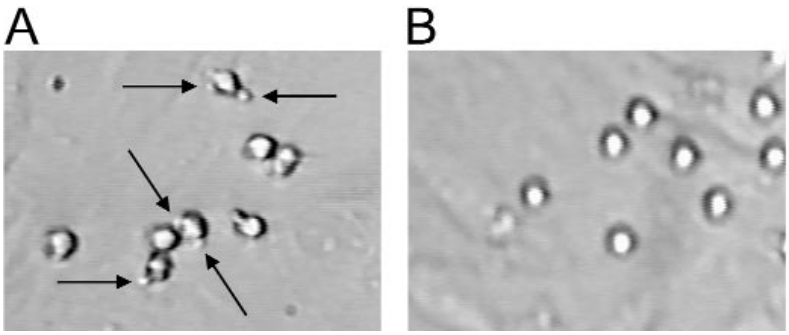


Figure 3. A) Eosinophils of allergic asthmatic patients and B) eosinophils of healthy controls were perfused on activated HUVEC cells. Platelets are present on the eosinophils of patients but not of healthy controls. Platelets are indicated by arrows.

Platelets, when activated, express P-selectin. This adhesion molecule has been shown to mediate initial tethering of leukocytes to platelets that are present and activated at injured vessel wall models (30;31). Moreover, binding of platelets to monocytes in the circulation has been shown to mediate rolling on activated HUVEC (32). The influence of platelet-decorated leukocytes (in this case eosinophils) on adhesion in the context of clustering behaviour has not been studied before. This secondary tethering process results in cells that cluster on the endothelial surface. To investigate if increased adhesion of eosinophils from patients is due to P-selectin that is expressed by adherent platelets, cells were isolated and perfused in the presence of a blocking anti-P-selectin monoclonal antibody (WASP12.2). Figure 4a depicts the total amount of adherent eosinophils from patients and control individuals treated with anti-P-selectin MoAbs.

Where the anti-P-selectin treatment did not significantly effect eosinophil adhesion from healthy controls (in the absence or presence of anti-P-selectin treatment the total adhesion was 887 ± 94 and 665 ± 121, respectively), the treatment did inhibit the adhesion of patient eosinophils by 50 % from 1237 ± 126 to 627 ± 166 ;the latter value being comparable to adhesion of control eosinophils. Additionally, when combining anti-P-selectin MoAbs, anti-α_4 integrin MoAbs and anti-E-selectin MoAbs, also the percentage of residual adhesion became comparable between controls and patients (15 ± 3 % and 15 ± 4 % respectively, figure 4b). The combination of anti-α_4 integrin MoAb, anti-P-selectin MoAb and anti-E-selectin MoAb (figure 4c) in perfusions with patient cells resulted in a cluster index of 0.24 ± 0.07, which was significantly different from 1.8 ± 0.3 when no anti-P-selectin was added (figure 2c). However, the additional presence of anti-P-selectin MoAb, besides blocking α_4-integrins and E-selectin, in healthy individuals did not result in a significant change in the cluster index (0.58 ± 0.25 to 0.78 ± 0.2). These results show that P-selectin-expressing platelets on eosinophils of patients are responsible for this phenomenon. Secondly, when the function of platelets is inhibited by blocking anti-P-selectin antibodies, the phenotype of increased adhesion and cluster index is abrogated.

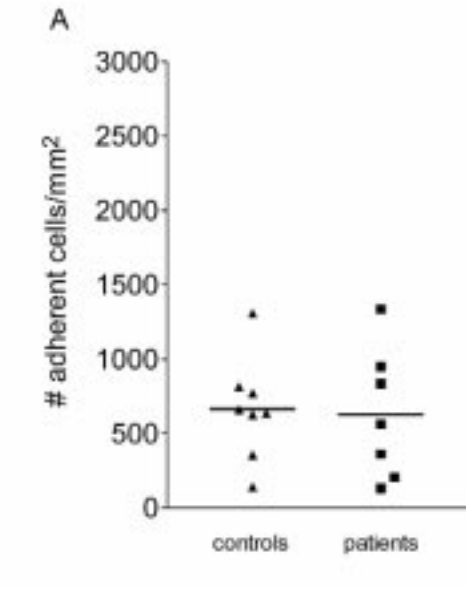


Figure 4. Adhesion behaviour of eosinophils of control vs. allergic asthmatic subjects when E-selectin on the endothelium and α_4 integrins and P-selectin was blocked in the eosinophil suspension. A) shows the amount of adherent eosinophils when all three adhesion molecules were blocked. B) shows the % adhesion of control situation (control MoAb-treated cells) and C) shows the cluster index. No significant differences were found between control vs. allergic asthmatic subjects.

In the presence of blocking antibodies against α_4 integrins, E-selectin and P-selectin, still 15 ± 3 % of the cells from healthy controls and 15 ± 4 % of the cells from patients adhered to activated HUVEC (figure 4b). When in addition β_2 integrins were inhibited, adhesion was abrogated both for cells of patients and of healthy controls to an average of less than 5 % of the control situation (=control antibody W6/32-treated cells, figure 5). When P-selectin was not inhibited but α_4 integrins and E-selectin were inhibited,

we showed that 28 % of the cells of patients and 15 % of the cells of healthy controls remained adhered (figure 1b). The question whether this residual binding was mediated by β_2 integrins was addressed. Figure 5 shows that in the presence of blocking MoAbs against β_2 integrins in addition to blocking MoAbs against α_4 integrins and E-selectin the residual binding of healthy controls and patients both decreased to less than 5 %. Thus, to reduce virtual all adhesion in patient cells, we need blocking of at least E-selectin, α_4 and β_2 integrins. When β_2 integrins on patient cells are not blocked but α_4 and E-selectin are blocked (figure 1b), then anti-P-selectin is needed to reduce the adhesion values to healthy control values (figure 4b). Most likely, P-selectin mediates tethering processes of eosinophil/platelet conjugates of patients to the endothelium and β_2 integrins mediate firm adhesion.

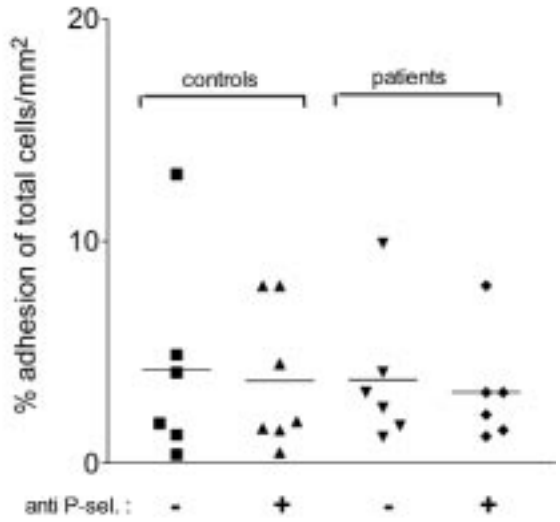


Figure 5. Adhesion behaviour of eosinophils of control vs. allergic asthmatic subjects when E-selectin on the endothelium and α_4- and β_2-integrins were blocked in the eosinophil suspension in the presence or absence of anti P-selectin MoAbs. Percentage adhesion of control situation (control MoAb-treated cells) is depicted.

Discussion

In this study we show that eosinophils of allergic asthmatic patients have an augmented adhesion to TNFα-activated HUVEC under flow conditions compared to eosinophils from healthy controls. Three observations suggest that platelets bound to eosinophils of asthmatic patients are causing the increased adhesion compared to eosinophils of healthy controls: I) eosinophils of patients but not of healthy individuals

bear platelets on their surface II) increased adhesion of patient eosinophils to activated HUVEC is prevented by treatment with anti P-selectin MoAbs III) cluster index of patient eosinophils on activated HUVEC is blocked by anti-P-selectin treatment. Figure 3 (chapter 7) illustrates the consequences of the presence of platelets on eosinophils of allergic asthmatics but not of healthy controls. It is known that eosinophils can bind P-selectin very well, especially under flow conditions (33). Recently, Woltmann *et al.* showed that eosinophils, but not neutrophils, rolled on P-selectin expressing HUVEC cells (34). In that study the asthma-related Th_2 cytokines IL-13 and IL-4 induced the P-selectin on the endothelium. We stimulated endothelial cells with TNFα, which is also a cytokine made by inflammatory cells in allergic sites (35). TNFα-activated HUVEC (passage 3) did not express P-selectin (data not shown, (34)). Therefore, we think that in our study the effect of anti-P-selectin is caused by platelet-derived P-selectin and not endothelial-derived P-selectin. Furthermore, only when the anti-P-selectin MoAbs were incubated during isolation and preparation of eosinophils of patients it had its inhibiting effect, suggesting that during isolation platelets already bound to eosinophils.

For leukocytes other than eosinophils an important role for platelets has already been established. Neutrophils and monocytes are known to bind platelets on damaged vessel wall models in a P-selectin-dependent manner. Moreover, circulating complexes of leukocytes with platelets on their membrane play an important role in the pathogenesis of atherosclerosis (30;31;36 and reviewed in 37). However, also in allergic asthmatic diseases platelets may play an important role (reviewed in 38). Wardlaw *et al.* showed that eosinophils of allergic asthmatic patients were positive for β_3 integrins. However, these were not β_3 integrins expressed on the eosinophils itself but on platelets that bound very tightly to the eosinophils. Electron microscopy studies demonstrated this tight interaction (39). Also, platelets have been shown to be in contact with the vasculature of the bronchi of asthmatic patients but not of healthy controls (40). Another study showed that inhalation of PAF-acether in a baboon model of asthma increased the amount of platelets in the pulmonary circulation in contrast to the periphery. This increase in platelets was followed by an increase in eosinophils (41). Furthermore, in a rabbit model of allergen-induced late asthmatic airway obstruction, Coyle *et al.* showed that platelet depletion by injecting anti-platelet serum reduced the eosinophil infiltration in the BAL 24 hours after allergen challenge and the bronchial hyperresponsiveness (42). Also, the release of platelet derived factors in allergic asthmatics has been reported (43). Although not consistently, platelet factor 4 (PF4) has been detected in the plasma of allergic asthmatic patients but not in healthy control subjects (44). For secreting PF4, platelets must be activated by stimuli like collagen, thrombin or ADP (adenosine diphosphate). Thus, a correlation between activated platelets and eosinophil infiltration exists. We suggest that P-selectin bearing platelets associated with eosinophils could be a mechanism for an increased recruitment of these cells.

Under physiological flow conditions anti-P-selectin MoAbs reduced the cluster index and the total adhesion of eosinophils to activated endothelial cells. Cluster formation occurs in several situations: I) already adhering eosinophil/platelet complexes serve as secondary tethering platforms recruiting fast-flowing cells II) free flowing eosinophil/ platelet complexes get recruited to eosinophils already adhering to the endothelial surface III) free flowing platelets adhere transiently to eosinophils, thereby recruiting them to the surface. The mechanism by which platelets and eosinophils bind to each other is partially clear. Since anti-P-selectin MoAbs blocked the enhanced adhesion and clustering, we conclude that P-selectin mediates the interaction of eosinophils and platelets. Indeed, it has been shown by FACS analyses that activated platelets bind to eosinophils in a P-selectin-dependent way (45). It is very likely that PSGL-1 is the ligand for P-selectin since it is expressed on eosinophils and has been shown to mediate interactions with P-selectin (33). However, we can not exclude that the interaction between eosinophils and platelets is mediated by other adhesion molecules as well. It is known that platelet-bound fibrinogen can serve as a ligand for β_2 integrins on leukocytes (29). Furthermore, the platelet adhesion molecule GP1bα has been identified as a counter receptor for Mac-1 ($\alpha_M\beta_2$ integrin, 46). Thus it seems possible that β_2 integrins on the eosinophils are able to bind to ligands on platelets.

It seems that the β_2 integrins in the patient group was slightly activated because the additional inhibition of β_2 integrins besides the inhibition of E-selectin and α_4 integrins reduced the adhesion to <5 % of the control situation (anti HLA MoAb), irrespectively of the presence or absence of anti-P-selectin MoAbs (figure 5). These findings thus suggest that the β_2 integrins are slightly activated on eosinophils of allergic asthmatic patients under resting, non-activating conditions. Studies (21) performed under static conditions showed that eosinophils from patients that were symptomatic (with a peak expiratory flow rate variability of >10 %) had an increased adhesion to the β_2 integrin ligand ICAM-1 compared to non-symptomatic patients (with a PEF variability of <10 %). The same group showed that eosinophils from patients allergic to birch pollen were primed in the adhesion to VCAM-1 and ICAM-1 during birch pollen season but not before season (47). This suggests that only in an active season, when these patients have complaints, an increased adhesion is present. This is comparable with our study because the allergic asthmatic patients all had complaints at the moment of drawing blood. Thus, it seems that eosinophils of symptomatic allergic asthmatic patients have increased adhesion to endothelial adhesion molecules.

The discrepancy in binding of platelets to eosinophils of allergic asthmatics but not of healthy individuals is not known. Different hypotheses can be postulated: I) the platelets of the allergic asthmatic patient are different or II) the eosinophils are different. AdI. Because the interaction between the platelet and eosinophil can be blocked by anti-P-selectin, it might be that platelets of allergic asthmatic patients are activated *in vivo*

therefore expressing P-selectin. Burgers *et al.* (48) suggested that activation of platelets can occur in asthmatics and that PAF is a candidate activator in mediating this response. Also, they showed a slight decrease in platelet blood count after allergen challenge in allergic asthmatic patients, which was also shown by others (49). This suggests that activated platelets leave the bloodstream, possibly leading to the persistence in airway inflammation.

AdII. It might be that the expression or functionality of PSGL-1 on the eosinophils of allergic asthmatic patients is higher than on cells of control subjects. For neutrophils, eosinophils and other leukocytes it is known that PSGL-1 on resting cells is located randomly on the cell at the tips of the microvilli. Upon activation of neutrophils the PSGL-1 molecules are redistributed to the uropod of the cell (50). Clustering of adhesion molecules is known to induce changes in avidity, thereby increasing the binding to their ligands. However, clustering of PSGL-1 leads to weakened interactions with P-selectin and a concomitant shift to integrin-mediated adhesions on activated neutrophils adhering to a surface. For eosinophils this has not been studied, but what is known is that PSGL-1 of eosinophils adhere better to P-selectin than PSGL-1 of neutrophils (33). Thus far, no differences have been described in PSGL-1 expression or function between eosinophils of allergic asthmatic patients versus healthy controls.

Another mechanism for platelet binding to eosinophils of allergic asthmatics might be due to the primed phenotype of these cells (22). Many effector functions of eosinophils, like respiratory burst (51), production of lipid mediators (52), degranulation (53) and migration (20) are sensitive for priming. The priming agents PAF (54) and IL-5 (55) can increase the affinity of β_2 integrins on eosinophils. This might subsequently lead to the increased binding to platelets. In this study we found a minor role for activated β_2-integrins that could be due to priming of the eosinophils *in vivo*.

In summary, platelets are prone to bind eosinophils of allergic asthmatic patients; a phenomenon that might influence secondary tethering and thereby eosinophil adhesion and migration to inflammatory sites. These processes can contribute to the increased eosinophils found in bronchial tissue in allergic asthmatic patients.

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CHAPTER 4

IL-8 induces a transient arrest of rolling eosinophils on human endothelial cells

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Abstract

Eosinophils exhibit a rolling interaction with E-selectin-expressing endothelium and need to be activated by inflammatory mediators to firmly adhere to this surface. This study shows that IL-8 induces a transient arrest of unprimed eosinophils that roll on E-selectin present on tumor necrosis factor α-activated human umbilical vein endothelial cells in an *in vitro* flow chamber. This process was antagonised by neutralising antibodies directed against IL-8, showing the specificity of the IL-8 effect. Furthermore, blocking antibodies against both α_4- and β_2-integrins inhibited the IL-8-induced transient arrest while these antibodies had no effect when they were added separately. The IL-8-induced arrest was Pertussis Toxin (PTX) sensitive. Studying the effect of IL-8 in more detail we evaluated putative changes in $[Ca^{2+}]_i$ in eosinophils induced by IL-8. We showed that IL-8 induces a transient increase in $[Ca^{2+}]_i$ in ~40 % of the cells, provided that the eosinophils are interacting with endothelial cells or fibronectin-coated surfaces. Together these data show that resting eosinophils respond to IL-8 provided that the cells adhere on physiological surfaces. The induction of a *transient* arrest provides a new level of chemokine-induced regulation of leukocyte adhesion under flow conditions.

Introduction

Eosinophils play an important role in allergic inflammatory diseases like asthma. Infiltrates of these cells are present in the structures of the airway wall and the lumen of the bronchi of allergic asthmatic patients (1). To enter the site of inflammation, eosinophils must leave the bloodstream and pass the endothelium. A widely accepted paradigm for leukocyte extravasation is referred to as the multistep model (2). In this model selectins and their carbohydrate-bearing ligands mediate rolling interactions between leukocytes and the endothelium. In this respect E-selectin, which is present on activated endothelium, has been shown to mediate rolling of neutrophils (3) and eosinophils (4). Subsequently, cells can be activated upon interaction with inflammatory mediators, resulting in the activation of integrins which bind to their ligands expressed on the endothelium. In this way, firm adhesion of the cells to the endothelium is established (5;6).

An important class of inflammatory mediators involved in the arrest of inflammatory cells are chemokines which are released at the site of inflammation. Chemokines can be divided in 4 different groups: CL, CCL, CXCL, and CX_3CL in which X is the number of amino acids in between cysteine residues at the NH_2-terminal site of the molecule. Of the CL and CX_3CL families, only one member of each group has been described,

namely lymphotactin (7) and fraktalkine (8), respectively. CCL chemokines, including eotaxin, RANTES and MCP-3 have been reported to be mainly chemotactic for monocytes, lymphocytes and eosinophils. CXC chemokines, including IL-8 and GRO-α, have been shown so far as more specific for neutrophils (see 9;10 for reviews).
However, several clinical studies indicate that expression of the CXCL chemokine IL-8 is enhanced both at the level of mRNA and protein in pulmonary "eosinophilic" diseases such as asthma (11-13). IL-8 has been shown to be produced by bronchial epithelium cells of asthmatic patients (11). Other sources for IL-8 include endothelial cells, fibroblasts, macrophages and mast cells (see 12 for review). These cells play a key role in the pathogenesis of allergic asthma. Many studies have been designed to link the production of different chemokines to the occurrence of inflammatory cells in the tissues. As allergic asthma is characterized by a clear eosinophilic inflammation in the bronchial tissue, several studies evaluated the role for IL-8 in eosinophil activation and migration processes *in vitro* and *in vivo*. No clear consensus exists concerning this issue. Some *in vitro* studies on IL-8 induced eosinophil chemotaxis failed to show an IL-8 dependent effect on eosinophil migration (14), whereas other reports did: some studies were performed with cytokine-activated or "primed" eosinophils (15;16) and other studies were performed with cells from allergic asthmatic (17) or eosinophilic (18) subjects. In most of these studies the effect of IL-8 on eosinophils have been determined by the use of chemotaxis assays such as the Boyden chamber. Subtle differences in experimental set up (e.g. choice of filters, medium etc.) might explain differences in outcome between the different studies. In addition to these *in vitro* studies the effect of IL-8 on eosinophils has also been suggested by an *in vivo* study that showed that provocation with IL-8 induces eosinophilia in the nasal epithelium (19).

Chemokines are ligands for G-protein-coupled serpentine receptors. On neutrophils, two high-affinity G-protein-coupled receptors for IL-8 have been described, CXCR1 and CXCR2 (20;21). When chemokines bind to their receptor, an increase in intracellular free Ca^{2+} ions $[Ca^{2+}]_i$ is elicited. Increased $[Ca^{2+}]_i$ leads to multiple downstream signaling events, and these have been correlated with a number of cellular functions (22). It has been questioned whether eosinophils can increase $[Ca^{2+}]_i$ upon IL-8 stimulation, because these changes in $[Ca^{2+}]_i$ were very small (15). Indeed, Petering *et al.* contributed these small changes to contamination of neutrophils. Remarkably, all studies that failed to show the effect of IL-8 on $[Ca^{2+}]_i$ mobilization in eosinophils were performed on cells in suspension. Migration studies, on the other hand, showed that eosinophils can respond to IL-8, and in these experiments cells adhered to substrates. We hypothesize that eosinophils that adhere to physiological substrates are more susceptible for IL-8 stimulation compared to cells in suspension. Therefore, the effect of IL-8 on eosinophils adherent to physiological relevant surfaces was evaluated.

Materials and Methods

Reagents

Percoll was obtained from Pharmacia (Uppsala, Sweden). N-formyl-methionyl-leucyl-phenylalanine (fMLP) was purchased from Sigma Chemicals (St. Louis, MO, USA). Human serum albumin (HSA) was purchased from the Central Laboratory of The Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands). Recombinant human TNFα was purchased from Boehringer Mannheim (Germany). IL-8 was purchased from Peprotech (72aa, Rocky Hill, NJ, USA) and Eotaxin-1 was purchased from R&D Systems (Minneapolis, MN, USA). Pertussis Toxin (50 μg/ml, Sigma, St. Louis, Missouri, USA). Incubation buffer contained 20 mM HEPES, 132 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.2 mM KH_2PO_4, supplemented with 5 mM glucose, 1.0 mM $CaCl_2$, and 0.5 % (w/v) HSA (Human Serum Albumin). All other materials were reagent grade.

Antibodies

The MoAb HP2/1 (anti VLA-4, CD49d) was purchased from Immunotech (Marseille, France). MoAb IB4 was isolated from the supernatant of a hybridoma obtained from the American Type Culture Collection (Rockville, MD, USA). The antibodies we used against α_4 integrins (HP2/1) and β_2 integrins (IB4) have been described as functional blocking antibodies (23-25;26) Therefore, antibody-induced differences in function of the eosinophils caused e.g. by crosslinking of integrins, seem to be unlikely. Control antibody W6/32 (anti HLA-A,B,C) was isolated from the supernatant of a hybridoma obtained from the American Type Culture Collection (Rockville, MD, USA). Activating antibody 8A2 (26) was kindly provided by dr. J.M. Harlan (University of Washington, Seattle, USA) and dr. T.W Kuijpers (Central Laboratory for Blood Transfusion (CLB), Amsterdam, The Netherlands). Anti-CXCR1 and anti-CXCR2 antibodies, 5A12 and 6C6 (Pharmingen, San Diego, USA), respectively, have been described as blocking MoAbs of neutrophil migration (27). Anti-E-selectin MoAb BBIG-E4 (5D11) was purchased from R&D systems (Abingdon, UK). MoAbs were incubated with eosinophils ($4x10^6$ cells/ml) at 10 μg/ml during 15 minutes before the experiments. The cell suspensions were diluted once with incubation buffer (final concentration of 5 μg/ml MoAb at $2x10^6$ cells/ml in incubation buffer), and the coverslips were placed directly in the system. Anti-IL-8 (clone B-K8, Biosource International, California, USA) was added to IL-8 10^{-8} M in a final concentration of 20 μg/ml.

Isolation of eosinophils

Blood was obtained from healthy volunteers from the Red Cross Blood Bank, Utrecht, The Netherlands. Mixed granulocytes were isolated from the buffy-coat of 500 ml blood anti-coagulated with 0.4 % (w/v) trisodium citrate (pH 7.4), as previously described (28). Mononuclear cells were removed by centrifugation over isotonic Ficoll (1.077 g/ml). After lysis of the erythrocytes with an isotonic ice-cold NH_4Cl solution, the granulocytes were washed and resuspended in RPMI 1640 (Gibco, Paisley, UK) with 0.5 % (w/v) HSA.
Granulocytes were incubated for 30 min at 37°C to restore the initial density of the cells. Thereafter, the cells were washed and resuspended in phosphate-buffered saline (PBS) supplemented with 0.5 % HSA and 13 mM trisodium citrate, and incubated with fMLP (10 nM) for 10 min at 37°C, to decrease the specific gravity of the neutrophils, but not that of the eosinophils. Subsequently, eosinophils were obtained by centrifugation (20 min, 1000 g) over isotonic Percoll (density 1.082 g/ml, layered on Percoll with a density of 1.1 g/ml), washed and resuspended in incubation buffer. Purity of eosinophils was >95 %. This procedure leads to the isolation of relatively unprimed eosinophils compared to conventionally used isolation procedures with immunomagnetic beads (29).

Endothelial cells

Human umbilical vein endothelial cells were isolated from human umbilical cord veins according to Jaffe *et al.* (30), with some minor modifications (31). The cells were cultured in RPMI 1640 containing 20 % (vol./vol.) heat-inactivated human serum, 200 µg/ml penicilin/streptomycin (GIBCO, Life Technologies, Breda, The Netherlands) and fungizone (GIBCO, Life Technologies, Breda, The Netherlands). Cell monolayers were grown to confluence in 5-7 days. Endothelial cells of the second passage or third passage were used in perfusion assays. HUVEC was activated by TNFα (100 U/ml, 7 hours, 37°C) prior to the perfusion experiments.

Perfusion chamber

Perfusions under steady flow were performed in a modified form of transparent parallel plate perfusion chamber (32) as previously described by Van Zanten *et al.* (31). This micro-chamber has a slit height of 0.2 mm and width of 2 mm. The chamber contains a circular plug on which a coverslip (18 mm x 18 mm) with confluent HUVEC was mounted. Immediately after mounting the HUVEC, which were activated with

TNFα for 7 hours, the flow chamber was flushed with HEPES buffer during 2 minutes to wash out residual TNFα.

Eosinophil perfusion and evaluation

Eosinophil perfusions were performed as described (4). In short, eosinophils in suspension ($2x10^6$ cells/ml in incubation buffer) were aspirated from a reservoir through plastic tubing and the perfusion chamber with a Harvard syringe pump (Harvard Apparatus, South Natic, MA). The individual runs all occurred under specific shear conditions in a 37°C temperature box. Perfusion experiments were recorded on video tape. The eosinophil suspension was perfused during 3 minutes at a shear stress of 0.8 dyn/cm^2 to obtain an endothelial surface with firmly adhering and rolling eosinophils (4). After these 3 minutes buffer was added and the shear stress was increased to 2 dyn/cm^2. After 20 seconds at a shear stress of 2 dyn/cm^2, recording of the images on video was started. Subsequently, HEPES buffer containing IL-8 (10^{-8} M) or eotaxin (10^{-8} M) was added to the flow chamber. During the whole perfusion experiment antibodies (IB4; 10 µg/ml, HP2/1; 10 µg/ml, anti IL-8; 50 µg/ml or W6/32; 10 µg/ml) were present. After ~30 seconds the cytokine buffer reached the cells and the cells arrested. Subsequently it took 30 seconds to ~2 minutes for the cells to start rolling again. Therefore the percentage rolling cells were determined at the following points: "before" stimulation: this is at time point 30 sec before addition of the cytokine-containing buffer; "during" stimulation: this is at the time point that cytokine-containing buffer has reached the cells and the cells arrest; "after" stimulation: this is at time point 1 minute after the cytokine-containing buffer reached the cells. One randomly chosen image per experiment was recorded. Cells which started rolling again after the IL-8-induced arrest rolled out of the recorded image, but also cells at the front of the image rolled into the recorded image. Therefore we calculated the total amount of adherent cells present in the field "before", "during" and "after" stimulation which did not differ significantly in all tested circumstances. More than 90 % of the firmly adherent cells under baseline conditions were stable throughout the whole observation period when no cytokine was added (results not shown). Also, of the stable stationary cells in the "before" period, less than 5 % of the cells started rolling again after addition of IL-8.

To automatically determine the percentage of rolling cells custom-made software was developed in Optimas 6.1 (4). In short, a sequence of 50 frames, representing an adjustable time interval, was digitally captured. The velocity of each cell was calculated. The cut-off value to distinguish between rolling and static adherent cells was set at 1 µm/sec. With this method, static adherent, rolling and freely flowing cells (which were not in focus) could be clearly distinguished.

Flow cytometry

Flow cytometry analysis was carried out as described before (33). As primary MoAbs were used antibodies against β_2 integrins (IB4), CXCR1 (5A12), CXCR2 (6C6) or a control antibody (5D11 anti-E-selectin). Granulocytes were analyzed with a FACSvantage flowcytometer (Becton Dickinson immunocytometry systems, Mountain View, CA).

Imaging of intracellular free Ca^{2+}

Imaging of intracellular free Ca^{2+} was performed with a custom-built setup, consisting of a computerized excitation filterswitcher (Lambda-10, Sutter Instrument Company, Novato, California, USA) with excitation filters (D340/10 and D380/13, Chroma Technology Corporation, McHenry, Illinois, USA) coupled to a Leica (Wetzlar Germany) Leitz DMIL inverted microscope, which was equipped with light filters appropriate for Fura-2 (D510/40 and 400 DCLP, Chroma Technology Corporation, McHenry Illinois, USA), high n.A Immersion objective (UV-F 40x nA 1.30 Glycerol Imm., Nikon Corporation, Tokyo, Japan) and a Xenon arc lamp (XBO 75 W/2, Osram, Berlin, Germany). Series of 50 image pairs (512 x 512, ~1 second apart) were sequentially grabbed with a black and white framegrabber (Pulsar, Matrox Electronic Systems Ltd., Dorval, Quebec, Canada) from an intensified video camera (LI-µCAM, Lambert Instruments, Leutingewolde, The Netherlands) directly into computer memory. Ratios and calcium values were computed off line with Image Analysis software (Optimas 6.1, Media Cybernetics, Silver Spring Maryland, USA) and custom-made macros (A.L.I.). Calcium levels were calculated with the standard calibration formula of Grynkiewics *et al.* (34):
$[Ca^{2+}]_i = K_d \times \beta \times ((R-Rmin)/(Rmax-R))$ with parameters K_d=225 nM, β=2.4, and were displayed in false colors (blue-red ramp). Cell calcium was separated from background by calculating the threshold mask from the mean of the two ratio images for each of the 50 ratio images in the series. Threshold level was obtained by choosing the first and the last level in the series, and then by linear interpolation for the rest of the series, thereby counteracting the effect of bleaching over time. Intracellular $[Ca^{2+}]$ levels are determined by calculating the mean $[Ca^{2+}]_i$ of all pixels in a cell in each of the 50 ratios taken. The cut-off value to distinguish between a responding and a non responding cell was set at a mean value of 200 nM. Figure 4 and 6 show the mean $[Ca^{2+}]_i$ of all cells exceeding this cut-off value. In the experiments investigating the IL-8-induced increase in $[Ca^{2+}]_i$, eotaxin was given after the IL-8. The cells which did not respond to eotaxin (likely to be neutrophils) were excluded from analyses.
Freshly isolated eosinophils adhere strongly to some glass substrates. Under these circumstances attachment induces cells to flatten out with activation and generation of

calcium signals (unpublished observations). To circumvent these problems we let eosinophils adhere to fibronectin-coated surfaces with the use of MoAb 8A2 or to 7h TNF-α-activated HUVEC. Addition of this MoAb leads to freezing of β_1 integrins in a high-affinity state. By this procedure, the cells strongly attach to fibronectin, but otherwise remain deactivated, keeping a round shape (35), thus allowing clear ratio imaging. Eosinophils attached in this way display low intracellular free Ca^{2+} levels under control conditions for long periods of time, up to 30 minutes. Hereby large numbers of cells could be imaged while being treated with IL-8 and eotaxin.
All washing and incubation steps were performed in incubation buffer. Eosinophils ($5x10^6$/ml) were loaded with 2.5 μM Fura-2 AM (Molecular Probes, Eugene, Oregon, USA) for 15 minutes. Hereafter, the cells were incubated in the absence or presence of the antibody 8A2 (10 μg/ml) for 15 minutes at 37°C in an agitated water bath. After washing, 8A2 treated cells ($1 x10^6$ to $2 x10^6$) were plated onto fibronectin (0.1 mg/ml in Hepes, 15 min, 37°C) coated 24 mm cover slips. 7h TNF-α-activated HUVEC cultured on 24 mm cover slips were extensively washed with PBS and non-treated, Fura-2 AM loaded eosinophils were plated on the endothelium. The eosinophils were allowed to settle for 15 minutes on the substrate, and non-adherent cells were removed by washing the cover slips. Cover slips were mounted in an open chamber and placed in a warmed (37°C) metal ring for Calcium Imaging. Stimulants were added from the top after two pre-stimulus image ratios had been obtained. Stimulants (37°C, at two times the concentration) were added to an equal volume (250 μl) of incubation buffer already present in the chamber to obtain a homogenous mixture at the start of the ratio imaging.

Measurement of oxygen consumption

Oxygen consumption was measured at 37°C with an oxygen electrode as described previously (36). In short, eosinophils were resuspended in incubation buffer ($2x10^6$ cells/ml). PMA (100 ng/ml) was added and oxygen consumption was measured for 5 minutes.

Statistical Analysis. Results are expressed as mean ± standard error of the mean (SEM). Statistical analysis of the data was performed using a Student T test for paired data; p values < 0.05 were considered to be significant.

Results

Eosinophils exhibit a transient arrest upon IL-8 addition during rolling adhesion along TNFα-activated endothelium.

To investigate the effect of IL-8 on rolling eosinophils, freshly isolated eosinophils were perfused over 7h TNFα-activated confluent HUVEC at a shear stress of 2 dyn/cm^2. When eosinophils were treated with a control anti-HLA-class-I antibody (W6/32, figure 1a, 2a) the percentage of rolling cells compared to the total number of adherent cells (rolling and firmly adherent cells) was 48 ± 3 %. Upon stimulation with IL-8 (10^{-8} M), the percentage of rolling cells decreased to 12 ± 3 %. After 1 minute, the percentage of rolling cells increased to 28 ± 5 % (movie of IL-8-induced transient arrest is available on CD-ROM). Addition of anti-IL-8 antibody (10 μg/ml) to the IL-8 suspension prevented this transition from rolling to a stationary arrest of W6/32 treated (control) eosinophils (figure 1a). When eotaxin (10^{-8} M), a potent chemokine for eosinophils, was added to the rolling, W6/32-treated eosinophils, the percentage rolling cells decreased from 56 ± 3 to 7 ± 3 %. The cells now bound stably and longterm because after 1 minute the percentage of rolling cells was still very low (8 ± 3 %, figure 1a, 2b) (movie of eotaxin induced firm arrest is available on CD-ROM). In addition, the eosinophils flattened upon eotaxin treatment, while this was not observed upon addition of IL-8. We also tested whether eotaxin could induce a stable and longterm arrest when applied to cells which started rolling again after the IL-8-induced arrest. Indeed, more than 97 % of the cells were longterm arrested after addition of eotaxin to W6/32-treated eosinophils which were rolling after the IL-8-induced transient arrest.

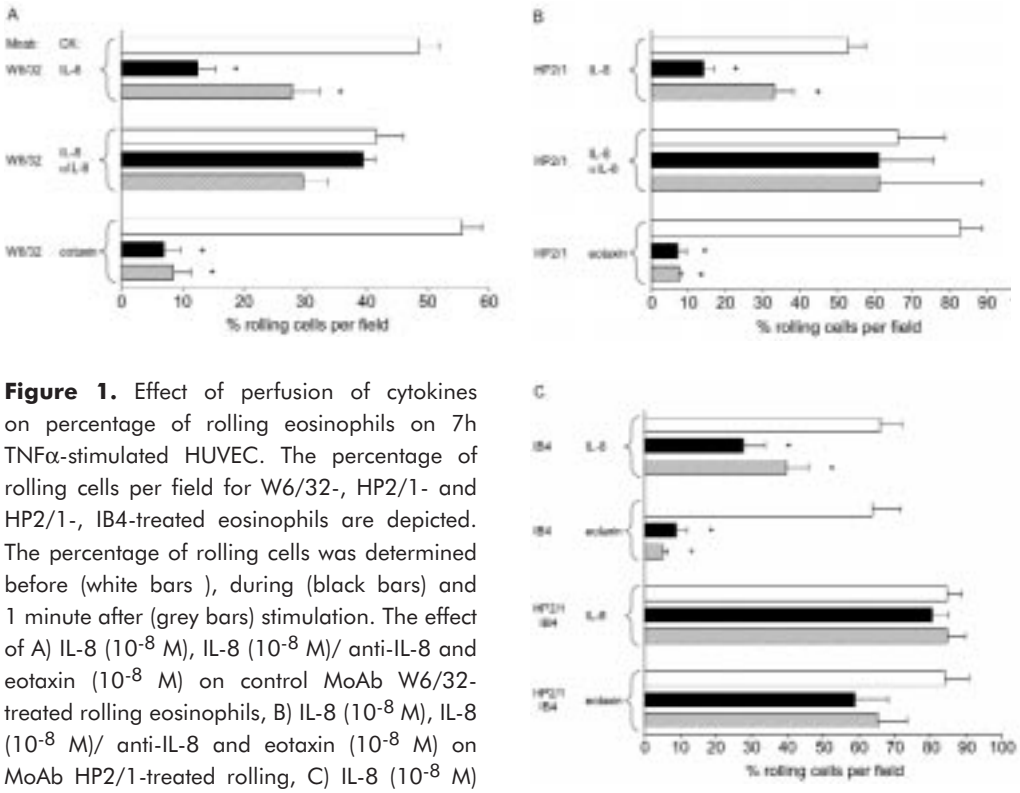

Figure 1. Effect of perfusion of cytokines on percentage of rolling eosinophils on 7h TNFα-stimulated HUVEC. The percentage of rolling cells per field for W6/32-, HP2/1- and HP2/1-, IB4-treated eosinophils are depicted. The percentage of rolling cells was determined before (white bars), during (black bars) and 1 minute after (grey bars) stimulation. The effect of A) IL-8 (10^{-8} M), IL-8 (10^{-8} M)/ anti-IL-8 and eotaxin (10^{-8} M) on control MoAb W6/32-treated rolling eosinophils, B) IL-8 (10^{-8} M), IL-8 (10^{-8} M)/ anti-IL-8 and eotaxin (10^{-8} M) on MoAb HP2/1-treated rolling, C) IL-8 (10^{-8} M) and eotaxin (10^{-8} M) on anti-β_2 integrin integrin-treated and both anti α_4- and β_2-integrin-treated eosinophils. Percentage rolling cells are plotted for 3 to 6 experiments ± SEM, except for W6/32 / IL-8 (n=10) and HP2/1 / IL-8 (n=16). The statistically significant effects of the different treatments against the situation before treatment were determined by paired Student T test (*: $p<0.05$).

To investigate whether α_4 integrins played a role in this IL-8-induced arrest, eosinophils were treated with anti-α_4 integrin antibody (HP2/1, figure 1b, 2c). The percentage of rolling cells of HP2/1-treated eosinophils on TNFα-activated HUVEC was 52 ± 5 % which was significantly higher than W6/32-treated eosinophils, as shown in a previous study (4). The rolling percentages of all groups are higher in this study compared to an earlier study (4). This is caused by the increase in the shear stress used in this study (2 dyn/cm^2) whereas the shear stress in the former study was 0.8 dyn/cm^2. Upon stimulation with IL-8 (10^{-8} M) the percentage of rolling cells decreased to 14 ± 3 %. After 1 minute, the percentage of rolling cells increased from 14 ± 3 % to 33 ± 5 %. Within 2 minutes almost all arrested cells (both W6/32- and HP2/1-treated) started rolling again. Addition of anti-IL-8 antibody (10 μg/ml) prevented the IL-8-induced transition from

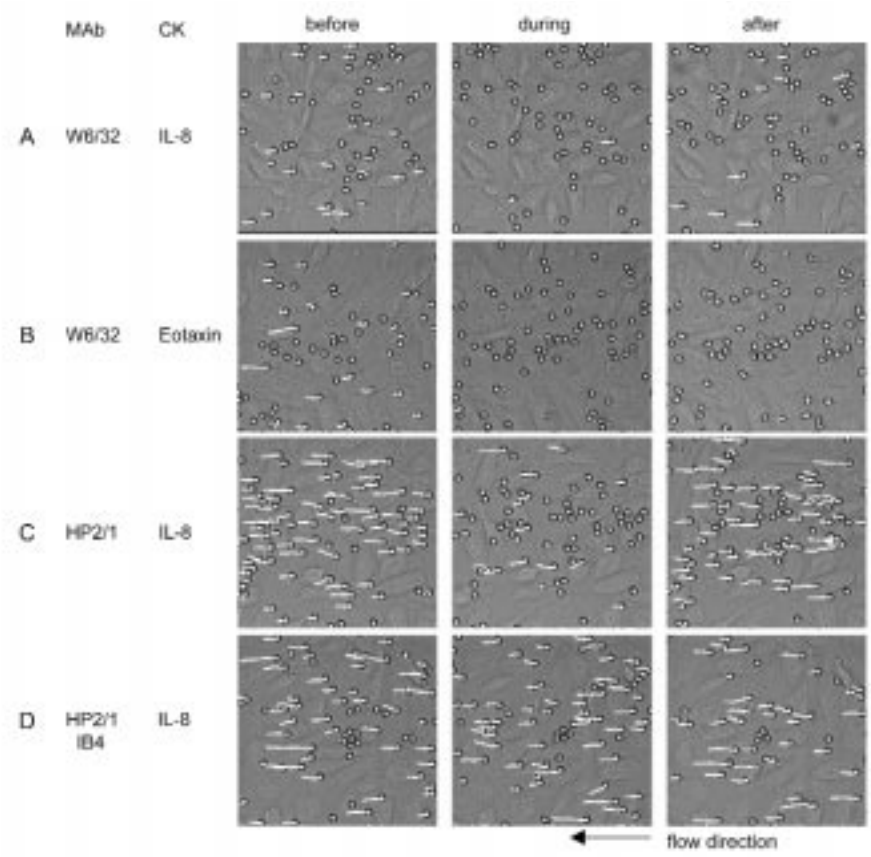

Figure 2. Computerized analyses of the effect of perfusion with cytokines on the percentage of rolling eosinophils on 7h TNFα-stimulated HUVEC. Eosinophils ($2x10^6$/ml) were incubated with 10 μg/ml of the indicated MoAbs before the assay. Lines indicate the rolling tracks of the cells in one field during 2 seconds. Cells are positioned at the beginning of the track. Images depict rolling tracks before, during and 1 minute after indicated cytokine stimulation. Data are depicted as representative experiments (see also figure 1).

rolling to stationary arrest of HP2/1-treated eosinophils (figure 1b). When eotaxin (10^{-8} M) was added to the rolling HP2/1-treated eosinophils, all cells bound stably and longterm (figure 1b). The experiment with the W6/32- and HP2/1-treated cells show that the IL-8 effect on eosinophils can occur independently of the interaction between $\alpha_4\beta_1$ integrin with its ligand VCAM-1 on activated endothelium.

To investigate whether β_2 integrins are implicated in the IL-8-induced arrest, eosinophils were incubated with an anti-β_2 integrin MoAb IB4. When β_2 integrins were blocked, the percentage of rolling cells per field was 66 ± 6%. Upon IL-8 stimulation, the percentage of rolling cells decreased to 28 ± 6 % and after 1 minute increased to 40 ± 6 % rolling cells again (figure 1c). When eotaxin was added to rolling IB4-treated

eosinophils, the percentage of rolling cells decreased from 64 ± 8 % to 9 ± 3 %. After 1 minute the cells were still firmly adhered to the endothelial cells.
Finally, we investigated the effect of blocking of β_2 integrins and α_4 integrins simultaneously. When both β_2 integrin and α_4 integrins were blocked, the percentage of rolling cells was 84 ± 4 %. Upon additon of IL-8 all cells kept rolling (80 ± 5 %). After 1 minute upon addition of IL-8 the percentage of rolling cells was still 85 ± 5 %. When eotaxin was added to IB4 and HP2/1 treated eosinophils, no significant changes in the percentage of rolling cells were observed (figure 1c). These data show that the IL-8-induced transient arrest and the eotaxin induced stable arrest can be mediated by either β_2 integrins or α_4 integrins.

To investigate whether the known IL-8 receptors CXCR1 and CXCR2 mediated the IL-8-induced response, eosinophils were incubated with antibodies against CXCR1 and CXCR2 (6C6 and 5A12, respectively). IL-8-induced transient arrest was not inhibited and the percentage of rolling cells decreased from 46 ± 10 % to 16 ± 5 % upon IL-8 stimulation (figure 3a). The IL-8 induced arrest was transient and the percentage rolling cells increased from 16 ± 5 to 27 ± 11 %. The functionality of these antibodies on IL-8 (10^{-8} M)-induced neutrophil chemotaxis in a Boyden chamber assay was confirmed by their ability to block the IL-8 induced migration by 74 %, as was also shown by others (27). In figure 3b and 3c it is shown that CXCR1 and CXCR2 are not present on isolated eosinophils whereas they are present on neutrophils (40). To address whether the IL-8-induced arrest was mediated by G-protein-coupled receptors, eosinophils were incubated with solvent (0,5 % glycerol), or PTX (100 or 500 ng/ml) for 2 hours. Control, glycerol-treated eosinophils arrested transiently upon IL-8 perfusion, comparable to W6/32- and HP2/1-treated eosinophils. In contrast, eosinophils treated with PTX showed a dose-dependent inhibition of IL-8-induced arrest (figure 3a). To control for possible negative effects of PTX on the normal physiology of the eosinophils, we performed respiratory burst experiments. Eosinophils incubated with glycerol (0.5 %) or PTX (500 ng/ml) for 2h at 37°C were tested for the induction of the respiratory burst upon activation with PMA. No differences were found in the PMA-induced oxidative burst of glycerol- vs. PTX-treated eosinophils (not shown).

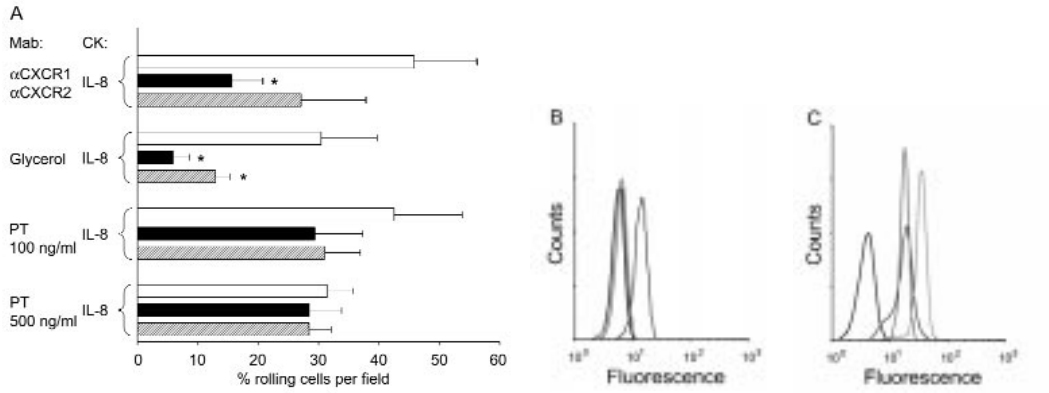

Figure 3. A) The effect of inhibition of serpentine receptors on the percentage of rolling eosinophils on 7h TNFα-stimulated HUVEC. The effect of IL-8 on anti-CXCR1 and anti-CXCR2 antibodies (5A12 and 6C6, 15 min, 37°C), control solvent (glycerol 0.5 %, 2h, 37°C), and Pertussis Toxin (100 and 500 ng/ml, 2h, 37°C)-treated eosinophils was determined before (white bars), during (black bars) and 1 minute after (grey bars) stimulation. Percentages of rolling cells are plotted for 3 to 6 experiments ± SEM. The statistically significant effects of the different treatments compared to the situation before treatment were determined by paired Student T test (*: $p<0.05$). In B) and C) granulocytes were stained for β_2 integrins (IB4, blue line), CXCR1 (5A12, green line), CXCR2 (6C6, red line) or an irrelevant antibody (black line) by FACS. B) and C) show the fluorescence for eosinophils and neutrophils, respectively. The experiment shown is representative for 3 independent experiments.

Eosinophils bound to fibronectin and endothelium show significant calcium responses upon IL-8 addition

To investigate the changes in intracellular free Ca^{2+} of adherent eosinophils upon IL-8 and eotaxin stimulation, cells were incubated with 8A2 and loaded on fibronectin-coated cover slips or non-treated cells were loaded on 7h TNFα-stimulated HUVEC (see Materials and Methods 35). After stimulation with IL-8 (10^{-8} M) a clear increase in $[Ca^{2+}]_i$ (>200 nM) was observed in 42 ± 7 % and 30 ± 7 % of the cells adherent to fibronectin and activated HUVEC, respectively (figure 4a and 4b showing a representative experiment). The increase in $[Ca^{2+}]_i$ of cells adherent to fibronectin is depicted in figure 5a. When neutralizing antibodies for IL-8 (clone B-K8) were added to the IL-8 solution before addition to the cells, the change in $[Ca^{2+}]_i$ response was blocked (figure 4a and 5b). A second IL-8 stimulation given did not elicit a $[Ca^{2+}]_i$ response, indicating homologous desensitization of the receptor (data not shown). Upon eotaxin (10^{-8} M) stimulation 97 ± 0.5 % and 98 ± 1.7 % of the cells adherent to fibronectin and activated HUVEC, respectively, increased their intracellular free Ca^{2+} concentration (figure 4 and 5c).

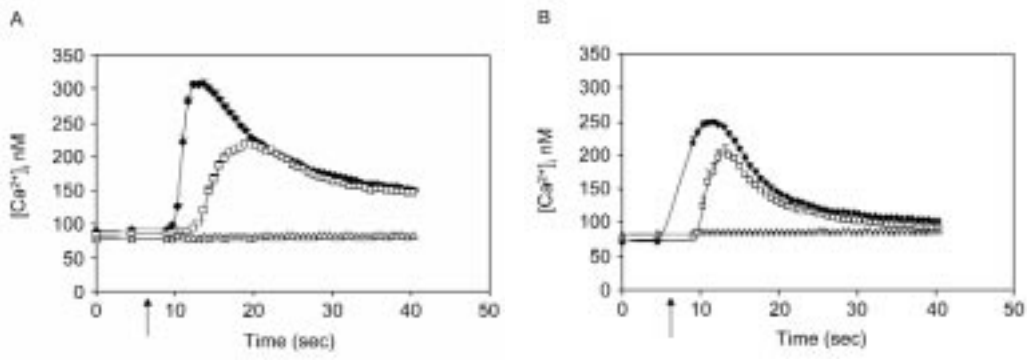

Figure 4. Effect of adding cytokines to adhering eosinophils on fibronectin. Eosinophils were loaded with 2.5 μM Fura-2 AM, incubated with MoAb 8A2 (35) and put on A) fibronectin-coated glasses for 15 minutes or put on B) 7h TNFα-activated HUVEC for 15 minutes. Non-adherent cells were washed away. IL-8 (open squares), eotaxin (solid diamonds) or IL-8/anti-IL-8 (open triangles) was added after 2 blanco images (arrow) and 48 images were taken at 340 and 380 nm after addition of cytokines. $[Ca^{2+}]_i$ was measured by calculating 340/380 ratios (see Materials and Methods). The figure is representative for 3 independent experiments.

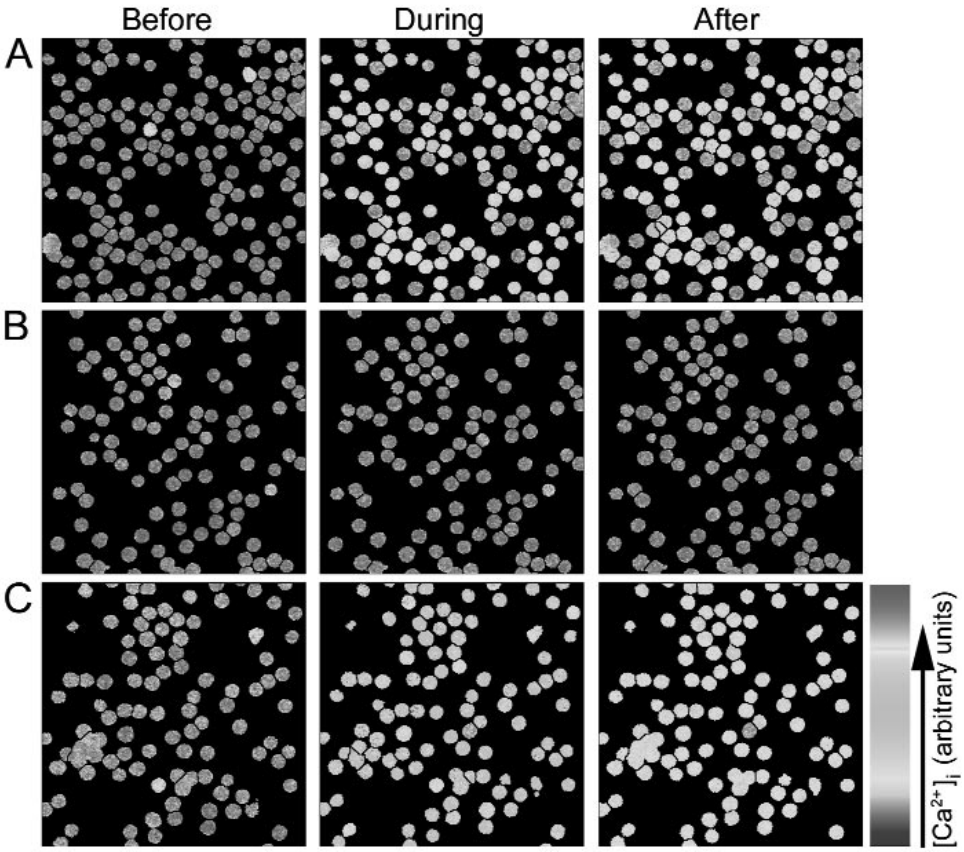

Figure 5. Computerised analyses of the effect of adding cytokines to fibronectin-adhering eosinophils. Eosinophils were loaded with 2.5 μM Fura-2 AM, incubated with 8A2 and put on fibronectin-coated glasses for 15 minutes. A) shows effect of IL-8 before, during and after addition. B) shows the effect of IL-8/anti-IL-8 before, during and after addition. C) shows the effect of eotaxin before, during and after addition. Data are depicted as a representative of 3 experiments (see also figure 4A).

To investigate whether the IL-8-induced $[Ca^{2+}]_i$ responses were sensitive to PTX, eosinophils were incubated with control solution (glycerol 0,5 %) or 500 ng/ml PTX for 2 hours at 37°C. Figure 6 shows that the $[Ca^{2+}]_i$ responses were blocked in PTX-treated cells adherent to fibronectin (figure 6a) or 7h TNF-α-activated HUVEC (figure 6b). Also, the positive control C5a induced a $[Ca^{2+}]_i$ response which was completely blocked by PTX (500 ng/ml).

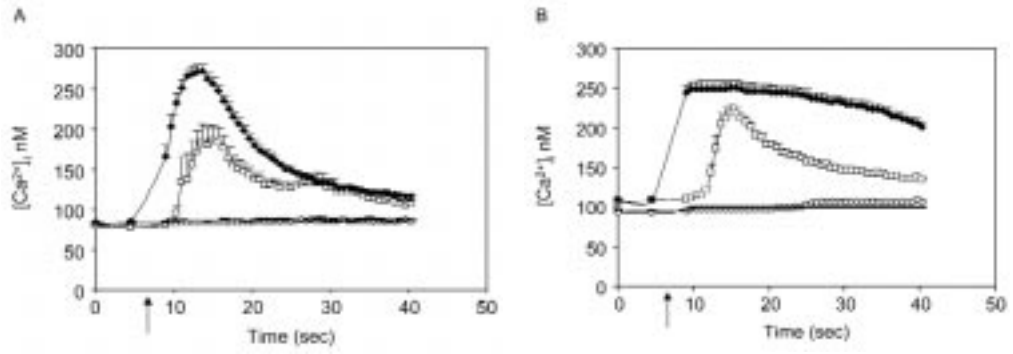

Figure 6. Effect of Pertussis Toxin on IL-8 (10^{-8} M) and C5a (10^{-8} M) induced $[Ca^{2+}]_i$ responses in eosinophils. Eosinophils were incubated with glycerol (0.5 %) or Pertussis Toxin (500 ng/ml, 2h, 37°C) and loaded with 2.5 μM Fura-2 AM. A) 8A2-treated cells were put on fibronectin-coated glasses or B) 8A2-untreated cells were put on 7h TNFα-activated HUVEC for 15 minutes. IL-8 (open squares) and C5a (solid diamonds) were added (arrow) to glycerol treated cells and IL-8 (open circles) and C5a (solid line) were added to Pertussis-Toxin-treated cells. Images were taken at 340 and 380 nm. $[Ca^{2+}]_i$ was measured by calculating 340/380 ratios (see Materials and Methods). The figure is representative for 3 independent experiments.

Discussion

In this article, the hypothesis was tested whether IL-8 can activate eosinophils when interacting with a physiological relevant substrate such as endothelial cells and/or fibronectin. Therefore, we first performed *in vitro* flow chamber experiments and evaluated the effect of IL-8 on rolling, non-stimulated eosinophils. We showed that IL-8 induced a transient arrest of eosinophils which were rolling on 7h TNFα-stimulated HUVEC even when α_4- or β_2-integrins were blocked. Only in the presence of blocking antibodies against both α_4- and β_2-integrins the IL-8-induced arrest was prevented. This shows that IL-8 can transduce signals leading to activation of α_4- as well as β_2-integrins. Furthermore we conclude that the chemokines eotaxin and IL-8 both act on α_4- and β_2-integrins. In contrast to IL-8 activation, eotaxin-induced activation of the integrins leads to firm adhesion and spreading. Also, Weber *et al.* concluded from static adhesion assays that chemoattractants, like RANTES, regulate the avidity of both β_1- and β_2-integrins expressed on the same eosinophil (37).

This is the first report showing I) that IL-8 affects resting, unprimed eosinophils in the transition from rolling to firm adhesion (figure 1 and 2) and II) that a chemokine can induce a transient arrest for a period of 0.5 to 2 minutes in the presence of the stimulus. Recently, Gerzten *et al.* (38) showed that monocytes, which are typical C-C chemokine

responders, firmly adhere to endothelium upon stimulation with the CXC chemokine IL-8. In contrast to eosinophils, monocytes adhered longterm to the endothelium upon IL-8 stimulation. These results suggest that the reaction to IL-8 is not restricted to the neutrophil lineage and can have different effects on different leukocyte subsets. We cannot exclude that activation of the endothelium by TNFα induces IL-8 secretion or presentation which could influence the eosinophil function. However, this seems unlikely because I) treatment of the endothelium by anti-IL-8 did not influence rolling velocity (data not shown) and II) the HUVEC was washed extensively before every experiment.

Our results also suggest that at least for unprimed eosinophils an additional stimulus besides IL-8 is needed to induce long term adhesion. This could be cytokines/ chemokines which are associated with allergic inflammation, like IL-5, IL-4 or eotaxin. Indeed, it is known from *in vitro* (15;16) studies that cytokine-activated eosinophils migrate in response to IL-8 in contrast to unactivated eosinophils. When eosinophils, which started rolling after the IL-8-induced transient arrest, were subsequently activated by eotaxin, the cells adhered firmly and longterm to the endothelium and spreading was visible. This indicates that IL-8 does not cross desensitise or modulate the eotaxin induced response. Longterm adhesion and spreading was also visible when eotaxin was administered directly to rolling cells (figure 1b, 1d, 2b). This transition between transient and longterm adhesion allows the control of leukocyte extravasation by integration of different signals induced by multiple cytokines. The effect of IL-8 on the arrest of cytokine-primed eosinophils cannot be addressed because cytokine-primed eosinophils showed static adhesion on activated endothelium, i.e. they did not roll because β_2 integrins were activated (39).

Activation of leukocytes by chemoattractants is often associated with an increase in the intracellular free Ca^{2+} concentration. However, many reports have only shown a small (if any) increase in $[Ca^{2+}]_i$ upon IL-8 stimulation of eosinophils (15;40;41). These studies measured the mean increase in $[Ca^{2+}]_i$ of a large population of cells in suspension. Indeed, Petering *et al.* showed increasing $[Ca^{2+}]_i$ responses in eosinophil suspensions to which increasing concentrations of neutrophils were added, suggesting that eosinophils in suspension do not raise $[Ca^{2+}]_i$ upon IL-8 stimulation (41). From a point of view it is more relevant to study changes in $[Ca^{2+}]_i$ in eosinophils adherent to natural relevant surfaces for several reasons: I) adhesion changes signaling in granulocytes (42), and II) chemokines are often presented by large carbohydrate structures on the surface of endothelial cells (43). Therefore, we investigated if IL-8 would elicit a change in $[Ca^{2+}]_i$ in eosinophils when attached to fibronectin or activated endothelium. We showed that ~40 % of the eosinophils adherent to fibronec-

tin (induced by β_1 integrin freezing antibody 8A2) and ~30 % of the eosinophils adherent to activated HUVEC exhibited an increase in $[Ca^{2+}]_i$ in response to IL-8. Petering *et al.* (41) concluded that contaminating neutrophils in the eosinophil suspensions caused the IL-8-induced increase in total $[Ca^{2+}]_i$. However, contaminating neutrophils cannot explain our results for varying reasons (I) our eosinophil populations contained less than 5 % neutrophils while 30 to 40 % of the adherent cells showed a $[Ca^{2+}]_i$ response upon IL-8 stimulation, (II) the contaminating effects of neutrophils were excluded by using single-cell measurement on adherent cells. Moreover, the eosinophil-specific eotaxin was added at the end of every experiment to show that the IL-8-responsive cells were indeed eosinophils.

In the static Ca^{2+} experiments only 30 and 40 % of the eosinophils adherent to activated HUVEC and fibronectin, respectively, were activated by IL-8. In contrast, almost all of the rolling eosinophils responded upon IL-8 in the flow chamber experiments. This discrepancy is consistent with the hypothesis that the subpopulation of IL-8-responsive cells are prone for an interaction with cytokine-activated endothelial cells under flow conditions.

We were not able to block the IL-8 induced transient arrest of eosinophils by antibodies against CXCR1 and CXCR2 (5A12 and 6C6, respectively) although the functionality of these antibodies was confirmed in migration assays. Using 5A12 and 6C6, the IL-8 receptors CXCR1 and 2 were not detected by FACS on eosinophils (figure 3b, c). This is analogous to the data of Petering *et al.* (41). Therefore, it is tempting to hypothesize the existence of an unknown IL-8 receptor on eosinophils. To show that a G-protein-coupled receptor is involved, PTX was added to the eosinophils, and this inhibited the IL-8-induced transient arrest and also the IL-8- and C5a-induced increase in $[Ca^{2+}]_i$. This indicates that a PTX sensitive G-protein-coupled receptor is mediating the effects of IL-8 on eosinophils.

Our experiments were performed on physiological surfaces expressing several integrin ligands, which might lead to cross-linking of integrins on the cell surface and concomitant cross-talk between these proteins (44). This putative cross-talk between integrins is not necessary per se for this transient arrest, because blockade of either Mac-1 or VLA-4 did not affect the IL-8 induced arrest in our flow chamber experiments. However, this does not mean that cross-talk did not occur. Interestingly, our experiments shown in figures 4 and 6 seem to indicate that possibly cross-linking of integrins by their ligands expressed by different surfaces influenced the kinetics of the IL-8- and/or C5a-induced changes in $[Ca^{2+}]_i$. The IL-8-induced changes in $[Ca^{2+}]_i$ are remarkably slow compared to eotaxin and C5a. These latter agonists were in contrast to

IL-8, very active in increasing $[Ca^{2+}]_i$ in eosinophils in suspension (41;15). Therefore, adhesion mediated by cross-linking of integrins might initiate a permissive signal for the IL-8-induced increase in $[Ca^{2+}]_i$ in adherent eosinophils. In addition, the C5a response had an unexpected sustained behaviour in eosinophils adherent on a surface, e.g. TNFα-activated endothelium, which is rich in different integrin ligands. Again, the kinetics of this response in adherent cells is different compared to the situation in suspension.

Summarizing, this study shows that resting, rolling eosinophils on 7h TNFα-stimulated HUVEC arrest transiently upon IL-8 stimulation at a shear rate of 2 dyn/cm^2. This α_4- and β_2-integrin-dependent process was probably not mediated by the known IL-8 receptors CXCR1 or CXCR2. In addition, ~40 and ~30 % of the adherent eosinophils (to fibronectin and activated endothelium respectively) increased their $[Ca^{2+}]_i$ in response to IL-8 stimulation. Our findings are consistent with a model that IL-8 can only transiently activate eosinophils, provided that they adhere to physiologically relevant surfaces. Transient arrest can easily be shifted to firm long-term arrest by additional chemokines. The transient arrest of eosinophils upon IL-8 exposure increases the time of contact between the cell and the endothelial lining, which potentiates the immunological surveillance.

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CHAPTER 5

Differential regulation of integrin-mediated leukocyte arrest by IL-8, eotaxin and C5a on activated endothelium

5

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In preparation

Abstract

The role of chemoattractants IL-8, eotaxin and C5a on integrin-dependent arrest of rolling leukocytes on activated endothelium was studied. Since changes in intracellular free Ca^{2+} levels ($[Ca^{2+}]_i$) have been implicated in the adhesive behaviour of cells, we focussed on the phospholipase C (PLC) family of enzymes that regulate $[Ca^{2+}]_i$ and which have been proposed to play a role in modulating integrin function. The effect of PLC inhibitor U73122 on chemoattractant-induced eosinophil adhesion under flow conditions was tested. IL-8-induced a β_2- and α_4-integrin-dependent transient arrest of unprimed eosinophils on TNFα-activated HUVEC under flow conditions. Interestingly, U73122 prevented the IL-8-induced arrest only when α_4- but not β_2-integrins were blocked. This suggests that the IL-8-induced activation of β_2 integrins but not of α_4 integrins is mediated via PLC. Eotaxin-1 induced an α_4- and β_2-integrin-dependent firm adhesion of eosinophils to activated endothelium under flow conditions. U73122 inhibited this arrest in the presence of blocking antibodies against β_2 integrins or against α_4 integrins. This suggests that the eotaxin-induced activation of both α_4 integrins as well as β_2 integrins are mediated via PLC. C5a, similar to eotaxin, induced a permanent firm arrest leading to spreading of the cells. U73122 prevented this arrest by 50 % in the presence of blocking antibodies against α_4 integrins or against β_2 integrins. When both α_4- and β_2- integrins were blocked, still 70 % of the cells arrested by C5a, in marked contrast to IL-8 and eotaxin, which have shown to be totally dependent on α_4- and β_2- integrins. Thus, it seems that C5a, unlike eotaxin, induced both PLC-dependent and independent adhesion of eosinophils under flow conditions. These data suggest that integrin receptors are differentially regulated through specific chemoattractant-receptor inside-out signal transduction pathways.

Introduction

The migration of leukocytes to inflammatory sites occurs in multiple steps. Cells roll along the endothelium, and when a chemoattractant is present, firm adhesion is induced and the migration through the endothelium starts (1). Firm adhesion is a result of activation of integrins that under resting conditions are not able to bind to their ligands, except for $\alpha_4\beta_1$ integrin, that binds its ligand VCAM-1 without activation. This chemoattractant-induced activation of integrins is called “inside-out” signaling and results in 1) a conformational change of the integrin which enhances affinity and/or 2) clustering of integrins, which enhances avidity of the integrin.

Chemoattractants can be divided in several classes. The classical peptide chemoattractants, like C5a (2), act on many leukocyte subtypes and are known to be very

potent. More specific chemoattractants are the chemokines that can be divided in CXCL, CCL, CL and CX_3CL families, based on the position of the cysteine motif (reviewed in (3)). CXCL chemokines are most active on polymorphonuclear cells, T and B cells, whereas CCL chemokines exert their action more on eosinophils, monocytes, basophils, T cells and NK cells. IL-8, a CXCL chemokine, is a very good chemoattractant for neutrophils and far less for eosinophils and monocytes (4). In contrast, eotaxin (5), a CCL chemokine, acts predominantly on eosinophils and basophils but not on neutrophils (6;7). Although there is specificity, no inflammatory chemokine is uniquely active on one population of leukocytes.

Chemoattractant-induced "inside-out" signaling results in affinity and/or avidity changes of integrins. Especially the activation of β_2 and β_1 integrins are important for adhesion to ICAM-1 and VCAM-1, respectively, expressed on activated endothelium. Of the β_2 integrins, LFA-1 (CD11a/CD18) which is highly expressed on lymphocytes, and Mac-1 (CD11b/CD18), which is highly expressed on granulocytes, can bind ICAM-1. Most of the β_1 integrins are ligands for extracellular matrix components, whereas only $\alpha_4\beta_1$ (VLA-4) has the capacity to bind VCAM-1. Under resting conditions, $\alpha_4\beta_1$ is present on eosinophils, monocytes and lymphocytes but not on neutrophils.

Binding of chemoattractants to their G-protein-coupled receptors results in the activation of phospholipase C, that cleaves phosphoinositol (4,5) bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 induces an increase in intracellular Ca^{2+} from internal stores, whereas DAG activates protein kinase C (PKC). $[Ca^{2+}]_i$ might play a crucial role in regulating avidity and affinity changes of integrins in leukocytes since it has been reported that an increase in $[Ca^{2+}]_i$ can activate integrins via inside-out signaling (8). Also, many cytoskeletal and signaling molecules are involved in regulating avidity changes of integrins and are themselves dependent on $[Ca^{2+}]_i$ increases in the cell (9;10).

In this study we compared the effects of different chemoattractants on changes in $[Ca^{2+}]_i$ and integrin activation. Since it was known from a previous study (11) that chemoattractant-induced arrest of rolling eosinophils on activated HUVEC was largely dependent on α_4- and β_2-integrins, we used this as a model sytem. Moreover, IL-8, eotaxin and C5a were compared in the context of their signaling routes to activate α_4 and β_2 integrins in an *in vitro* flow chamber model.

Materials and Methods

Reagents

CD16, CD14 and CD3 beads and isolation tools were purchased from Miltenyi, Biotec (Germany). Human serum albumin (HSA) was purchased from the Central Laboratory of The Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands). Recombinant human TNFα was purchased from Boehringer Mannheim (Germany). Isolation buffer contained PBS supplemented with pasteurized plasma solution (10 %) and sodium citrate (10 %). IL-8 (72aa, Peprotech INC., Rocky Hill, NJ, USA), Eotaxin-1 was purchased from R&D Systems (Minneapolis, MN, USA) and C5a was purchased from Sigma (St. Louis, USA). The Phospholipase C (PLC) inhibitor U73122 (1-{{6-{[(17β-3-methoxestra-1,3,5(10)trien-17-yl]amino}hexyl}}-1H-pyrrole-2,5-dione) and its inactive congener U73343 (1-{{6-{[(17β-3-methoxestra-1,3,5(10)trien-17-yl]amino}hexyl}}-2,5-pyrrolidine-dione) were from Calbiochem (La Jolla, CA, USA). Incubation buffer contained 20 mM HEPES, 132 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.2 mM KH_2PO_4, supplemented with 5 mM glucose, 1.0 mM $CaCl_2$, and 0.5 % (w/v) HSA. All other materials were reagent grade.

Antibodies

The MoAb HP2/1 (anti VLA-4, CD49d) was purchased from Immunotech (Marseille, France). MoAb IB4 was isolated from the supernatant of a hybridoma obtained from the American Type Culture Collection (Rockville, MD, USA). MoAbs mentioned above are functionally blocking antibodies. Control antibody W6/32 (anti HLA-A,B,C) was isolated from the supernatant of a hybridoma obtained from the American Type Culture Collection (Rockville, MD, USA). MoAbs were incubated with eosinophils ($4x10^6$cells/ml) at 10 μg/ml during 15 minutes before the experiments. The cell suspensions were diluted once with incubation buffer (final concentration of 5 μg/ml MoAb at $2x10^6$ cells/ml in incubation buffer), and the coverslips were placed directly in the system.

Isolation of eosinophils

Blood was obtained from healthy volunteers from the Red Cross Blood Bank (Utrecht, The Netherlands) anti-coagulated with 0.4 % (w/v) trisodium citrate (pH 7.4). Mixed granulocytes were isolated as described previously (12). Mononuclear cells were

removed by centrifugation over isotonic Ficoll (1.077 g/ml). After lysis of the erythrocytes with an isotonic ice-cold NH_4Cl solution, the granulocytes were washed and resuspended in isolation buffer. Eosinophils were purified from granulocytes by negative immunomagnetic selection with anti-CD16-conjugated microbeads (MACS; Miltenyi Biotec, 13). To avoid mononuclear cell contamination also anti-CD3- and anti- CD14-conjugated microbeads were added to the granulocyte suspension. Purity of eosinophils was >97 %.

Endothelial cells

Human umbilical vein endothelial cells (HUVEC) were isolated from human umbilical cord veins according to Jaffe *et al.* (14), with some minor modifications (15). The cells were cultured in Endothelial cell Growth Medium-2 (EGM-2) (Biowhittaker, Walkersville, MD, USA). Cell monolayers were grown to confluence in 5-7 days. Endothelial cells of the second passage or third passage were used in perfusion assays. HUVEC was activated by TNFα (100 U/ml, 5-7 hours, 37°C) prior to the perfusion experiments.

Perfusion chamber

Perfusions under steady flow were performed in a modified form of transparent parallel-plate perfusion chamber (16) as previously described by Van Zanten *et al.* (15). This micro-chamber has a slit height of 0.2 mm and a width of 2 mm. The chamber contains a circular plug on which a coverslip (18 mm x 18 mm) with confluent HUVEC was mounted.

Eosinophil perfusion and evaluation

In vitro flow chamber experiments were performed as described (11;17). In short, eosinophils in suspension ($2x10^6$ cells/ml in incubation buffer) were aspirated from a reservoir through the perfusion chamber. Eosinophil perfusions were performed as individual runs under specific shear conditions by 37°C. During the perfusion, the flow chamber was mounted on a microscope stage (DM RXE, Leica, Weitzlar, Germany) which was equiped with a B/W CCD-video-camera (Sanyo, Osaka, Japan), coupled to a VHS video recorder. Perfusion experiments were recorded on video tape. Video images were evaluated for the percentage of rolling cells by dedicated routines made in

the image-analysis software Optimas 6.1 (Media Cybernetics systems, Silverspring, MD, USA). The eosinophils which were in contact with the surface appeared as bright white-centered cells after proper adjustment of the microscope during recording. The adherent (rolling or firmly attached) cells on the HUVEC were detected by the image analyzer. The eosinophil suspension was perfused during 3 minutes at a shear stress of 0.8 dyn/cm^2 to obtain an endothelial surface with firmly adherent and rolling eosinophils (17). Shear stress was increased to 2 dyn/cm^2, and recording of the images on video was started. Subsequently, the cytokines IL-8 (10^{-8} M), eotaxin (10^{-8} M) or C5a (10^{-8} M) were added in the presence or absence of antibodies (IB4, HP2/1 or W6/32; 10 μg/ml) or in the presence of the PLC inhibitor U73122 or the control analogue U73343. To automatically determine the velocity of rolling cells, custom-made software was developed in Optimas 6.1. For determining the percentage of rolling cells and the rolling velocity sequence of 50 frames representing an adjustable time interval (δt, with a minimal interval of 80 milliseconds, ~30 sec before, during and 1 minute after stimulus addition) was digitally captured. Within each frame, the position of every cell was detected and for all subsequent frames the distance moved by each cell and the number of images in which a cell appeared in focus was measured. The velocity of a cell (v) in micrometers per sec was calculated from the equation: $v = L/\delta t(x-1)$ in which L is the covered distance (μm), δt is the time interval between images (seconds), and x is the number of images in which a cell appears. The cut-off value to distinguish between rolling and static adherent cells was set at 1μm/sec. With this method, static adherent, rolling and freely flowing cells (which were not in focus) could be distinguished clearly.

Imaging of intracellular free Ca^{2+} on adherent cells

Imaging of intracellular free Ca^{2+} was performed with a custom-built set-up as described before (11). Series of 50 image pairs (512 x 512, 0.6 second apart) were sequentially grabbed with a black and white framegrabber (Pulsar, Matrox Electronic Systems Ltd., Dorval, Quebec, Canada) from an intensified video camera (LI-μCAM, Lambert Instruments, Leutingewolde, The Netherlands) directly into computer memory. Ratios and calcium values were computed off-line with Image Analysis software (Optimas 6.1, Media Cybernetics, Silver Spring, MD, USA) using custom-made macros (A.L.I.). Calcium levels were calculated with the standard calibration formula of Grynkiewics *et al.* (18):

$[Ca^{2+}]_i = K_d \times \beta \times ((R-Rmin)/(Rmax-R))$ with parameters K_d=225 nM, β=2.4 and displayed in false colors (blue-red ramp). Cell calcium was separated from background by calculating the threshold mask from the mean of the two ratio images for each of the 50 ratio images in the series. The threshold level was obtained by choosing the first and

the last level in the series, and then by linear interpolation for the rest of the series, thereby counteracting the effect of bleaching over time. Intracellular [Ca^{2+}] levels were determined by calculating the mean [Ca^{2+}]$_i$ of all pixels in a cell in each of the 50 ratios taken. The cut-off value to distinguish between a responding and a non-responding cell was set at a mean value of 200 nM. The insert in figure 1 shows the mean [Ca^{2+}]$_i$ of all cells exceeding this cut-off value. All washing and incubation steps were performed in incubation buffer. Eosinophils ($4x10^6$/ml) were loaded with 1.25 μM Fura-2 AM (Molecular Probes, Eugene, OR, USA) for 15 minutes, then washed and kept at room temperature. 7h TNF-α-activated HUVEC cultured on 24 mm cover slips were extensively washed with PBS and non-treated, Fura-2 AM- loaded eosinophils were plated on the endothelium. The eosinophils were allowed to settle for 15 minutes on the substrate, and no adherent cells were removed by washing the cover slips. Cover slips were mounted in an open chamber and placed in a warmed (37°C) metal ring for Calcium Imaging. Stimulants were added from the top after two pre-stimulus image ratios had been obtained. Stimulants (37°C, at 2 times the concentration) were added to an equal volume (250 μl) of incubation buffer already present in the chamber to obtain a homogenous mixture at the start of the ratio imaging.

Imaging of intracellular free Ca^{2+} on non-adherent cells

Cells were loaded with Fura-2AM as described above and were kept at room temperature until use. Cells were put in a quartz cuvette (400 μl, $2x10^6$/ml) and stimulated with indicated chemoattractants. The fluoresence was measured under stirring conditions at 37°C in a Hitachi F-4500 fluorescent spectrophotometer (Hitachi Ltd, Tokyo, Japan), using a multi-wavelength timescan program. Fura-2 fluorescence was measured at 340 nm (F1) and 380 nm (F2) at 510nm emission. [Ca^{2+}]$_i$ was calculated as described above.

Statistical Analysis. Statistical analysis of the data was performed using the Student T test, p values < 0.05 were considered to be significant.

Results

Different effects of IL-8, eotaxin and C5a on $[Ca^{2+}]_i$ in both adherent and non-adherent eosinophils

An important difference between IL-8, eotaxin and C5a is that the IL-8 induced increase in $[Ca^{2+}]_i$ occurs only when eosinophils adhere to a substrate but not when cells are in suspension, whereas eotaxin and C5a induce an increase in $[Ca^{2+}]_i$ in both adherent and non-adherent cells (figure 1, 2 and also a previous study (11)). The previous study also showed that IL-8-induced an activation of β_2- and α_4-integrins, resulting in an arrest of eosinophils on activated endothelial cells. Therefore, we hypothesize that upon IL-8 activation the integrins are cross-linked to their ligands on the endothelium. And subsequently, the integrin cross-linking might result in the observed release of $[Ca^{2+}]_i$. It is known that cross-linking of β_2 integrins phosphorylates PLC-γ which results in an increase in $[Ca^{2+}]_i$ (19). Since this isoform of the PLC family is dependent on tyrosine phosphorylation, we investigated whether a tyrosine kinase inhibitor could block the IL-8-induced Ca^{2+} signal. Figure 1b shows that genistein, an inhibitor of tyrosine kinases, blocks the IL-8 induced increase in $[Ca^{2+}]_i$ but had no effect on eotaxin- and C5a-induced increase in $[Ca^{2+}]_i$. This suggests that the IL-8, but not the eotaxin- or C5a-induced increase in $[Ca^{2+}]_i$ is dependent on a tyrosine kinase. Next, the PLC inhibitor U73122 was used to show that IL-8, eotaxin and C5a absolutely depend on functional PLC activity. Figure 2 shows that on adherent cells (2a, 2b) and cells in suspension (2c) U73122 (2a, 2c), but not the control compound U73343 (2b, 2c), abrogates the increase in $[Ca^{2+}]_i$. Remarkably, the Ca^{2+} experiments on adherent cells showed that U73122 not only abrogated the chemoattractant-induced increases in $[Ca^{2+}]_i$ but also interfered with adhesion. Cells were attached very loosely to the endothelial surface. Thus, PLC appears to be critical for changes in $[Ca^{2+}]_i$ but also for adhesion events.

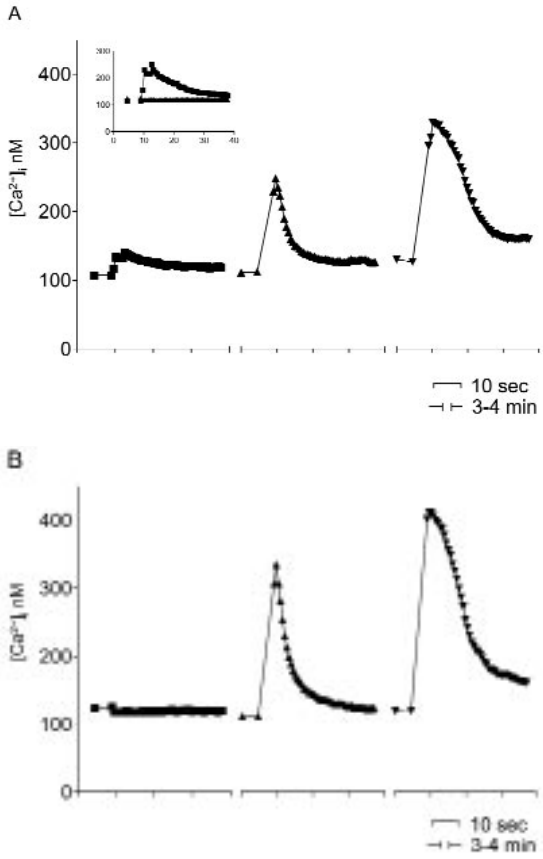


Figure 1. IL-8-, eotaxin- and C5a-induced Ca^{2+} response in the presence of control solvent treatment (A) or genistein (B). Fura-2AM-loaded eosinophils were incubated with DMSO (0.1 %) or genistein (50 μM) for 15 minutes and loaded on activated endothelial cells. IL-8 (10^{-8} M, solid squares), eotaxin (10^{-8} M, solid triangles, point up) and C5a (10^{-8} M, solid triangles, point down) were applied with 3-4 minutes recovering time in between. Mean Ca^{2+} tracks are shown of cells in one image. The insert depicts the mean track of the cells that responded to IL-8 (15 % of the cells responded, cut-off value $[Ca^{2+}]$ >200 nM) in the presence of control solvent (solid squares). Genistein treated cells (insert, flat line) showed no increase in $[Ca^{2+}]$ > 200 nM upon IL-8. This is a representative experiment out of 3.

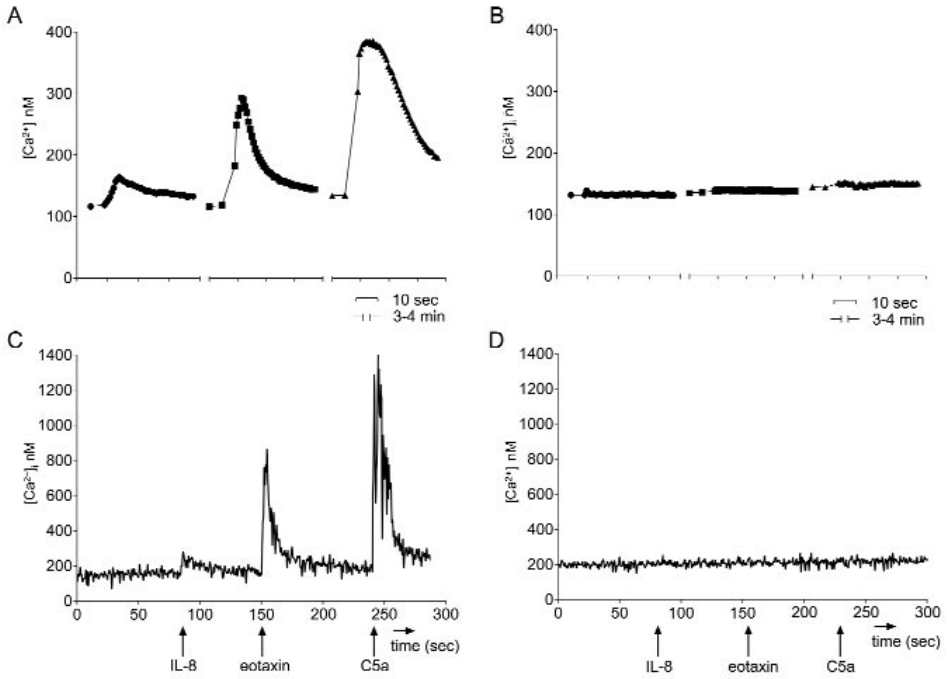


Figure 2. IL-8-, eotaxin- and C5a-induced Ca^{2+} response on adherent eosinophils in the presence of A) control compound (U73343, 10 μM) or B) PLC inhibitor (U73122, 10 μM). Fura-2AM loaded eosinophils were incubated with U73343 or U73122 for 15 minutes and loaded on activated endothelial cells. IL-8 (10^{-8} M, solid circles), eotaxin (10^{-8} M, solid squares) and C5a (10^{-8} M, solid triangles) were applied with 3-4 minutes recovering time in between. Mean Ca^{2+} tracks are shown of cells in one image. This is a representative experiment out of 3. IL-8-, eotaxin- and C5a-induced Ca^{2+} responses in the presence of U73343 (C) or U73122 (D) are shown for eosinophils in suspension. Peak Ca^{2+} responses are depicted. This is a representative experiment out of 2.

Effect of U73122 on adhesive behaviour of eosinophils under flow

Since the Ca^{2+} experiments revealed that U73122 interfered with adhesion, we first examined the effect of U73122 on the rolling behaviour of eosinophils on activated endothelial cells. Eosinophils were incubated with the indicated inhibitors and monoclonal antibodies (MoAbs) and perfused for 3 minutes, and the percentage of rolling cells was determined at a shear rate of 2 dyn/cm^2. Figure 3 shows that 35 ± 6 % of the eosinophils treated with the control compound U73343 and W6/32 (control/non-blocking MoAb) rolled on the activated endothelium. Addition of the active compound U73122 and W6/32 showed an increase in rolling eosinophils to 81 ± 3 %. Treating the eosinophils with HP2/1 (a blocking MoAb against α_4 integrins) together with the control

compound U73343 resulted in a percentage of rolling cells of 74 ± 4 %. Addition of U73122 and HP2/1 to eosinophils even further increased the percentage of rolling cells to 88 ± 3 %. IB4 (a blocking β_2 integrin MoAb) together with the control compound U73343 resulted in a percentage rolling of 44 ± 5 %, whereas the combination of IB4 with U73122 further increased rolling to 81 ± 4 %. We conclude that U73122 as well as inhibiting intracellular Ca^{2+}, also decreased the basal activity of α_4 integrins. Furthermore, comparing W6/32-treated cells and IB4-treated cells both in the presence of U73433, the percentage of rolling cells is 35 ± 6 and 44 ± 5 %, respectively. Although these values do not differ significantly, it seems that β_2 integrins on resting eosinophils are slightly activated. This slight activation is further addressed by the observation that in the presence of blocking antibodies against α_4 integrins, and thus focussing on β_2 integrin mediated processes, U73122 leads to a higher percentage of rolling cells than U73343 (88 ± 3 % compared to 74 ± 4 %, respectively, $p<0.01$).

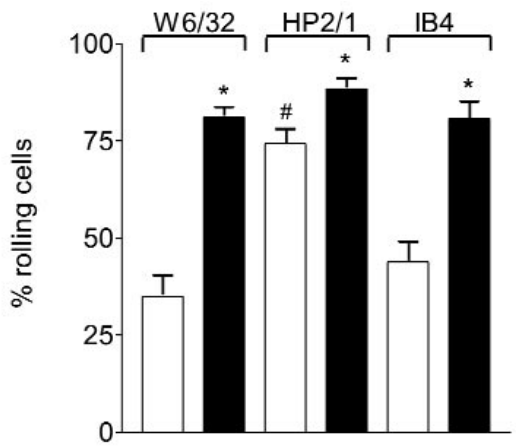


Figure 3. Effect of inhibition of PLC on the percentage of rolling eosinophils on activated HUVEC in the presence of different MoAbs. Eosinophils were incubated with indicated MoAbs against HLA(A,B,C), (W6/32, control), β_2 integrins (IB4) or α_4 integrins (HP2/1) and in the presence of the control compound U73343 (10 µM, white bars) or the PLC inhibitor U73122 (10 µM, black bars) for 15 minutes. Cells were perfused over 5-7 hours TNFα-activated HUVEC and the percentage of rolling cells was determined at a shear rate of 2 dyn/cm^2. *: $p<0.01$, #: $p<0.001$, p values were determined by an unpaired Student T-Test.

IL-8, eotaxin and C5a induced arrest of rolling eosinophils

Previous results have shown that IL-8 induced a transient arrest of eosinophils on TNFα-activated HUVEC that is α_4- and β_2- integrin dependent (11). Remarkably, cells remained round upon IL-8-induced arrest (figure 4a and 4b) suggesting that IL-8 can activate integrins that does not result in firm adhesion and spreading. In contrast to IL-8, eotaxin (figure 4c and 4d) and C5a (figure 4e and 4f) induced a firm arrest of eosinophil to activated HUVEC followed by spreading of the cells. To address the differences in integrin modulation by these chemoattractants we focussed on chemoattractant-induced intracellular signals in eosinophils upstream of $[Ca^{2+}]_i$.

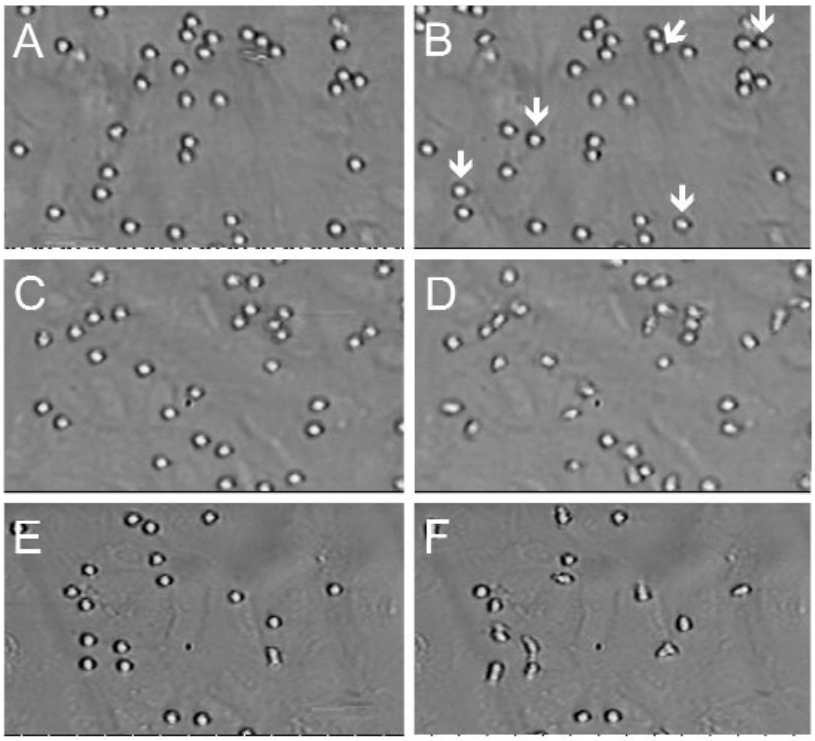
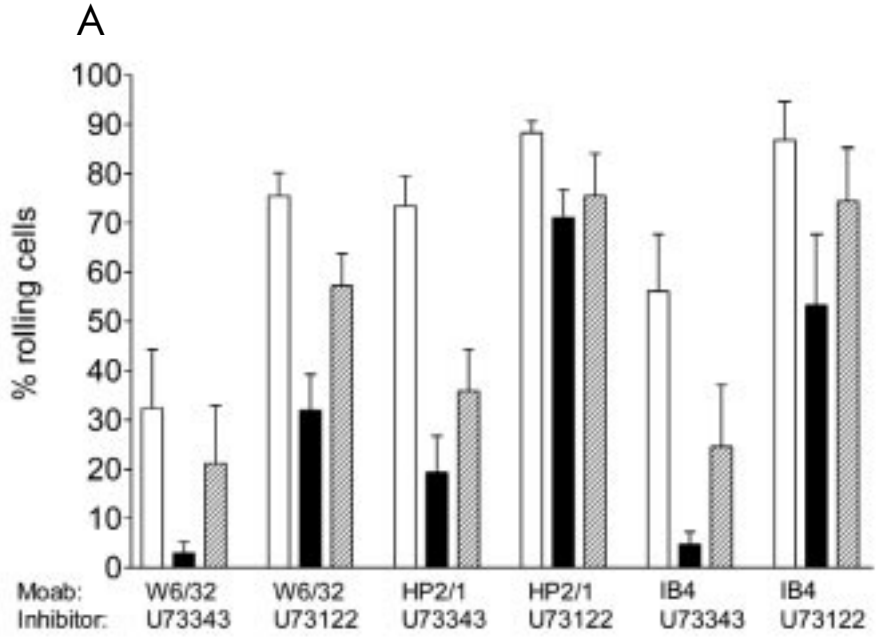


Figure 4. Effect of IL-8, eotaxin and C5a on the transition from rolling to arrest of eosinophils on TNFα-activated HUVEC under flow conditions (2 dyn/cm^2). Panel A, C, and E show the effects of IL-8 (A), eotaxin (C) and C5a (E) the moment the cells arrest. Cells were round. Panel B, D and F show the effects of IL-8 (B), eotaxin (D) and C5a (F) one minute after the chemoattractants induced the arrest. In panel B (IL-8) the cells remained round and some started rolling again, as shown by the different positions the cells had compared to panel A (arrows indicate rolling cells). In panel D (eotaxin) and F (C5a) the cells spreaded and were at the exactly same place as in panel C and E, respectively.

Effect of inhibition of PLC on the IL-8-, eotaxin- and C5a-induced arrest of rolling eosinophils

U73122 and U73343 were used as a tool to study chemoattractant-induced inside-out signaling in eosinophils. Cells were incubated with the indicated inhibitors and MoAbs and perfused for 3 minutes. The percentage of rolling cells was determined at a shear rate of 2 dyn/cm^2. Subsequently chemoattractants were perfused over the cells and the percentage rolling was determined ~0.5 minute and ~1 minute after adding the chemoattractant. Figures 5a, 5b and 5c depict the effects of IL-8 (10^{-8} M), eotaxin (10^{-8} M) and C5a (10^{-8} M), respectively. Figure 5a shows that eosinophils treated with the control compound U73343 in combination with W6/32, HP2/1 or IB4 showed a transient arrest upon IL-8 addition. A transient arrest upon IL-8 stimulation was also observed when eosinophils were incubated with W6/32 and U73122. However, HP2/1- and U73122-treated cells showed no arrest upon IL-8 stimulation, the cells kept rolling. IB4- and U73122-treated cells did arrest transiently upon IL-8, comparable to the control (W6/32) treated cells. This suggests that the IL-8-induced activation of β_2 integrins, but not of the α_4 integrins, is dependent on PLC. Figure 5b shows that the control compound U73343 has no effect on the eotaxin-induced firm arrest in the presence of W6/32, HP2/1 or IB4. However, when cells were incubated with U73122 in the combination with either W6/32, HP2/1 or IB4, the cells did not arrest but kept rolling. This suggests that the eotaxin induced α_4- and β_2-integrin activation are both dependent on PLC.
The conclusions for IL-8- and eotaxin-induced effects can be drawn because we recently showed that IL-8- and eotaxin-induced arrest was totally dependent on α_4- and β_2-integrins (11). For C5a this is not yet known. In figure 5 (last set of data) it is shown that C5a induced arrest of eosinophils is for 30 % dependent on the combined action of β_2 and α_4 integrins. The control compound U73343 in combination with either W6/32, HP2/1 or IB4 induced firm adhesion of eosinophils on activated HUVEC. In all three cases, U73122 in combination with either W6/32, HP2/1 or IB4 induced a 50 % decrease in rolling cells.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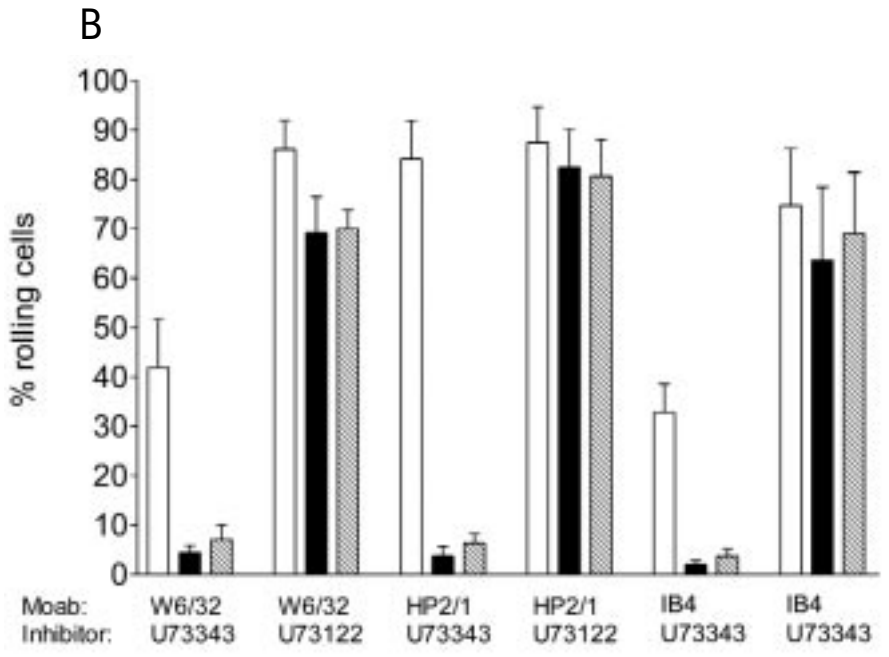
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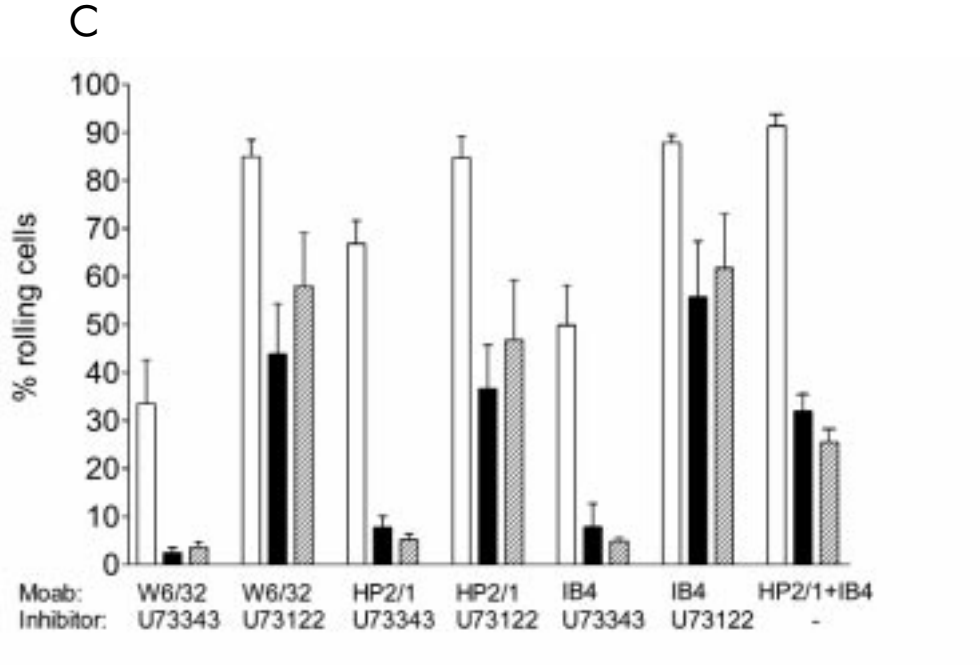

Figure 5. Effect of inhibition of PLC on chemoattractant-induced arrest of rolling eosinophils on activated HUVEC in the presence of different MoAbs. Eosinophils were incubated with the indicated MoAbs against HLA(A,B,C) (W6/32, control), β_2 integrins (IB4) or α_4 integrins (HP2/1) and in the presence of the control compound U73343 (10 μM) or the PLC inhibitor U73122 (10 μM) for 15 minutes. Cells were perfused over 5-7 hours TNFα-activated HUVEC, and the percentage of rolling cells was determined before (white bars), during (black bars) of one minute after addition of chemoattractant (hatched bars). The chemoattractants IL-8 (10^{-8} M, A), eotaxin (10^{-8} M, B) and C5a (10^{-8} M, C) were tested. In panel 5C the last set of bars represents the effect of C5a on HP2/1- and IB4-treated eosinophils. Values are displayed as mean ± SEM of at least 3 experiments.

Discussion

Most of the chemoattractant receptors on granulocytes belong to the serpentine family. Signaling via these receptors induced by the corresponding chemoattractants, such as bacterial products, bioactive lipids and chemokines, follows a general theme. Upon receptor engagement high-molecular trimeric G-proteins are activated, which in turn activate phospholipase-Cβ, leading to liberation of inositol-1,4,5-trisphosphate and 1,2-diacylglycerol. These second messengers induce an increase in intracellular free Ca^{2+} ($[Ca^{2+}]_i$) and activation of protein kinase C (PKC), respectively (20). These signaling molecules are thought to play an important role in the movement of the cells (10;21) by activating adhesion molecules, integrins, on the surface of the leukocytes (8;9;22;23). Several lines of evidence, obtained from experiments employing cells adherent to several surfaces, are in support of this hypothesis (24-26).

General validity of this mechanism is hampered by an important conceptual problem, namely how specificity is achieved. If all chemoattractant receptors utilize similar signaling routes in controlling the affinity/avidity state of the integrins, the cell cannot discriminate between the presence of one chemoattractant from the other. We set up experiments to test an alternative hypothesis, postulating that different chemoattractants modulate the functionality of integrins on eosinophils in different ways. We tested this hypothesis with eosinophils adherent to endothelial cells under flow conditions. This adhesion system resembles the *in vivo* situation more than models under static conditions. We chose three eosinophil chemoattractants that have unique characteristics: IL-8 only activating adherent and/or primed eosinophils (11;27;28), eotaxin, a more specific chemokine for eosinophils (29) and C5a, a very potent chemoattractant (30). Eosinophils were activated with these three chemoattractants and evaluated in the context of activation of PLC and increased $[Ca^{2+}]_i$ and adhesion under flow conditions to TNFα-activated endothelial cells.

An important difference between the three chemoattractants was found in their ability to increase the $[Ca^{2+}]_i$ in eosinophils. In marked contrast to eotaxin and C5a, IL-8 induced an increase in $[Ca^{2+}]_i$ only after adhesion of the cells to a physiological surface (figure 2 and (11)). These increases in $[Ca^{2+}]_i$ must have been mediated by activation of PLC, because the PLC inhibitor U73122 completely blocked these responses, whereas the inactive analogue U73122 did not have an effect. These findings are consistent with the hypothesis that both CCR3 and C5aR signal via a G-protein-dependent activation of PLC-β, as has been shown by others (20;31). The situation with IL-8 is more complex. The IL-8-induced increase in $[Ca^{2+}]_i$ in eosinophils is mediated by a mechanism involving a tyrosine-kinase-mediated activation of PLC (figure 1).

Together with the finding that the increase is late (optimal only after several seconds, see figure 1 and reference 11) it is tempting to speculate that PLC-γ is activated under these conditions. It is not likely that this isoform is directly activated by a chemokine receptor. A more attractive hypothesis is that PLC-γ is activated as a result of cross-linking of integrins on the cell surface. Indeed, cross-linking of β_2 integrins in several cell systems induces a clear increase in $[Ca^{2+}]_i$ that is mediated by PLC-γ (19). This also explains why IL-8 can only induce changes in $[Ca^{2+}]_i$ in adherent cells.
Activation of PLC is the common denominator in activation of eosinophils by the three different chemoattractants. Therefore, we evaluated whether inhibition of PLC has a consequence on the adhesion behaviour of human eosinophils to activated endothelial cells under flow conditions. It has been reported that PLC plays a role in granulocytes and platelets as determined by static adhesion assays (32;33). We found that IL-8, eotaxin- and C5a-induced adhesion events were differentially regulated by PLC. IL-8 induced a transient arrest of eosinophils that had been incubated with blocking antibodies against β_2 integrins and with the PLC inhibitor. When α_4 integrins and PLC were inhibited in eosinophils, IL-8 did not induce an arrest. In an earlier study we showed that the IL-8- as well as the eotaxin-induced arrest of eosinophils to activated endothelium were both totally dependent on α_4- and β_2- integrins (11). Thus, IL-8 induced a PLC-dependent β_2 integrin but a PLC-independent α_4 integrin-mediated arrest. For eotaxin a different phenotype was observed. Eotaxin did not induce an arrest of eosinophils that were incubated with the PLC inhibitor and a control antibody (W6/32). Concomitant with this observation we showed that the PLC inhibitor in combination with blocking antibodies against α_4- or β_2-integrins also prevented an eotaxin induced arrest. This indicates that eotaxin activates β_2 integrins and α_4 integrins in a PLC dependent manner.

The situation for C5a is more complex. In contrast to IL-8 and eotaxin, the C5a-mediated arrest of eosinophils was only inhibited by 30 % when both α_4- and β_2-integrins were inhibited. This suggests that additional adhesion molecules may exist. Furthermore, inhibition of PLC resulted in a C5a-induced arrest of 50 % of the cells, irrespective of whether α_4 integrins or β_2 integrins were blocked simultaneously. From this observation we can conclude that PLC-dependent processes are involved in C5a-induced arrest. We showed that different chemoattractants may use PLC in different ways and we postulate that this leads to specificity in chemoattractant-induced integrin modulation.

We hypothesize that the order of potency of the three chemoattractants in inducing eosinophil adhesion is C5a > eotaxin > IL-8. Possible mechanisms explaining this phenomenon are: 1) additional, but undefined, adhesion mechanisms that can be initiated by C5a but not by eotaxin and IL-8 and 2) C5a and eotaxin activate

intracellular pathways that lead to spreading of the cells, whereas IL-8 does not. The observed differences between the studied chemoattractants in inducing the transition from the rolling to the firm adhesion state may predict differences in the migratory capacity of these chemoattractants. Indeed, IL-8 was only able to induce a transient arrest of rolling eosinophils and IL-8 appeared to be a weak inducer of eosinophil migration. Only when eosinophils are primed, IL-8 was only able to induce small migratory responses (27). Eotaxin appeared to be a strong inducer of the migratory reponse of eosinophils over endothelial cells (34;35;29) that equals the C5a-induced transmigration (J. Alblas, unpublished observations). However, eotaxin-induced eosinophil migration over bare filters only succeeded using thin (10 μm) filters (36). In thick (150 μm) bare filters, eotaxin only induced migration in the presence of 10 % human pooled serum (J. Alblas, personal observations). Furthermore, eosinophils isolated from IL-5 transgenic mice migrated equally well in response to eotaxin in the presence or absence of endothelial cells (34). This indicates that eosinophils need an additional stimulus (provided by endothelial cells, serum or cytokines) besides eotaxin to induce migration over long distances. On the contrary, C5a induced eosinophil migration on bare, thick filters (30) without additional signals needed. Thus, the potency of IL-8, eotaxin and C5a to induce an arrest of eosinophils may equal their potency in inducing chemotaxis. This might be a result of the differences between the chemoattractants in activating adhesion molecules on the cells.

In summary, this study showed that phospholipase C plays an important role in integrin-mediated arrest of leukocytes. The α_4 integrin-mediated arrest is dependent on functional PLC only for the chemoattractants eotaxin and C5a, but not IL-8. This IL-8 induced PLC-independent pathway for activating α_4 integrins is intriguing but the mechanism is not known for the moment. The β_2 integrin-mediated arrest is dependent on PLC for the chemoattractants IL-8 and eotaxin, and possibly also for C5a. Therefore, different chemoattractant receptors can induce different inside-out signaling pathways for regulating the same integrin. Also, one chemoattractant can use different inside-out signaling pathways for activating different integrins on the same cell. In conclusion, each chemoattractant may lead to activation of specific intracellular pathways leading to specific control of adhesion processes.

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CHAPTER 6

Activation of RhoA and ROCK are essential for detachment of migrating leukocytes

6

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Abstract

Detachment of the rear of the cell from its substratum is an important aspect of locomotion. The signaling routes involved in this adhesive release are largely unknown. One of the few candidate proteins to play a role is RhoA, since activation of RhoA in many cell types leads to contraction, a mechanism probably involved in detachment. To study the role of RhoA in detachment regulation, we analyzed several subsets of migratory leukocytes by video microscopy. In contrast to fast migrating neutrophils, eosinophils did not detach the rear of the cell unless stimulated with serum. When measuring the amount of active RhoA, using a GST-Rhotekin pull-down assay, we found that serum is an excellent activator of RhoA in granulocytes. Inhibition of RhoA or one of Rho's target proteins, the kinase ROCK, in neutrophils resulted to the phenotype observed in eosinophils: the rear of the cell was firmly attached to the substratum, whereas the cell body was highly motile. ROCK inhibition resulted to impaired migration of granulocytes in filters, on glass and through endothelial monolayers. Also, the ROCK signaling pathway is involved in changes in integrin-mediated adhesion. Eosinophil transduction by a tat-fusion construct containing active RhoA resulted in detachment stimulation in the presence of chemoattractant. From these results we conclude that activation of the RhoA-ROCK pathway is essential for detachment of migratory leukocytes.

Introduction

When an eukaryotic cell migrates, the leading lamella protrudes, then the cell body moves forward and finally the rear of the cell (or uropod) releases the binding from the extracellular environment. When the process of rear release is slow compared to the protrusion of the leading lamella, it will determine the migration rate (1). The mechanisms by which adhesions release are largely unknown. First, cytoskeletal contraction may overcome the negative force that is excerted by the adhesion molecules bound to the extracellular matrix. Contraction of actin filaments can pull on filaments connected to integrins that link the cell to the extracellular matrix (2). Alternatively, ligand-induced signaling has been proposed to play a role in adhesive release. Calcineurin, a calcium-regulated serine-threonine phosphatase, plays a role in recycling of integrins in neutrophils (3). Also implicated in adhesive release is RhoA: inhibitors of RhoA induce cytoskeletal breakdown and cell rounding (4) and inhibit migration in several cell types (5).

RhoA is a member of the Ras superfamily of small GTP-binding proteins that regulate formation of actin stress fibers and focal adhesions. RhoA activation can be blocked by the ADP-ribosyltransferase from Clostridium botulinum, C3 exoenzyme. In this way the importance of RhoA in several cellular processes, such as cell morphology, migration, cytokinesis, DNA synthesis and cell growth, has been established (reviewed in 6). Like Ras, RhoA cycles between a GDP-bound inactive state and a GTP-bound active state. RhoA-bound nucleotides are regulated by several groups of proteins: guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs). When GTP-bound, RhoA binds to and activates a number of downstream effectors such as Rho kinase, lipid kinases and Rhophilin (7). Rho kinase, also known as ROCKII or ROKα is closely related to p160ROCK (ROCKI) or ROKβ (8). ROCKII is implicated in mediating actomyosin-based contractility stimulated by RhoA. It phosphorylates myosin phosphatase, resulting in elevated myosin light chain (MLC) phosphorylation (9). MLC phosphorylation is correlated with myosin II filament assembly and actin-activated myosin ATPase activity (10). Apart from this, Rho kinase may also phosphorylate MLC directly (11). Using the specific inhibitor Y27632, ROCKs were shown to be important for many of the Rho-mediated cellular processes, including smooth muscle contraction (12), myosin light chain phosphorylation (13), tumor cell invasion (14) and motility (15).

Until recently, RhoA activity was established by the appearance of stress fibers or focal adhesions. In leukocytes, however, stress fibers and focal adhesions are not formed. Several groups have now published an activation assay for RhoA, using the RhoA-binding domain of Rhotekin (16), and were able to show RhoA activation upon stimulation of 3T3 cells with the integrin ligand fibronectin or with LPA (17;18).

We have studied the role of RhoA and its downstream kinase ROCK in the process of detachment of highly motile leukocyte subsets. Serum stimulation of detachment-defective eosinophils leads to RhoA-activation and enhanced migration. Inhibition of RhoA or ROCK in motile neutrophils results in a phenotype similar to eosinophils: the leading lamella is protruding, whereas the rear of the cell remains firmly attached to the substratum. Furthermore, we investigated the effects of ROCK activity on adhesion. The results are discussed with respect to the theoretical background of rear release and its role during cell migration.

Materials and Methods

Reagents and antibodies

Eotaxin was obtained from R&D Systems; PAF (1-O-hexadecyl-2-acetyl-sn-glycero-3-phosphoryl-choline) and fMLP were from Sigma; recombinant human IL-5 was a gift from M. McKinnon (Glaxo-Wellcome, Stevenage, UK); Ficoll-paque and Percoll were from Pharmacia (Uppsala, Sweden); human serum albumin (HSA) and pasteurized plasma solution were from CLB (Amsterdam, The Netherlands); Calcein-AM and Alexa-phalloidin were from Molecular Probes; VCAM-1 and ICAM-1 were from R&D Systems (Abingdon,UK); neutralizing antibodies against α_4-integrin (HP2/1) were from Immunotech (Marseille, France); antibodies against β_2-integrins (IB4) were isolated from culture medium of hybridoma obtained from the ATCC (Rockville, USA); anti-RhoA was from Santa Cruz, anti-phospho-Erk1/2, anti-phospho-p38 and anti-phospho-PKB were from BioLabs. The ROCK-inhibitor Y27632 was kindly provided by Welfide Corporation (Osaka, Japan).

Cell isolation

Blood was obtained from healthy volunteers via the Red Cross Blood Bank (Utrecht, The Netherlands). Eosinophils were isolated as previously described (19). In short, buffy coats of citrate-anticoagulated (0.4 % w/v) blood were subjected to Ficoll centrifugation. Erythrocytes were removed by isotonic shock (NH_4Cl), and the mixed granulocyte fraction, after 10 min treatment with fMLP (10^{-8} M), was subjected to discontinuous Percoll gradient (1.082/1.1 g/ml) centrifugation. Dense eosinophils were washed once with PBS containing 0.3 % citrate and 10 % pasteurized plasma, then taken up in RPMI-Hepes supplemented with 0.5 % HSA. The eosinophils were more than 95 % pure and more than 98 % viable. Cell viability was not affected by treatment with Y27632 (100 μM) for up to 2.5 hours.

Video microscopy and tracking of granulocytes

Glass coverslips (0.3 mm) were coated with a solution of 1 % human serum albumin (HSA). Purified neutrophils or eosinophils, suspended in RPMI-Hepes with 0.5 % HSA, were attached to the coverslip for 10-15 min at room temperature.

The coverslip was then inverted in a droplet of medium containing the desired ligands or antibodies and sealed with a mixture of beeswax, paraffin and Vaseline (1:1:1, w/w/w). Cell migration at 37°C was monitored by time-lapse microscopy and analyzed by custom-made macro (A.L.I.) in image analysis software (Optimas 6.1, Media Cybernetics, Silverspring, USA). Cells were followed for 8 minutes (figure 1 and 3) or 20 minutes (figure 7). As expected with the use of primary cells, a heterogeneous population of different age and priming, there is a variation in the rate and the amount of movement observed in the figures. However, the results shown are representative for a larger field of cells (usually 50-100 cells) and, the experiments were repeated at least three times for every assay condition. For figures 1, 3 and 7 video material is available on CD-ROM.

RhoA activation assay

RhoA activity assay was performed as described (18). Neutrophils or eosinophils were stimulated in suspension, then lysed in (50 mM Tris pH 7.5, 150 mM NaCl, 10 % glycerol, 1 % NP-40, 0.1 % Triton X-100, 5 mM $MgCl_2$, 0.1 mM PMSF, 10 µg/ml leupeptine, 10 µg/ml aprotinin). Cleared lysates were incubated with bacterially produced GST-RBD[Rhotekin] (16) bound to glutathion-agarose beads for 45 min at 4°C. The beads were washed 3 times with lysis buffer, then bound proteins were eluted in SDS-sample buffer and analyzed by Western blotting using anti-RhoA MoAb from Santa Cruz.

Migration assay in the Boyden chamber

Eosinophil migration was measured in the modified Boyden chamber assay as described (20). Cellulose nitrate filters (pore width 8 µm, thickness 150 µm; Sartorius) were soaked in 0.5 % HSA. The assays were performed in Hepes-buffered RPMI supplemented with 0.5 % HSA for 2.5 hours at 37°C in a CO_2 incubator. Filters were fixed, stained with haematoxilin (Weigert's method) and embedded in malinol. Analysis of the filters was done by an image analysis system (Quantimet 570 C) and an automated microscope to score the number of cells at 15 intervals of 10 µm in the Z-direction of the filters. The results are expressed as the chemotactic index, indicating the mean migrated distance, excluding cells with migration 0.

Transendothelial migration

Human umbilical vein endothelial cells (HUVEC) were plated in Transwell chambers (Costar; pore width 8 μm; diameter 6 mm), coated with fibronectin. When confluence was reached, purified eosinophils (10^5/well) were calcein-AM (Molecular Probes) loaded according to the manufacturer, washed, resuspended in RPMI-Hepes and placed in the upper wells. The lower wells were filled with RPMI-Hepes containing the chemoattractant. The chambers were placed for 1 hour at 37°C in a CO_2 incubator. At the end of the experiment, the migrated cells in the lower wells, the inserts as well as the cells from the upper wells were lysed in 1 % Triton-X100-containing buffer. Fluorescence intensities were measured with a FluorImager. Standard curves with fixed amounts of calcein-AM-loaded cells were prepared each time. Values are given as percentage migrated cells of total cells loaded.

Adhesion assay

Static adhesion of eosinophils was performed in 96-well Elisa plates (Nunc), coated for 1 hour at room temperature with 0.1 % HSA or VCAM-1 (5 μg/ml) in PBS. Cells were calcein-AM loaded, incubated with antibody or inhibitors and added to the wells already containing medium with or without serum. After 5 min, the plates were placed at 37°C for 5 min, then they were washed once with PBS containing 0.1 % BSA and lysed in TX-100 containing buffer. Remaining cells were quantified using a FluorImager.

Tat-fusion constructs

PCR products comprising the coding region of constitutively active V14RhoA, dominant negative N19RhoA or dominant negative N17Rac-1 (6) were cloned into the pTAT-HA vector (31), sequenced and transformed into the BL21(DE3) strain. Bacteria were obtained from an overnight culture, resuspended and sonicated in Z-buffer (8 M Urea, 100 mM NaCl and 20 mM Hepes, pH 8.0), and supernatants containing 10 mM imidazole were loaded onto a Ni-NTA column (Qiagen). Tat-fusion proteins were eluted with 1 M imidazole in Z buffer, diluted 5 times with 20 mM Hepes buffer pH8.0 and applied to a Source 30Q column (Amersham). After washing, bound proteins were eluted with 1 M NaCl, desalted on a PD-10 column into PBS/1 mM $CaCl_2$, flash frozen in 10 % glycerol and stored at -80°C.

When using tat-V14RhoA constructs in eosinophil migration assays, such as shown in figure 7, we found no effect of the tat-constructs in a minority of the experiments, which we cannot explain. The effects of tat-V14 Rho decreased after incubations of more than 45 minutes.

Results

Imaging of cellular detachment

To study the migration of migratory leukocytes, we performed video microscopy on human eosinophils. Tracking of cells attached to albumin-coated glass-slides allows the study of speed, direction and morphology of each cell. Figure 1 (video available on CD-ROM) shows the tracks of eosinophils and neutrophils migrating in the absence or the presence of stimulus. Eosinophils in the control situation or when stimulated with the chemoattractant platelet activating factor (PAF), were highly motile. Under these circumstances eosinophils moved the cell body, but were not able to detach the uropod and therefore hardly migrated (figure 1G). While the addition of chemoattractants had no effect on eosinophil detachment, serum-stimulation of the cells resulted to rear release. More cells were able to detach their uropod, resulting in a higher mean migration speed, as can be concluded from the longer tracks. Neutrophils however, were optimally stimulated with the chemoattractant fMLP and serum did not further enhance neutrophil migration (figure 1D-F).

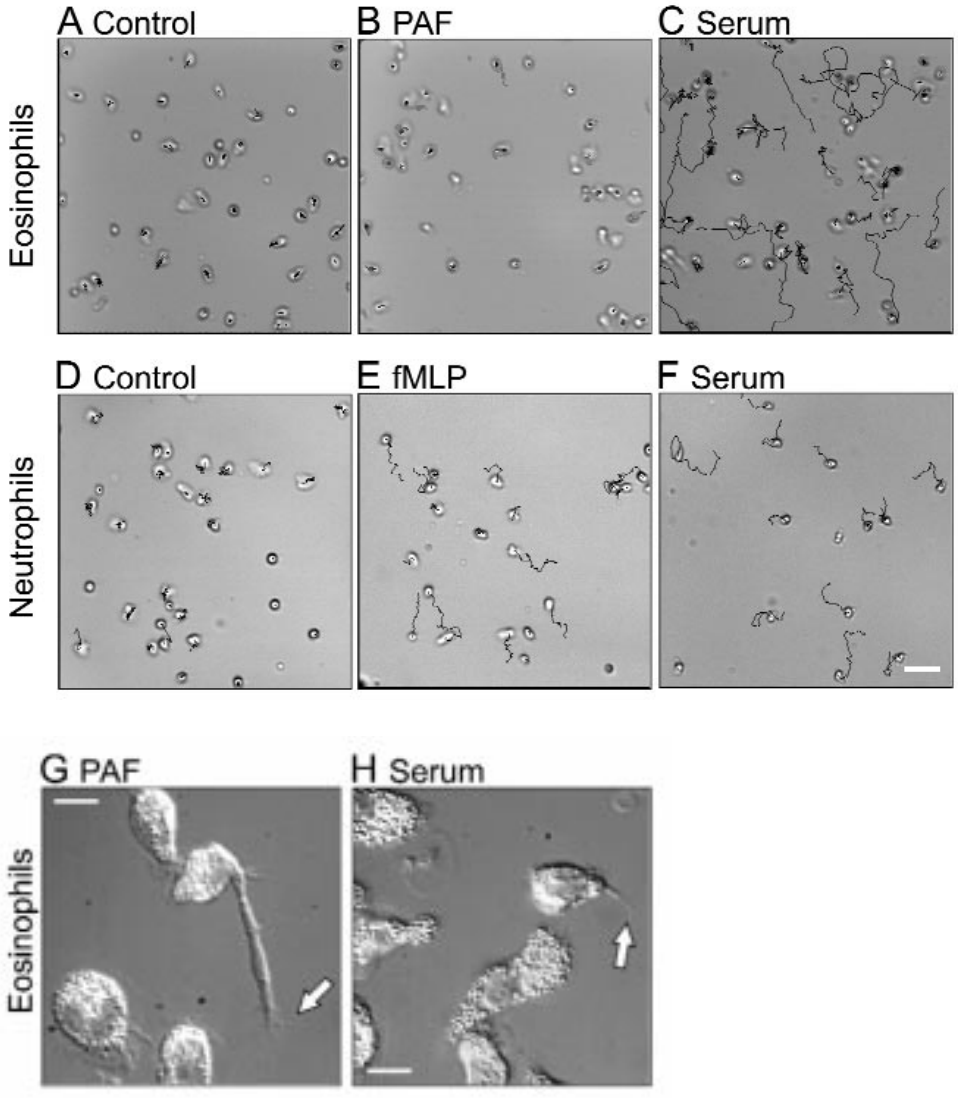

Figure 1. Cell tracks of randomly moving eosinophils (A,B,C,G,H) and neutrophils (D,E,F). The cells were attached to albumin-coated glass coverslips in RPMI-Hepes containing 0.5 % HSA. Then they were transferred to the same buffer complemented as indicated without stimulus (A,D) with PAF (10^{-7} M; B,G), fMLP (10^{-8} M; E) or 10 % human pooled serum (C,F,H). The cells were warmed to 37°C and then monitored for 20 min (A-C), 8 min (D-F) or 30 min (G-H). Bar: 30 μm (A-F) or 10 μm (G-H). Arrows indicate a non-detached uropod (G) and a detached uropod (H). Video material is available on CD-ROM.

Involvement of RhoA in detachment of the uropods

Because serum is able to stimulate detachment, we hypothesize that serum may activate the RhoA-ROCK pathway. We used a recently developed RhoA activation assay (18) to test this hypothesis. In this assay a GST-Rhotekin pull-down assay is performed on lysates of activated leukocytes, then the amount of active RhoA present in the cells is determined by Western blotting. Figure 2 shows that serum is an excellent activator of RhoA, both in neutrophils and eosinophils. Compared to serum, the activity of RhoA after stimulation by the chemoattractant fMLP (formyl-Met-Leu-Phe) and PAF was rather weak. However, these latter experiments show that a relatively small and transient activation of RhoA per se is not sufficient for rear release (see below). These results indicate that increased detachment observed after serum stimulation correlates with strong RhoA activation. This RhoA activation was not dependent on a cycle of attachment and detachment because the experiments were performed with cells in suspension.

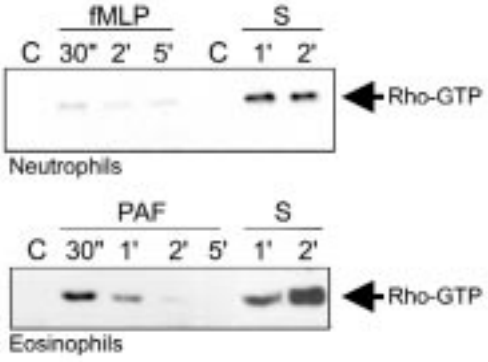

Figure 2. RhoA activation assay in neutrophils and eosinophils. The cells were stimulated with fMLP (10^{-7} M), PAF (10^{-7} M) or serum (S) for the time-periods indicated, lysed and subjected to GST-Rhotekin pulldown as described in Materials and Methods. Western blots of active RhoA (arrow) are shown.

To determine whether RhoA is involved in stimulation of detachment, we used a specific inhibitor of RhoA; C3-exoenzyme. We studied the migration of neutrophils, because these cells have no rear release problems when monitored on a glass coverslip in the presence of chemoattractant. Figure 3 (video available on CD-ROM) shows tracks of neutrophils that were control-treated (3A) or preincubated with C3-exoenzyme (3B). Those cells showed a morphology similar to eosinophils: the uropod was attached, but the cell body was still highly motile, as can be seen in the videos. These results indicate that RhoA is involved in cellular detachment of the uropod.

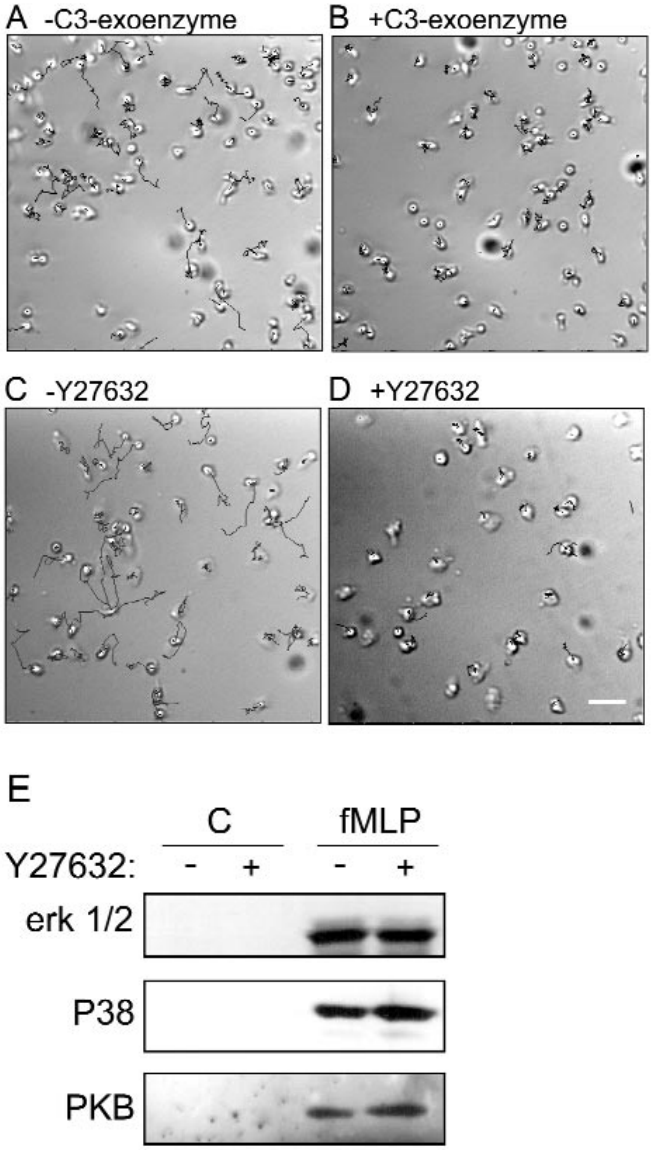

Figure 3. Migration of neutrophils on albumin-coated glass coverslips. The cells were untreated (A,C) or pre-treated with C3-exoenzyme (10 µg/ml; 4 h, B) or Y27632 (10^{-5} M; 30 min, D) then attached to the coverslips. The coverslips were inverted in assay medium containing fMLP (10^{-8} M) and embedded. Random movement of the cells was recorded at 20 sec intervals. Tracks recorded during an 8-min period are shown. Bar: 30 µm. (E) fMLP-induced activity of Erk1/2, p38 and PKB in neutrophils in the absence or presence of Y27632 (10^{-5} M; 30 min) using phosphospecific antibodies. Video material for 3 A -D is available on CD-ROM.

ROCKs are involved in rear release regulation

ROCKI and ROCKII are downstream targets of RhoA, involved in MLC phosphorylation and thereby in force generation and contraction of cells. To investigate whether or not ROCKs are involved in detachment, we used the ROCK-inhibitor Y27632 (12) in several migration assays. Figure 3 (video available on CD-ROM) shows tracks of neutrophils that were stimulated with fMLP either in the absence (3B) or in the presence (3C) of Y27632. After ROCK-inhibition by Y27632, the cells showed a decreased net cell translocation and thus a decreased mean migration speed, which can be concluded from the track length. However, these cells were still highly motile, as can be seen in the videos provided with figure 3B. The uropod of the cells was again attached to the substratum, while the cell body moved around it. It is obvious that the cells treated with Y27632 were not able to detach. The specificity of the ROCK inhibitor Y27632 has been tested *in vitro* by Uehata *et al.* (12). Several important kinases were tested: PKC, PKA, MLCK, PAK. Furthermore, Y27632 did not have any effect on RhoA-dependent transcription and Rac-dependent membrane ruffling. In neutrophils, we tested fMLP-stimulated activation of PKB, Erk1/2 and p38 (figure 3E). We found no inhibitory effect of Y27632 on these key signaling routes.

The importance of ROCKs during transendothelial migration

Migration of eosinophils across a monolayer of endothelial cells is complicated by the fact that the endothelial cells not only form the substrate over which the eosinophils must move and the barrier they must cross, but the endothelial cells also provide signals to the eosinophils, for instance by secreted chemokines. In the past, attention has focussed on the role of RhoA and Rho kinase in the contraction and permeability of endothelial cell layers (21). We investigated the involvement of ROCK in eosinophils when transmigrating an endothelial cell layer in the absence of serum.

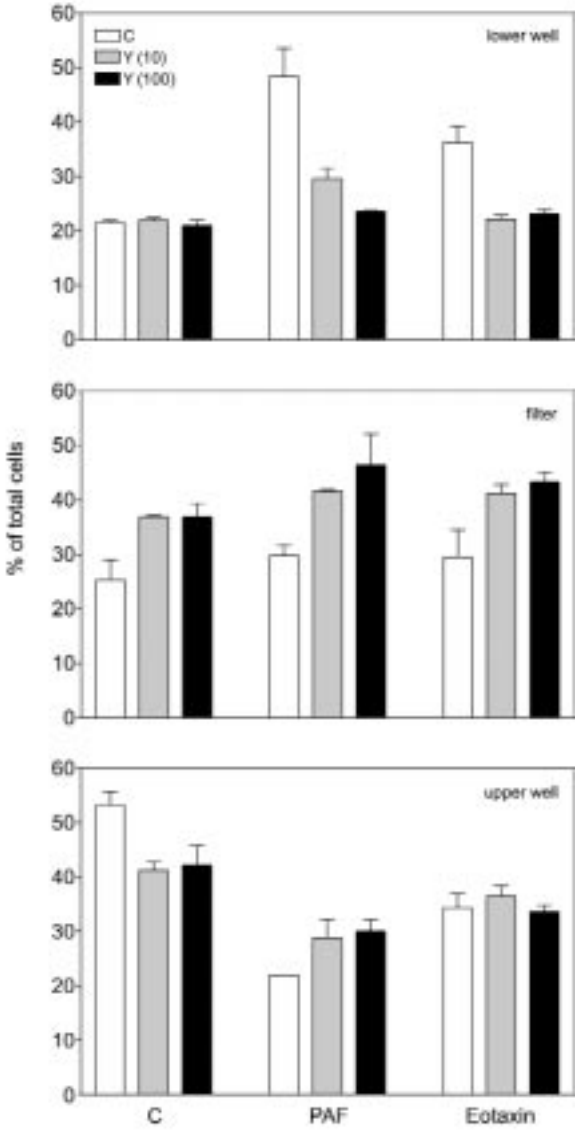

Figure 4. Transendothelial migration of eosinophils in the presence of Y27632. Eosinophils migrating over confluent monolayers of HUVEC (see Materials and Methods for details) were stimulated with PAF (10^{-7} M) or eotaxin (10^{-7} M). Pre-treatment of the eosinophils with Y27632 (in μM) was for 30 min as indicated. After 1 hour of migration, the cells were taken out of the lower wells, filters and upper wells and quantified separately with the use of calcein. The results are expressed as % of total cells (mean ± SEM) of a representative experiment performed in duplicate.

Figure 4 shows a transwell assay performed on eosinophils pretreated with Y27632. The transmigration (top) induced by PAF or eotaxin was completely blocked by Y27632 at 100 μM, whereas the control values were hardly affected. Analysis of the filters (middle) shows that after inhibition of ROCK by Y27632, a considerable number of cells remained present in the filter, independent of the stimulus. This could indicate an increase in adhesion of eosinophils to the endothelial cells or a decreased detachment. To rule out the first possibility, we performed adhesion assays of eosinophils to endothelial

monolayers. We never observed an increased adhesion after Y27632 treatment (results not shown). Therefore, we conclude that the high number of Y27632-treated cells present in the filter is likely the result of a defect in detachment.

ROCK involvement in chemotaxis and chemokinesis

In the Boyden chamber assay, neutrophils or eosinophils migrate through a 3-dimensional filter of nitrocellulose. Migration of cells in filters occurs under less stringent circumstances. The main difference between these experimental set-ups is the presence of a 2- versus a 3-dimensional substrate. Eosinophils migrating through filters showed efficient migration after stimulation with PAF or IL-5, which was not the case when migrating over albumin-coated glass coverslips. Preincubation of eosinophils with the ROCK-inhibitor Y27632 resulted in a dose-dependent inhibition of chemotaxis towards PAF (figure 5A). When serum was present in both the upper and the lower wells of the chemotaxis chamber, chemokinesis was observed. The ROCK-inhibitor was effective in inhibition of chemokinesis stimulated by IL-5 or serum as well as chemotaxis stimulated by PAF. The observed IC_{50} for the different stimuli was 3-10 μM, which is in range with the published value in smooth muscle cells (12). Similarly, chemotaxis of neutrophils induced by fMLP is inhibited by Y27632 (figure 5B). Thus, activation of ROCK is necessary for the migration of granulocytes.

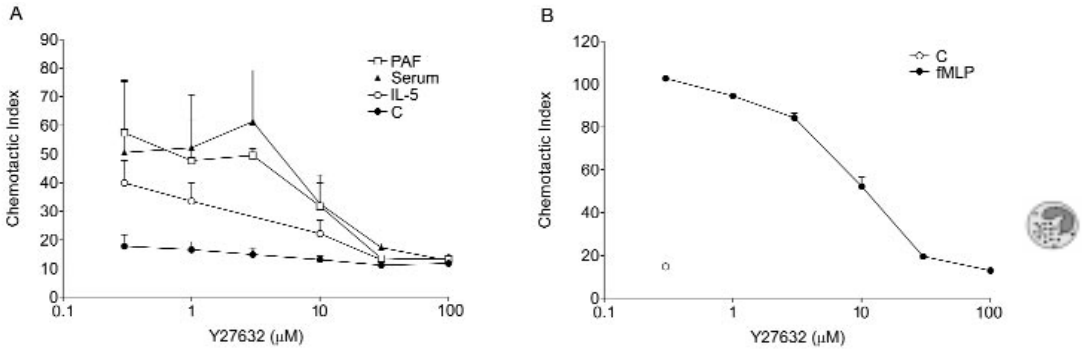

Figure 5. Granulocyte chemotaxis in the presence of ROCK-inhibitor Y27632. A) Eosinophils migrating in the Boyden chamber were stimulated with PAF (10^{-7} M) present in the lower wells, IL-5 (10^{-10} M) in the upper well or with serum (10 %) in both wells. B) Boyden chamber chemotaxis assay of neutrophils stimulated with fMLP (10^{-8} M). Y27632 pre-treatment was in all cases for 30 min. The results are expressed as chemotactic index (mean ± SEM of 2-6 experiments performed in duplicate).

ROCKs influence attachment

Activation of ROCK leads to phosphorylation of myosin light chain (MLC), an event that is correlated with force generation and contraction of cells. It can be envisioned how a higher contractility of a cell leads to better detachment from its substrate. Alternatively, RhoA activation is implicated in adhesion of neutrophils (22). Therefore, RhoA's downstream kinase ROCK could elicit a signal towards adhesion molecules, thereby regulating the affinity for ligands present on the substrate. To address this hypothesis, we measured the effect of ROCK-inhibitor on the activity of two important adhesion

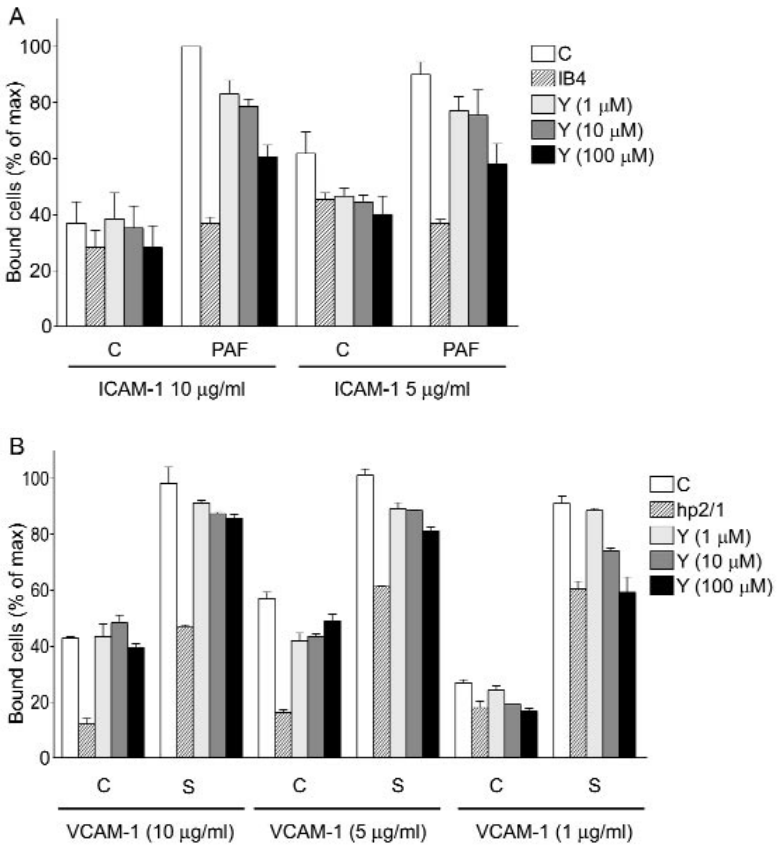

Figure 6. Adhesion of eosinophils to ICAM-1 or VCAM. Eosinophils were calcein-loaded, incubated for 30 min with Y27632 at the indicated concentrations (between brackets, in μM), or with neutralizing antibodies against α_4- (HP2/1) or β_2- (IB4) integrins. Then the cells were placed in 96-well plates coated with ICAM-1 (5-10 μg/ml) or VCAM (0.1-5 μg/ml) containing control buffer or serum-containing buffer and incubated for 5 min at 37°C. The cells were washed and lysed and fluorescence intensity was measured. The results are expressed as % of maximal binding (mean ± SEM of 2-5 experiments performed in duplicate).

molecules present on eosinophils (23): $\alpha_4\beta_1$-integrin (binding to VCAM-1) and β_2-integrins (binding to ICAM-1). Figure 6 shows that Y27632 had an inhibitory effect on the activity of α_4- as well as β_2-containing integrins. The effects of Y27632 are rather small, as observed by the partial inhibition of cells binding to ICAM and the fact that inhibition of binding to VCAM-1 is only observed at limiting ligand concentration. This means that ROCK activation does influence attachment as well as detachment.

Is RhoA-activity sufficient to stimulate detachment?

To investigate whether RhoA activation is the primary mechanism of detachment, we transduced eosinophils with activated RhoA (V14RhoA). As classic transfection procedures are unsuccessful on these primary cells, we made use of the cell permeability of tat-fusion proteins (31). When tat-V14RhoA was applied to the cells in the presence of the chemoattractant PAF, tat-V14RhoA was able to replace serum and stimulate detachment (figure 7B, video is available on CD-ROM). The experiments were performed in the continuous presence of tat-fusion protein to avoid diffusion out of the cell. The stimulation of detachment increased in time, probably due to a higher concentration of tat-V14RhoA in the cell, but decreased again after about 45 minutes (figure 7E). As a control, a fusion-protein containing syntenin was used, which had no effect on detachment (figure 7C, D). To substantiate the hypothesis we did the reverse experiment. Tat-N19RhoA was applied to the cells in the presence of serum. This resulted in an inhibition of detachment (figure 7F). In the control situation, eosinophils incubated with tat-syntenin in the presence of serum showed normal detachment. From these results we conclude that activation of the Rho-ROCK pathway alone is necessary but not sufficient to stimulate detachment. Other, as yet unidentified signals, elicited by the stimulus PAF may contribute to the stimulation of detachment.

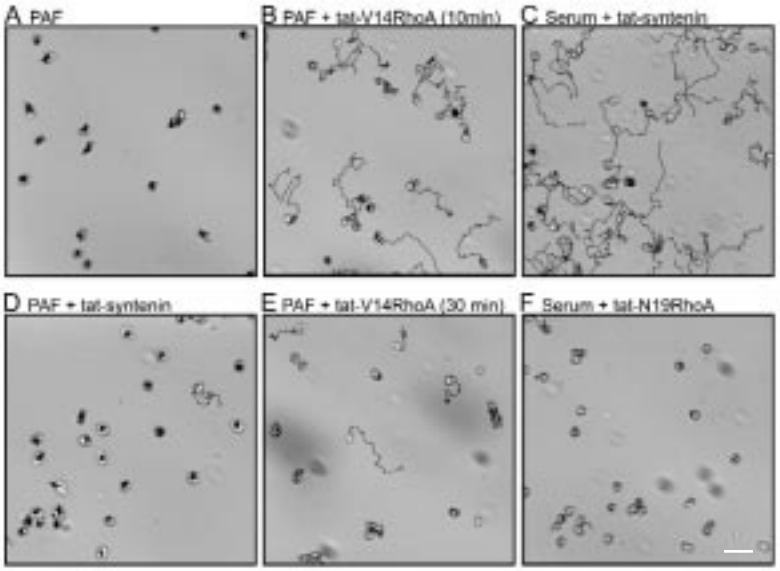

Figure 7. Video microscopy of migrating eosinophils in the presence of tat-fusion proteins. Cells were attached to albumin-coated glass coverslips, transferred to medium containing 1 µM of the indicated tat-fusion proteins and/or PAF (10^{-7} M) or human pooled serum (10 %) and embedded at t=0. Images were recorded with 20 sec intervals during 20 min at 37°C. Cell tracks of a representative experiment are shown. Bar: 30 µm. Video material is available on CD-ROM.

Discussion

We have shown that the Rho-ROCK pathway is essential in the regulation of the process of uropod detachment or rear release, a field in migration research that has not received much attention until now. One of the possible explanations is the difficulty in monitoring rear release. The only method to appreciate cellular rear release is through time-lapse (video) microscopy of cells migrating on a two-dimensional substratum. Highly motile cells that are detachment-defective can easily be recognized by direct viewing of the cells. Such a cell has a uropod that is firmly attached to the substratum. Under high magnification viewing it becomes clear that the cell body is moving actively around the cell.

We found that high motility and stimulation of rear release can be separated. In eosinophils, a situation of high motility and no detachment was found in the absence of serum. This situation can be mimicked in neutrophils by inhibition of RhoA or ROCK. This leads us to the conclusion that motility and rear release have a different molecular basis.

The mechanisms underlying rear release are largely unknown. Based on our findings, ROCK-mediated cytoskeletal contraction turns out to be very important. The measurement of force generation in cells is expected to correlate with rear release stimulation.

A second mechanism that may explain rear release involves integrin modulation. In this model, ligand-stimulated signaling is responsible for changes in affinity or avidity of integrins (1;24). It is clear that the strength of adhesions determines the migration speed of cells (25): at decreased adhesive strength migration speed is enhanced. This occurs in a situation without addition of external stimuli, which implies no changes in cytoskeletal contraction. Whether or not RhoA signaling plays a role in integrin modulation is still in debate. RhoA was found to be important for β_2 integrin-dependent lymphocyte aggregation (26) and β_1- and β_2-integrin dependent leukocyte adhesion (22) but not for inside-out signaling towards platelet $\alpha_{IIb}\beta_3$ (27).

From previous work we know that interference with the ligand binding capacity of α_4-containing integrins can improve rolling of eosinophils (28) and rear release and migration of the cells (unpublished results). We found a contribution of Rho's immediate downstream target ROCK in signaling towards $\alpha_4\beta_1$- or β_2-integrin-dependent attachment. This means that at the same time ROCK stimulated detachment and attachment. Detachment of the rear of the cell resulted in to migration, not necessarily in detachment of the entire cell. This can be observed in figure 6: serum stimulation, which very efficiently stimulated detachment of the uropod, resulted in increased adhesion, i.e. attachment. Adhesion per se does not correlate with migration properties of cells. Whether or not integrins are turned off during rear release has to be further explored.

In migrating cells, Rac and Cdc42 are responsible for lamellipodial protrusions, whereas RhoA regulates uropod detachment by stimulation of actomyosin filament contraction. Coordination of the activity of these three GTPases is necessary for optimal migration. In adherent cells that contain focal adhesions, Rac was able to counteract Rho-stimulated formation of stress fibers and focal adhesions directly or through activation of PAK (18;29). In leukocytes however, focal adhesions are not present. Here, the formation of contractile forces appeared to be enough to ensure adhesive release, and Rac or Cdc42 may have been activated in concert with RhoA (30). Clearly, the adhesive state of cells influences RhoA activation by soluble factors (17).

In conclusion, our results point to an essential role for the Rho-ROCK pathway in migration of leukocytes, namely in the relatively unknown process of rear release.

Acknowledgements

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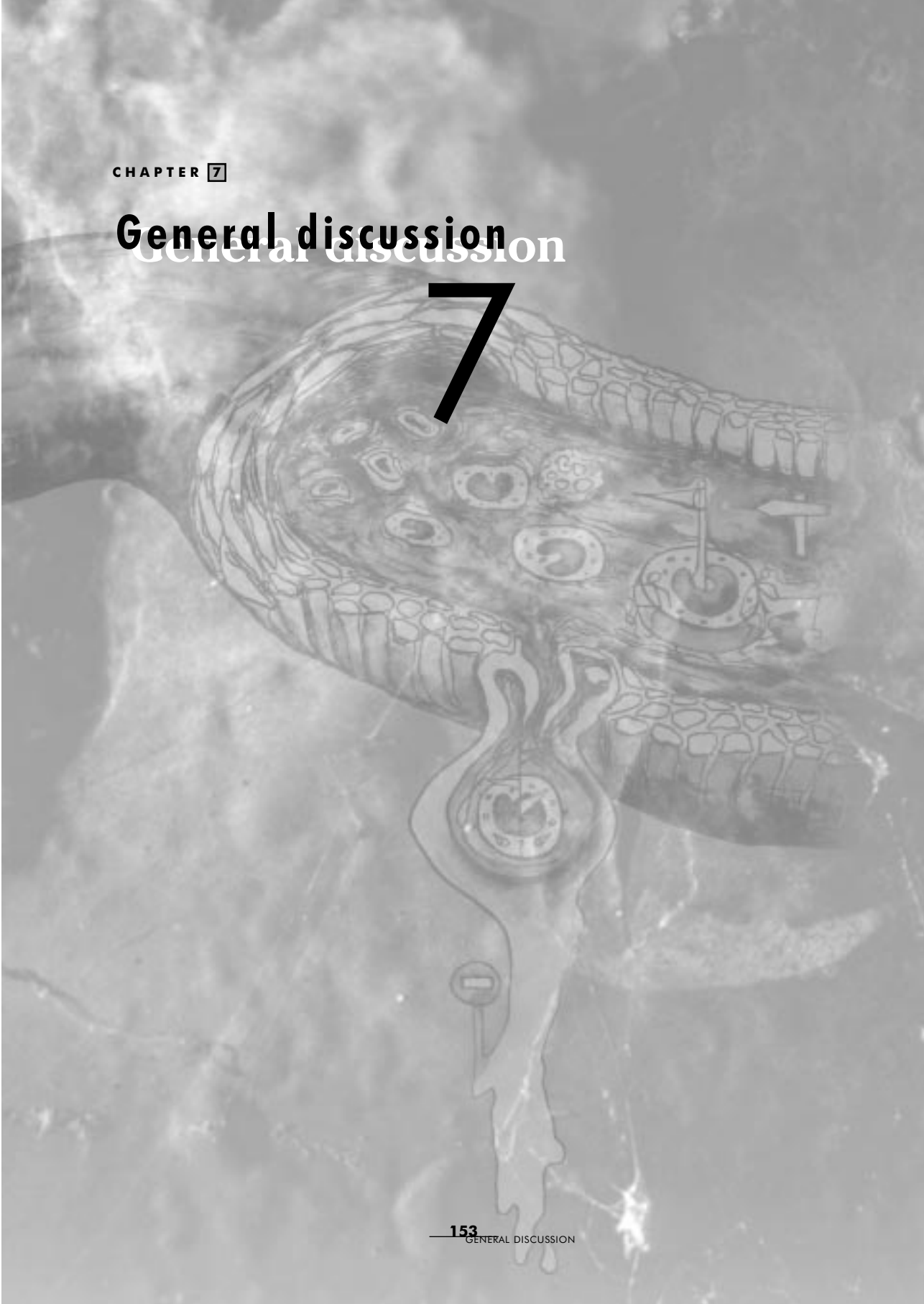

CHAPTER 7

General discussion

General discussion

7

Two ways for leukocyte recruitment to lung tissue

Biopsies of patients suffering from asthma or deceased from a status asthmaticus have shown that infiltration of eosinophils occurs in the bronchial compartment (1). This part of the lung is supplied by blood from the bronchial circulation, as part of the systemic circulation. Only 1 % (~0.05 liters/min) of the cardiac outflow is supplying this site. In the pulmonary circulation, on the other hand, approximately 5 liters of blood are passing per minute. In the dense alveolar capillary network of the pulmonary circulation oxygen uptake takes place. Moreover, emigration of leukocytes occurs mainly in the capillaries of the pulmonary circulation. In contrast, in the systemic circulation leukocyte emigration is thought to occur mainly in the postcapillary venules and not in the capillaries (2).

The mechanisms of influx of inflammatory cells to the alveolar compartement are different from those to the bronchial compartement. It is known that induction of an asthma phenotype is characterized by influx of inflammatory cells (especially T cells and eosinophils) to the bronchial tissue (reviewed in (3)). The mechanism is largely dependent on adhesion molecules, since animal models have shown that inhibition of multiple integrins (for example CD11/CD18) and Ig-superfamily members (for example ICAM-1) abrogates the influx of cells (4-7). This is in contrast to what is found in animal models in which the mechanisms of leukocyte influx upon infection of the lower airways have been investigated. Emigration of neutrophils into the alveolar space in response to certain bacterial stimuli (e.g. S. pneumoniae) is independent of adhesion molecules such as CD11/CD18 (8-10). It is thought that emigration of neutrophils from the cappillaries into the lumen of the alveoli is facilitated by mechanical changes in leukocyte shape. Typically capillaries are very small in diameter (4-9 μm). Neutrophils (6-8 μm diameter) likely pass these capillaries at very low velocity and are already in close contact with the endothelial layer, without any need for selectin function. Doerschuk *et al.* have suggested that a stimulus-induced decrease in leukocyte deformability can increase the time a leukocyte remains in close contact with the endothelium in the capillaries (reviewed in (2)). This might be an alternative for rolling events. However, the exact mechanism of emigration of neutrophils to the alveolar space remains to be determined. The possibility that unidentified adhesion molecules at capillary endothelial cells mediate the migration is not excluded yet, and might still be an alternative mechanism for this phenomenon.

Allergic asthma is a disease of the tracheobronchial tree, and the blood flow to this site is part of the systemic circulation in which leukocyte emigration mainly occurs in postcapillary venules. Therefore, we used a model system under flow conditions in

which HUVEC cells were activated with TNFα-stimulating inflamed postcapillary venules. TNFα was chosen because it can be produced by many cells that play an important role in allergic diseases (11-15). Recently, Broide *et al.* (16) showed that rolling and firm adhesion of leukocytes to the endothelium was inhibited in TNFα-receptor p55/75 knock-out mice compared to wild-type mice after allergen challenge. It was suggested that this effect was due to the inhibiton of adhesion molecules on the endothelium. Besides its effect on endothelial cells, TNFα can also activate eosinophils from allergic asthmatic patients but not those from healthy controls (17). This further indicates that TNFα may be an important cytokine in allergic diseases.
We focused on the multistep paradigm of leukocyte extravasation, because it is thought to be valid for eosinophil extravastion to the bronchial compartement of patients with allergic asthma. The *in vitro* flow chamber model was used to investigate eosinophil tethering, rolling and adhesion processes.

Eosinophil tethering and rolling by selectins and α_4 integrins

The mechanism of recruitment of leukocytes to inflammatory sites is tightly regulated and ensures the temporal and spatial distribution of cells through the body. A first prerequisite for extravasation is the presence of selectins and their counter structures that mediate rolling (18). In chapter 2 it was shown that eosinophils can use E-selectin on the endothelium for rolling interactions. Although many studies suggest that P-selectin is the preferred selectin used by eosinophils (reviewed in 19), we clearly show that eosinophil roll stably on E-selectin expressed by activated endothelial cells. Furthermore, the α_4 integrins on freshly isolated eosinophils mediate initial attachment (chapter 2) to the endothelium, without the need for activation of the eosinophils. Indeed, *in vitro* (20;21 and *in vivo* 22;23) studies have shown that α_4 integrins on several leukocyte subsets can bind to their ligand VCAM-1 without prior activation of the cells. While all these studies (including ours) used isolated cells, it can not be excluded that leukocyte isolation might lead to activation of these cells and thereby induce the activation of α_4 integrins. A reasonable assumption is that leukocytes in whole blood are less manipulated than isolated leukocyte subsets from whole blood. Therefore, studies with whole blood might shed light on the activation status of $\alpha_4\beta_1$ integrins. Such studies demonstrated that lymphocytes express a pool of α_4 integrins that exist in an intermediate affinity state, and this pool has been shown to bind to high concentrations of VCAM-1 (24). Also, Patel (25) showed by *in vitro* flow chamber experiments with whole blood that eosinophils preferentially accumulated in an α_4 integrin dependent fashion on IL-4-activated HUVEC. Furthermore, a study on E-,

P- and L-selectin triple knock-out mice revealed that the residual rolling interactions in the venules of the cremaster muscle were dependent on α_4 integrins. In this experimental set-up, autologous peripheral leukocytes in mice were observed without manipulation of the cells (26). Thus, it is tempting to speculate that α_4 integrins display an intermediate activation state and thus are functionally active *in vivo*. At least two factors might be needed *in vivo* to establish that the basal $\alpha_4\beta_1$ integrin activity leads to leukocyte migration, 1) the presence and the density of the ligand and 2) the presence of chemoattractants for further activation of integrin-mediated migration.
Ad1, VCAM-1 expression on endothelial cells needs cyokine-induced upregulation so in a normal non-inflammatory situation, VCAM-1 is not present on endothelial cells. An exception is the bone marrow, because venules at this site constitutively express VCAM-1 (22). In this respect, tethering and rolling interactions are probably not sufficient to ensure migration of $\alpha_4\beta_1$ integrin-bearing cells. Ad 2) Mazo and von Andrian (27) suggested that the chemokine SDF1α might play an important role in further activation of the integrins and induction of migration. This illustrates that chemokines are needed to increase the activity of $\alpha_4\beta_1$ integrins for their ligands and also to activate other integrins, for example the β_2 integrins. Indeed, activation of eosinophils can further increase the affinity of $\alpha_4\beta_1$ for its ligands and can also activate β_2 integrins (chapter 4 and 5). Recently, it was shown that chemokines activate α_4 integrins on lymphocytes in sub-seconds, leading to small clusters of α_4 integrins and thereby increasing the tethering efficiency to the surface (28). This illustrates that chemokines can already interfere with the first step of extravasation; tethering and rolling processes, in addition to their already established role in the second, third and fourth step; i.e. activation, firm adhesion and migration, respectively. Moreover, α_4 integrins that exist in an intermediate activation state on leukocytes *in vivo* might increase immuno-surveillance, because when ligand is present α_4 integrins increase tethering and rolling interactions. This is in contrast to other integrins like β_2 integrins, which have not been described to mediate initial tethering or rolling processes.

Eosinophil arrest: an interplay between serpentine receptors and adhesion molecules

A prerequisite for succesful migration is chemokine-induced arrest of eosinophils. The types of the chemoattractants that are present on the apical surface of the endothelial cells and the ones that create a soluble gradient from the site of inflammation towards the lumen of the blood vessel determine the type of adhesion of leukocytes to the endothelium. For eosinophils this is illustrated in chapters 4 and 5. IL-8 induces a transient arrest of eosinophils, in contrast to eotaxin and C5a that induce

a firm and longterm arrest. Although IL-8 is not potent enough to induce longterm arrest, it does increase the time of interaction of the eosinophil with the endothelium. In this way, the chance to respond to additional stimuli increases. When eotaxin or C5a was applied after the IL-8-induced transient arrest, the rolling cells arrested and longterm arrest was induced (chapter 4 and data not shown). For monocytes a similar observation has been described (29). In this study it was shown that immobilized Gro-α on endothelial cells via heparan-sulfate proteoglycans induced the transition from rolling to firm adhesion of monocytes but did not induce transmigration. Secreted MCP-1, in contrast, did not induce a transition from rolling to firm adhesion but did induce migration. This suggests that chemokines can act in different ways and on different levels in the multistep model. The combination of chemoattractants present at the site of inflammation and the set of adhesion molecules expressed might determine the specificity in the extravasation of the different leukocyte subpopulations.

Control of integrin function

Chemoattractants induce signals into the cell that lead to activation of integrins (inside-out signaling). Two modes of integrin activation have been described, 1) a change in conformation of the heterodimer, leading to a high-affinity state of the integrin and 2) induction of integrin clustering in the plasma membrane, leading to increased avidity. Integrins in high-affinity and/or avidity state bind to their substrates, and this leads to the transmission of signals back into the cell (outside-in signaling). The mechanisms how activators induce integrin affinity and/or avidity changes of integrins, and the consequences this has for the binding of the cell, are getting more clear. Recently, it was shown that chemokines such as SDF-1α induce rapid integrin affinity changes and lateral mobility changes of integrins on lymphocytes (30). The affinity change was transient and was thought to be important for fast binding to ligand. The avidity change was more sustained, and it was shown that at low ligand concentration an increase in avidity is needed to maintain stable adhesion. The authors further postulated that the binding of clustered integrins to their ligands is needed to maintain a high-affinity state of the integrins. For eosinophils it has been shown that chemoattractants such as RANTES and C5a can induce a prolonged integrin-affinity state (31), as determined by an antibody recognizing the high affinity epitope on Mac-1. This might lead to the maintenance of a stable integrin cluster and, as a consequence, firm adhesion of the leukocyte to the substrate. For a cell to migrate on a surface it subsequently has to protrude at the front but also detach at the back of the cell. Integrin binding to ligand can induce outside-in signals that may contribute to this adhesion and de-adhesion process.

In chapter 4 we show that when rolling eosinophils encounter IL-8 the α_4- and β_2-integrins become activated and bind to endothelial cells. This binding is a prerequisite for an IL-8-induced increase in $[Ca^{2+}]_i$, because in suspension IL-8 does not induce an increase in $[Ca^{2+}]_i$. Thus, IL-8 induces an activation of integrins (inside-out signaling). Subsequently, ligand engagement of integrins activates a tyrosine-kinase-dependent signal that induces an increase in $[Ca^{2+}]_i$ (outside-in signaling) (chapter 5). Besides the observed increase in $[Ca^{2+}]_i$, integrin-induced outside-in signaling might also lead to other effector functions. Many studies suggest that outside-in signals can lead to a shift in the type of integrin used by the cell for movement processes. It has been documented that activation of $\alpha_4\beta_1$ integrins by ligand binding (32) or by the use of an activating antibody (33) induces signals leading to activation of β_2 integrins. Also, it has been shown that activation of β_2 integrins leads to a decrease in $\alpha_4\beta_1$ integrin activation (34). The same mechanism has been shown for mouse T cells and monocytes: ligand binding of $\alpha_v\beta_3$ leads to reduced $\alpha_4\beta_1$ integrin functions and LFA-1 integrin function, respectively (35;36). For eosinophils differences in kinetics of integrin binding to ligands have been shown for different chemoattractants (31). Also, evidence exists that β_2 integrin activation leads to modulation of α_4 integrins. Blom *et al.* (37) showed that IL-5 activates β_2 integrins and that this resultes in a decrease in the binding of 8A2-treated eosinophils to fibronectin. This IL-5-induced effect required an intact cytoskeleton, since cytochalasin B in combination with IL-5 and 8A2 increased the binding to fibronectin, which was totally α_4 integrin-dependent. From this study it is tempting to speculate that stimulus-induced activation of β_2 integrins may lead to de-activation of $\alpha_4\beta_1$ integrins via a cytoskeleton-dependent mechanism. Furthermore, it has been shown that eotaxin-2 and other chemokines that bind CCR3 lead to detachment of eosinophils from endothelial cells (38). Whether or not this is via activation and ligand binding of one type of integrin that shuts down $\alpha_4\beta_1$ integrins is not known. It would be interesting to investigate whether chemoattractant-induced activation of integrins on eosinophils leads to outside-in signaling and subsequent effects on integrin-mediated function. Chapter 4 and 5 show that chemoattractants induce the arrest of eosinophils on activated endothelium via α_4 and β_2 integrins. Eotaxin and C5a induce spreading of eosinophils on the endothelial cells, and when cells were recorded for a longer time, we observed migrating cells on the surface of endothelial cells (data not shown). For this process, eosinophils need to control the ligand binding of their integrins. Outside-in signaling of integrins leading to a decrease in activity of another type of integrin is an attractive model to regulate adhesion and migration processes, and we do not exclude cross-talk to have occurred in our experiments.

Integrin regulation by Phospholipase C

In chapter 5 we describe that inhibition of PLC in resting cells leads to a decrease in integrin activity. This was similar to the results of Smith *et al.* (39), who showed that adhesion of resting, non-activated neutrophils to TNFα-activated endothelial cells was inhibited by U73122 because of the inhibition of Mac-1 ($\alpha_M\beta_2$ integrins) avidity. Furthermore, we describe in chapter 5 that chemoattractants activate integrins via PLC-dependent and -independent intracellular pathways. Figure 1 shows a working model how the different chemoattractants might affect integrin activation in eosinophils. IL-8 can activate α_4 as well as β_2 integrins, as was shown in the flow chamber experiments with blocking antibodies (chapter 4). The IL-8-induced transient arrest via β_2 integrins did not occur when PLC was inhibited, whereas the α_4 integrin mediated arrest did occur when PLC was inhibited. Thus, the IL-8 induced β_2 integrin activation, but not the α_4 integrin activation, was dependent on PLC function. Furthermore, we showed that IL-8 cannot induce an increase in $[Ca^{2+}]_i$ in cells in suspension. However, IL-8 does induce an increase in $[Ca^{2+}]_i$ on adherent cells that was late in onset and tyrosine-kinase dependent. Thus, we speculate that upon IL-8-induced activation of α_4 integrins and subsequent binding to ligand, outside-in signals lead to PLC-γ activation. Indeed, it has been shown that crosslinking integrins can induce tyrosine phosphorylation and activation of PLC-γ (40). Activated PLC-γ might subsequently activate β_2 integrins. However, we cannot exclude that IL-8 directly activates β_2 integrins via a PLC-dependent mechanism since we showed that 1) eosinophils in the presence of blocking antibodies against α_4 integrins arrest upon IL-8 addition in a β_2 integrin-dependent way and 2) the IL-8-induced β_2 integrin-mediated arrest was dependent on PLC function.

Eotaxin and C5a can both induce increases in $[Ca^{2+}]_i$ in cells in suspension. Furthermore, the increase in $[Ca^{2+}]_i$ on adherent cells by these stimuli was not sensitive to tyrosine-kinase inhibitors and was rapidly induced. Thus, it is very likely that PLC-β is activated by these two chemoattractants. However, it does not exclude that PLC-γ is activated as well. It might be that the effect of eotaxin and C5a on PLC-β is dominant over PLC-γ in the induction of $[Ca^{2+}]_i$, whereas it is the PLC-γ isotype that is more important in integrin activation. Indeed, PLC-γ has been shown to be present in focal adhesions and it contains many domains by which it can interact with signaling molecules (see below). For eotaxin we found that the α_4- and β_2-integrin dependent arrest both were dependent on PLC function. For C5a this is different. The C5a-induced arrest was only for 30 % dependent on the combined function of α_4- and β_2-integrins, suggesting that other factors might also play a role (see below). Furthermore, inhibition of PLC resulted in a prevention of C5a-induced arrest of 50 % of the cells. Surprisingly, preliminary experiments suggest that inhibition of α_4 integrins, β_2 integrins and PLC

prevents C5a-induced arrest. This might lead to the hypotheses that 1) PLC-independent α_4- and β_2-integrin activation leads to the activation of an unknown factor that mediates the eosinophil arrest to the endothelium. 2) C5a activation in the presence of blocking antibodies against α_4 and β_2 integrins leads to PLC activation and subsequent activation of the unknown factor. This unknown factor might be a highly organized adhesisome with α_4 integrins and β_2 integrins clustered in such a way that the blocking antibodies cannot recognize it anymore. Whether PLC functions upstream or downstream of the eotaxin- and C5a-induced integrin activation or both remains to be established.

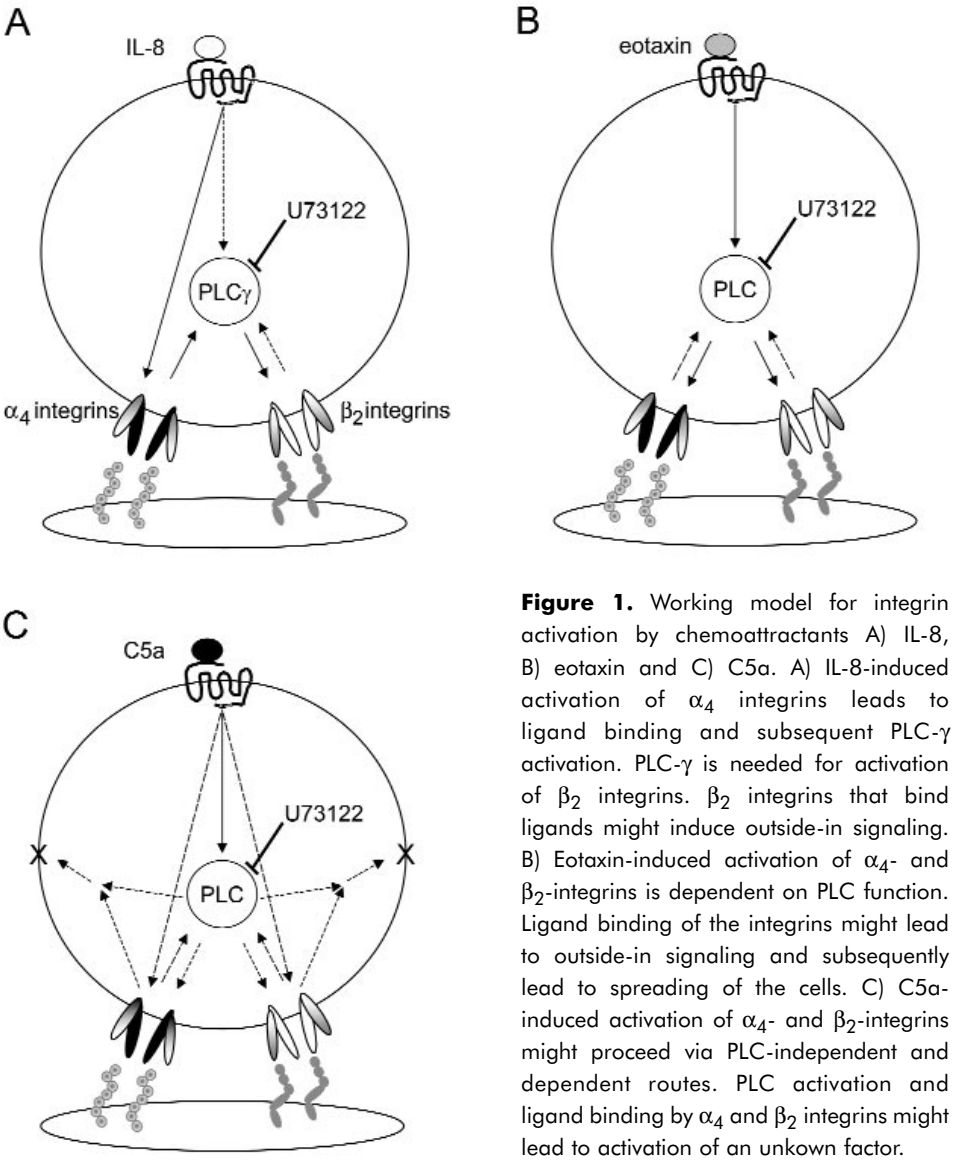

Figure 1. Working model for integrin activation by chemoattractants A) IL-8, B) eotaxin and C) C5a. A) IL-8-induced activation of α_4 integrins leads to ligand binding and subsequent PLC-γ activation. PLC-γ is needed for activation of β_2 integrins. β_2 integrins that bind ligands might induce outside-in signaling. B) Eotaxin-induced activation of α_4- and β_2-integrins is dependent on PLC function. Ligand binding of the integrins might lead to outside-in signaling and subsequently lead to spreading of the cells. C) C5a-induced activation of α_4- and β_2-integrins might proceed via PLC-independent and dependent routes. PLC activation and ligand binding by α_4 and β_2 integrins might lead to activation of an unkown factor.

Two major questions arise from chapter 5 which remain to be resolved. 1) How can inhibition of PLC function lead to a decreased integrin activity in resting eosinophils? 2) How does PLC regulate chemoattractant-induced integrin activity? Different hypotheses can be put forward that address these questions.

How can inhibition of PLC function lead to a decreased integrin activity in resting eosinophils?

Chapter 6 shows that inhibition of PLC leads to a decreased affinity of α_4 integrins on resting eosinophils. We hypothesize that PLC itself is needed to keep the integrin linked to the cytoskeleton, to preserve the configuration state of the integrin. It is known that the PLC-γ isoforms, but not the PLC-β and δ isoforms, contain PH, SH2 and SH3 domains (41). Thereby, PLC-γ might function as an adaptor molecule and link the enzyme to the plasma membrane and/or to proteins with phosphotyrosine motifs or proline-rich domains, respectively. Of the many proteins present in focal sites, paxillin has been shown to bind to PLC-γ1 directly (42). Paxillin was immunoprecipitated in a complex with PLC-γ1 in fibroblasts adhering to fibronectin. In non-adherent cells the interaction between paxillin and PLC-γ1 could not be detected. Furthermore, it has been shown that the intracellular tail of the α_4 integrin binds to the signaling adaptor protein paxillin (43;44). This interaction ensures that spreading of fibroblasts is limited because cells remained in a round shape. In contrast, cell spreading of α_4 containing fibroblasts on VCAM-1 is induced in the absence of paxillin (43). It might be that inhibition of PLC interferes with paxillin binding to the α_4 integrin, thereby mediating a decrease in adhesion of eosinophils to the activated endothelium.

How does PLC regulate chemoattractant-induced integrin activity?

Different hypotheses can be put forward for a working mechanism for PLC in the regulation of chemoattractant-induced integrin activation. Affinity/avidity changes of integrins can be induced by 1) PLC itself 2) the PLC product IP_3 and subsequent Ca^{2+} release from intracellular stores, 3) the PLC product DAG and subsequent PKC activation. A fourth hypothesis for the working mechanism of PLC is that U73122 inhibits the expression of new integrins on the cell surface, thereby inhibiting adhesion.

Ad1) It is known that a functionally active cytoskeleton is needed for integrin avidity changes in leukocytes. Disruption of the cytoskeleton by agents such as cytochalasin D are known to prevent integrins from clustering in the cell membrane. However, at low

concentrations cytochalasin D can stimulate integrin clustering (45-47). It has been reported that PLC-γ is present in focal adhesions (48) and that it can regulate the actin cytoskeleton by degradation of PIP_2, that is a binding partner of profilin and gelsolin (reviewed in 48). In fibroblasts it has been shown that activation by EGF induces PLC-γ activation, leading to dissociation of PIP_2 from profilin and possibly from gelsolin (49). The product IP_3 that is generated by PIP_2 hydrolysis leads to an increase in $[Ca^{2+}]_i$. Ca^{2+} activates profilin and gelsolin and these proteins start severing and capping actin filaments, which is required for spreading of cells. It might be that, when PLC is blocked, PIP_2 remains associated with gelsolin and profilin, thereby inhibiting actin filament turnover and in this way inhibiting integrin avidity changes in the cell membrane and spreading of the cells. It could be that comparable mechanisms for PLC function in focal adhesions apply to focal sites or adhesisomes in leukocytes. These structures are formed upon cellular activation and contain many signaling and structural proteins.

Ad2) Changes in $[Ca^{2+}]_i$ might influence integrin affinity/avidity changes. Although no increase in $[Ca^{2+}]_i$ is observed when U73122 is present, IL-8 still induces activation of α_4 integrins (chapter 5 figure 5). This at least suggests that $[Ca^{2+}]_i$ is not necessary to activate α_4 integrins upon IL-8 stimulation. A role for Ca^{2+} in β_2 integrin activation cannot be excluded from this observation, and experiments in which intracellular Ca^{2+} mobilization is inhibited could shed light on this question. How $[Ca^{2+}]_i$ may act on integrin affinity/avidity changes in eosinophils is not known. It is known that $[Ca^{2+}]_i$ and the Ca^{2+}-dependent phosphatase calcineurin are important in the recycling of integrins from the rear to the front of a migrating neutrophil. Buffering $[Ca^{2+}]_i$ led to an accumulation of $\alpha_v\beta_3$ integrins in the uropod of neutrophils, thereby inhibiting detachment (50). It has been postulated that Ca^{2+} acts on other Ca^{2+}-dependent proteins that are involved in regulation of integrin-avidity changes such as calpain (51). In T cells it is known that upon T-cell receptor engangement the activated tyrosine kinase ZAP70 induces PLC-γ activation, leading to increased β_2 integrin mediated adhesion. This event was abrogated by treating the cells with the PLC inhibitor U73122 and also with the calpain inhibitor calpeptin (52). Preliminary results, however, showed that eosinophils incubated with calpeptin and anti- α_4 integrin antibodies did not inhibit the eotaxin-induced arrest, suggesting that calpain is not neccesary for β_2 integrin-mediated eotaxin-induced arrest. Another Ca^{2+}-dependent protein, L-plastin, is important in diffusion of integrins in the plasma membrane (53). It might be that changes in Ca^{2+} in cells lead to dissociation of L-plastin from actin bundles, preventing integrins from free diffusion.

Ad 3) The third hypothesis is that PLC-induced DAG generation and subsequent PKC activation influences integrin activation. Indeed, it is known that PKC can activate

β_2 integrins (54). PMA is a potent stimulus for PKC, that phosphorylates and binds RACK, and subsequently RACK binds to the intracellular domain of the β_2 integrin LFA-1 (55). Furthermore, PKC can activate MacMarcks, which subsequently induces an increase in β_2 integrin avidity via phosphoylation of paxillin, leading to spreading of macrophages (56). However, preliminary results show that incubation of eosinophils with staurosporine, which is a very potent but not specific inhibitor of PKC, does not inhibit the eotaxin-induced arrest of eosinophils (data not shown). This suggests that inhibition of this pathway is not the working mechanism of U73122. This is comparable to the study of Soede *et al.* (52) who showed that the activated PLC-γ-induced increase in β_2 integrin-mediated adhesion in T cells was abrogated by U73122 but was not inhibited by a PKC inhibitor.

Ad 4) The fourth hypothesis that U73122 inhibits the expression of new integrins on the cell surface is not very likely, for the following reasons. 1) Stimulus induced expression of new integrins on the cell surface is thought not to contribute to adhesion events (57) and 2) U73122 can also inhibit stimulus-induced β_2 integrin avidity changes on cytoplasts, which lack integrin-containing vesicles and only contain integrins on the cell membrane (39).

Further studies will focus on these working hypotheses. Special attention will be given to the possibility that PLC itself directly acts on integrin affinity and/or avidity changes. Techniques like Western blotting and immunohistochemistry on adherent eosinophils will shed light on the isotype of PLC that is implicated in integrin regulation and the type of adhesion (affinity and/or avidity changes) that is induced by the different chemoattractants. Next, protein transduction technology renders it possible to fuse the tat-protein of the HIV virus to recombinant PLC, thereby creating a fusion protein that can enter primary leukocytes. Also, enzyme-dead mutants or in the case of PLC-γ, protein with mutations in the SH2, SH3 or PH domains, can be used for further studying the role of PLC in integrin regulation.

Control of eosinophil migration by regulating detachment

Especially in fibroblasts the function of the different GTP-ases in the migration process has been well studied (58;59). Cdc42 and Rac1 mediate filopodia and lamellipodia formation, respectively, and RhoA induces stress fiber and focal adhesion formation. For leukocytes, the Cdc42- and Rac1-mediated processes seem to be

similar (60-62). On the other hand, the RhoA-dependent processes seem to differ from fibroblasts. Leukocytes move much faster that fibroblasts (~1-10 μm/min, vs. ~0.1-1 μm/min respectively 63) and have no large focal adhesions but instead have smaller adhesion sites. Also, no stress fibers can be detected in leukocytes. Therefore, the role of RhoA in leukocytes is thought to be different from that in fibroblasts. In chapter 6 we found a role for RhoA in the detachment of the uropod of leukocytes (figure 2). Recently, a similar role for RhoA has been found in monocytes (64) and tumor cells (65). We showed that neutrophils migrate very well on albumin-coated glass in response to fMLP. However, when RhoA or its downstream effector Rho-kinase was inhibited, migration was inhibited. Eosinophils did not migrate well on albumin when stimulated with PAF. On the other hand, serum stimulation induced migration and this correlated with RhoA activation. Also, introducing dominant active RhoA (tat-V14RhoA protein) in the eosinophils induced migration on an albumin surface in the presence of PAF. We showed that PAF only transiently activates RhoA, whereas serum leads to a sustained stimulation of RhoA. Probably, activation by PAF (chapter 6) or eotaxin (data not shown) alone is not sufficient to give a sustained activation of RhoA in eosinophils needed for detachment from albumin. Preliminary experiments showed that eotaxin can induce migration when eosinophils were put on fibronectin coated glass (1 and 0.1 mg/ml). Therefore, it is tempting to speculate that binding of $\alpha_4\beta_1$ integrins to fibronectin leads to a sustained activation of RhoA. Indeed, Cox *et al.* (66) have shown that binding of cells to high concentrations of fibronectin (0.1 mg/ml) induced RhoA activation. Different hypotheses can be put forward to explain how RhoA induces detachment. 1) RhoA is known to act on the actinomyosin protein motor in non-muscle cells, therefore, activation of RhoA could lead to retraction forces that stimulate detachment. 2) RhoA could act on integrin activation and/or recycling processes of integrins in the rear of the cell, leading to detachment. Worthylake *et al.* (64) showed for monocytes that RhoA and p160/ROCK activation is needed for tail (uropod) retraction. Inhibition of RhoA (by C3) or p160/ROCK (by Y-27632) induced the formation of long tails that inhibited the monocytes from migration. While contractility inhibitors that act on myosin light chain kinase (which is a target of RhoA) could not induce tail formation, RhoA and Rho-kinase inhibitors were able to do this. Furthermore, the tails of the C3 and Y-27632 treated monocytes contained high concentrations of β_2 integrins, in contrast to tails of control-treated cells. Another study (67) showed that inhibiton of RhoA and Rho kinase in T cells resulted in an increased avidity of β_2 integrins. From these studies it was concluded that RhoA acts on integrin dependent processes. However a role for retraction forces or alternative mechanisms cannot be excluded.

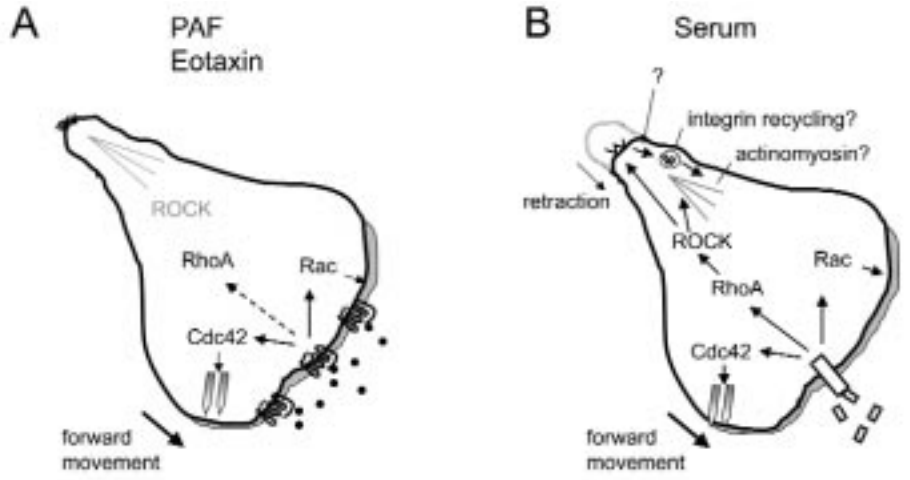

Figure 2. Model of the proposed mechanism of eosinophil migration on albumin-coated glass. A) The PAF and eotaxin-induced activation of RhoA is not strong enough to let the cells detach from the surface. B) The serum-induced potent activation of RhoA induces detachment. This might lead to a) integrin recycling processes that subsequently lead to less adhesion at the uropod and detachment and/or b) actinomyosin-based contraction of the uropod.

Interference with adhesion: a tool to combat asthma?

A characteristic of the chronic inflammation that is present in the airways of allergic asthmatics is the infiltration of the bronchial tissue by leukocyte subsets, especially eosinophils. It is thought that the presence of eosinophils contributes to the severity of the disease. Therefore, eosinophil extravasation to the bronchial tissue might be an interesting target to interfere with. A clear characterization of the molecular interactions controlling eosinophil extravasation will shed light on possible target molecules. The results described in this thesis address the importance of some of these molecules. Chapters 2 and 3 show that eosinophils of healthy individuals and allergic asthmatic patients use an E-selectin ligand and α_4 integrins for initial attachment and rolling interactions with activated endothelium. Chapter 3 further shows that eosinophils of allergic asthmatics but not of healthy individuals are prone to bind platelets. Consequently, a P-selectin-dependent secondary tethering mechanism results in increased adhesion to and cluster index on activated endothelium (figure 3). So far, this is the first report describing platelet-mediated adhesion events for eosinophils. Binding of platelets to patient eosinophils might have contributed to the observed β_2 integrin activity (chapter 3), though we did not address this possibility directly.

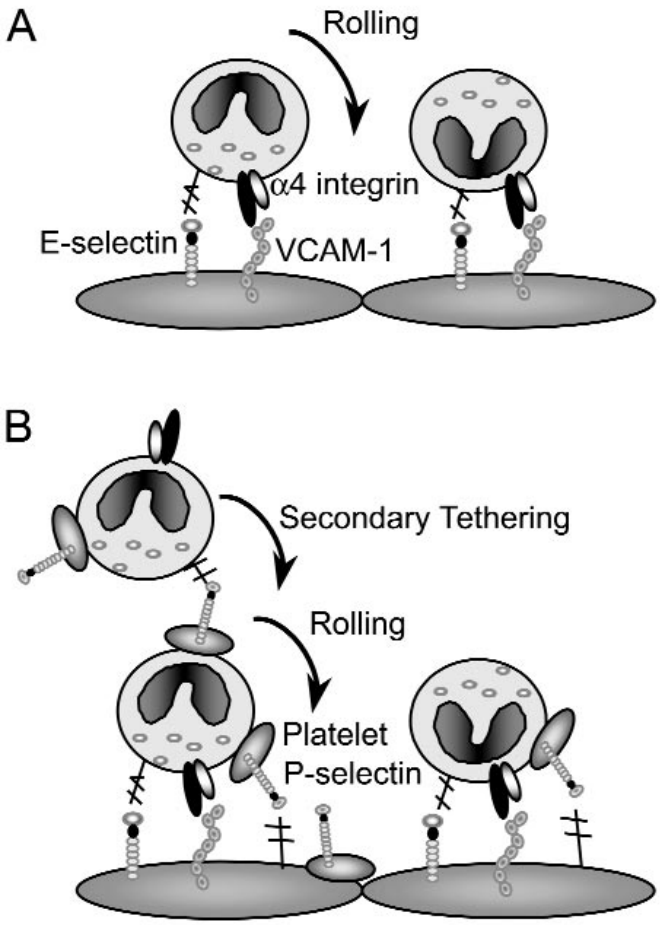

Figure 3. Platelet-mediated secondary clustering of eosinophils of allergic asthmatic patients. A) Eosinophils of healthy controls do not bind platelets. Adhesion to the endothelium is mediated via primary tethering processes. B) Eosinophils of allergic asthmatic patients bind platelets. Platelet-derived P-selectin-dependent secondary tethering processes increase the number of cells that are recruited to the surface.

We tried to address the question whether eosinophils from allergic asthmatic patients were primed. It is known from previous studies that eosinophils from allergic asthmatic patients migrate well towards low concentrations of PAF due to their primed phenotype (68). We tested whether an activated phenotype could be deduced in the transition from rolling to firm adhesion. PAF was perfused over rolling eosinophils in the presence of blocking antibodies against α_4-integrins in an attempt to study β_2 integrin-mediated arrest. More eosinophils from allergic asthmatic patients than from healthy controls arrested at a concentration of PAF of 10^{-10} M but the difference was only small (figure 4a). The PAF-induced arrest was dependent on β_2 integrins (figure 4b), suggesting that indeed the eosinophils of allergic asthmatic patients were primed. A drawback of these experiments is that they were performed with eosinophils isolated and perfused in the presence of anti-P-selectin since eosinophils from healthy controls that do not bind platelets are not a good control for eosinophils of allergic asthmatic with platelets. It is very likely that the eosinophils that bind platelets are the eosinophils that are primed most. Since these eosinophils were blocked in our set-up, a possible strong effect of PAF could have been missed.

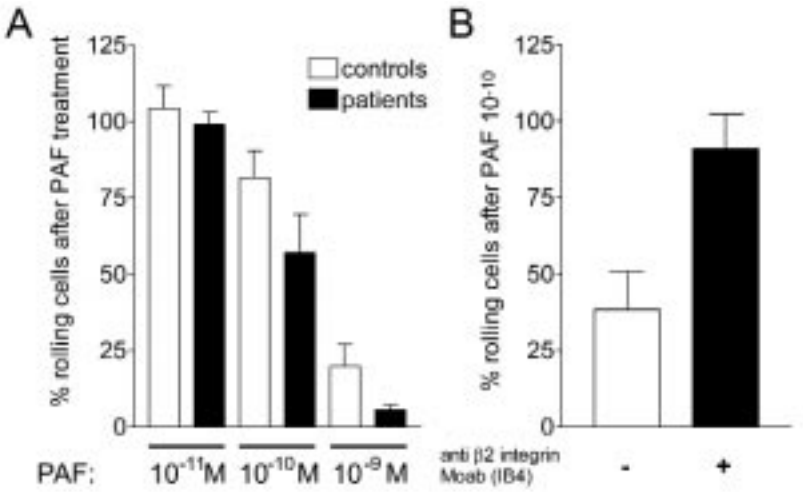

Figure 4. PAF-induced arrest of eosinophils of healthy controls vs. allergic asthmatic patients on TNFα-activated HUVEC. Eosinophils were isolated in the presence of anti-P-selectin antibodies and perfused in the presence of both anti-P-selectin and anti-α_4-integrin functional blocking MoAbs. In A) rolling cells after PAF treatment as a percentage of the amount of rolling cells before treatment is depicted. At PAF 10^{-10} M and 10^{-9} M more eosinophils of patients arrest compared to cells of controls. In B) rolling cells in the absence (white bar) or presence (black bar) of blocking anti β_2 integrin MoAbs after PAF treatment (10^{-10} M) as a percentage of the amount of rolling cells before treatment is depicted for eosinophils of allergic asthmatic patients. Data are presented as mean ± SEM of at least 4 experiments.

The molecules that mediate adhesion of eosinophils to activated endothelium are E- and P-selectin ligands, α_4 integrins and β_2 integrins. Because many adhesion molecules are involved in eosinophil recruitment to activated endothelium and often show overlapping function, the question arises to which target molecule therapy should be directed. Inhibition of the selectins might be a tool to decrease tethering and rolling interactions and prevents thereby subsequent adhesion and migration processes. Indeed, E- as well as P-selectin-dependent adhesion processes play a role in eosinophil adhesion (chapter 2 and 3). However, these selectins have also been shown to be important for homing of leukocytes to e.g. skin tissue. E- and P-selectin double knock-out mice show recurrent bacterial skin infections, implying that these selectins are very important for normal homeostasis and immuno-surveillance (69). β_2 integrins seem an attractive target for inhibition since many studies have shown the need for this type of integrin in migration processes of eosinophils. However, not only eosinophils but all other leukocyte subsets use β_2 integrins for migration. Inhibition or dysfunction of β_2 integrins leads to severe bacterial infections, as illustrated by the disease LADI, in which β_2 integrins are absent (70). Thus, the concept that β_2 integrins are a possible target for therapy can be ruled out. The α_4 integrin might be an attractive candidate, since this molecule is involved in both tethering and firm adhesion processes of eosinophils. Furthermore, lymphocytes also bear α_4 integrins and play a dominant role in allergic asthma, too. Indeed, α_4 integrin antagonists are being developed by pharmaceutical companies (71). A drawback of the use of α_4 integrin antagonists might be that this is an important molecule for the homing of leukocytes to the bone marrow, although selectin mediated rolling interactions can also occur at this site (22). The results of adhesion molecule antagonist therapies for diseases like asthma, but also for other chronic inflammatory diseases such as rheumatoid arthritis, are to be awaited. The eventual conclusions of these integrin-antagonist therapies might be that the working mechanism is too broad. It may be worthwhile to develop α_4 integrin antagonists that block either VCAM-1 or fibronectin binding to circumvent the problem of possible side effects. For this possibility a more detailed knowledge will be needed concerning the binding sites of integrins, which can be obtained by crystal structure analysis. Recently, the crystal structure of the extracellular domain of the $\alpha_v\beta_3$ integrin has been determined (72). This is the first integrin that has been characterized at this level, and the knowledge of the quaternary arrangements will contribute to new insights in integrin function and regulation. Furthermore, it may contribute to the development of drugs that are directed against integrins.

Concluding remarks

Eosinophil rolling, adhesion and migration processes are under control of numerous adhesion molecules and chemoattractants, as outlined in this thesis. A clear picture is present of the type of adhesion molecules and chemoattractants that can mediate the different steps of eosinophil extravasation. Although the exact mechanisms how different chemoattractants communicate with adhesion molecules at the molecular intracellular level in primary cells are not known, a start has been made in this field. We showed that under flow conditions different chemoattractants can regulate integrins on eosinophils in different ways, and that PLC is involved in this process. It is important for further research that the inside-out signaling pathways leading to integrin activation and subsequent adhesion and migration processes of leukocytes are performed under flow conditions. Only at these more physiological situations the subtle differences between integrin functions will be found. When specificity in the mechanisms of the different chemoattractants on adhesion events is found, the way opens that leads to more specific therapeutic strategies.

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Nederlandse Samenvatting

Een stappenmodel voor de uittreding van witte bloedcellen

Witte bloedcellen kunnen reizen door het lichaam. Dit is van belang voor de continue bewaking die het immuunsysteem uitvoert om eventuele gevaren in het lichaam direct te kunnen waarnemen. Wanneer er gevaar is, hetgeen bijvoorbeeld een binnengedrongen worm of bacterie (ook wel pathogeen genoemd) kan zijn, dan moet dit opgeruimd worden. Een vereiste is dat de witte bloedcellen vanuit het bloed naar de plek waar de pathogeen zich bevindt moeten reizen. De cellen doen dat door met hechtingsmoleculen (adhesiemoleculen) die op de celmembraan (= het "jasje van de cel") zitten te hechten aan hechtingsmoleculen die op de binnenbekleding van de bloedbaan (de endotheelcellaag) zitten. Als de cellen vastzitten aan de endotheelcellen dan beginnen ze vervolgens tussen de endotheelcellen door te kruipen en zo komen ze uiteindelijk in de weefsels terecht. Daar zoeken ze de pathogeen op die ze vervolgens vernietigen.

De uittreding vindt plaats in verschillende stappen. *Stap 1*. Omdat het bloed continu stroomt moet de cel eerst afgeremd worden voordat ze kan hechten. Het afremmen gebeurt doordat de cel met specifieke adhesiemoleculen (selectines) bindt aan suikerstructuren op de endotheelcellen. Dit resulteert in het rollen van de witte bloedcel over de endotheelcellen. *Stap 2*. Als er signaalmoleculen (chemoattractanten) aanwezig zijn dan passen deze als een sleutel in een slot. Het slot (op de witte bloedcel) is een specifiek eiwit ook wel receptor genoemd. De receptor kan vervolgens signalen via de celmembraan naar binnen toe doorgeven, waardoor de cel wordt geaktiveerd. *Stap 3*. Aktivatie van de cel resulteert in het aktiveren van een andere klasse adhesiemoleculen, de integrines. Deze zitten altijd op de witte bloedcel maar kunnen alleen door aktivatie binden aan hun tegenstructuren op de endotheelcellen, de vasculaire adhesiemoleculen. Dit resulteert in de overgang van een rollende cel naar een stilzittende cel en vervolgens in een spreidende cel. *Stap 4*. De witte bloedcel gebruikt nu de geaktiveerde integrines en haar cytoskelet (de "botten" van de cel) om over het endotheel te kruipen en een plek te zoeken waar ze tussendoor kan glippen richting de weefsels. Dit wordt migratie genoemd. Chemoattractanten helpen ook bij deze stap omdat de cel richting de hoogste concentratie chemoattractant kruipt welke aanwezig is rondom de pathogeen die zich in het weefsel bevindt. (Deze stappen zijn geïllustreerd in figuur 1 van hoofdstuk 1; stap 1 is rolling, stap 2 is activation, stap 3 is firm adhesion en stap 4 is migration).

Dit algemene mechanisme van uittreding van witte bloedcellen van de bloedbaan naar het weefsel treedt op bij verschillende ziektes. Sommige uitgetrede cellen doen goede zaken door b.v. het opruimen van pathogenen, maar andere uitgetrede cellen doen slechte zaken omdat ze niets te zoeken hebben op de plek waar ze naar toe gaan. Dit laatste kan negatieve gevolgen hebben voor het weefsel en zodoende kan een goed bedoelde immuunreaktie leiden tot een bepaald ziektebeeld.

Allergisch astma

De ziekte allergisch astma onstaat doordat het immuunsysteem een fout maakt. Bij mensen met allergisch astma kunnen onschadelijke stoffen in het milieu (b.v. huisstofmijt, kattestof, pollen) door het immuunsysteem als schadelijk worden gezien. Dit kan leiden tot een reaktie in het bronchiale weefsel van de long. Een scala aan immunologische reakties vindt plaats welke tot uiting komen in de klinische symptomen benauwdheid en piepen. Een van de meest opmerkelijke immunologische reakties die plaatsvindt is dat een bepaalde witte bloedcel, de eosinofiele granulocyte, in een groot aantal naar het bronchiale weefsel van de long reist. Er is aangetoond dat deze cellen mede verantwoordelijk zijn voor het verslechteren van de longfunctie doordat ze het epitheel (de cellen die de barrière vormen tussen buitenlucht en bronchiaal weefsel) kunnen beschadigen.

De eosinofiele granulocyte

De eosinofiele granulocyte (afgekort eo) is een witte bloedcel die haar naam heeft gekregen toen onderzoeker Paul Ehrlich eind 19de eeuw met de kleurstof eosin dit celtype aan kon kleuren. Eosin kan basische stoffen aankleuren die in de blaasjes (granula) van de eo's te vinden zijn. Deze basische stoffen gebruikt de cel om pathogenen op te ruimen. Er wordt gedacht dat vooral wormen kunnen worden gedood en opgeruimd door eo's. Aangezien er amper nog schadelijke wormen voorkomen in het milieu van de westerse wereld heeft dit geleid tot werkeloosheid onder de eo's. Werkeloosheid leidt tot verveling en dat leidt tot ongewenste aktiviteiten die eo's uitoefenen in personen met de ziekte allergisch astma. Zoals gezegd verplaatsen de eo's zich vanuit het bloed naar het longweefsel waar ze vervolgens schade berokkenen. In dit proefschrift worden experimenten beschreven waarin is onderzocht hoe de eo's vanuit het bloed naar het longweefsel kunnen komen. Als je weet welke hechtingsmoleculen de eo gebruikt om aan het endotheel te hechten en vervolgens naar

het longweefsel te reizen, zou daar een stof voor kunnen worden ontwikkeld om dat tegen te gaan. Want zolang de eo's in het bloed blijven zijn ze niet schadelijk.

Het onderzoeksmodel

De experimenten beschreven in dit proefschrift zijn niet in een heel organisme uitgevoerd (*in vivo*) maar in een testsysteem buiten het lichaam (*in vitro*). Het rollen, de aktivatie en de hechting van eo's *in vivo* vindt plaats in het bloed dat continu stroomt. Omdat de stroming een essentiële factor is voor het optreden van de verschillende stappen, werd de stroming in het *in vitro* testsysteem nagebootst. Een andere factor is de endotheelcellaag. De endotheelcellen werden geïsoleerd uit navelstrengen (die bevatten een prachtig dik bloedvat) en werden in de proefopzet geaktiveerd zodat ze rolmoleculen (E-selectine) en hechtingsmoleculen (VCAM-1, ICAM-1) op hun oppervlak kregen. Uit bloed werden eo's geïsoleerd en deze werden over de endotheelcellaag gestroomd. Door gebruik te maken van stoffen die specifiek adhesiemoleculen kunnen remmen (monoclonale antistoffen) werd de functie van elk adhesiemolecuul bestudeerd.

De resultaten

In hoofdstuk 2 wordt beschreven dat eo's (van gezonde donoren) rollen over E-selectine. Daarnaast speelt de integrine $\alpha_4\beta_1$ die op eo's zit ook een voorname rol in de eerste hechting van de eo aan het endotheel. Als de E-selectine moleculen en de α_4 integrines tegelijkertijd werden geblokkeerd dan konden de eo's niet meer rollen en hechten. Ze stroomden langs het endotheel zonder een interactie aan te gaan. De rol voor α_4 integrine in de eerste hechting was onverwacht omdat de integrines over het algemeen alleen hechting mediëren **na** aktivatie van witte bloedcellen. Maar in de experimenten werden de eo's niet geaktiveerd. De hypothese die hieruit volgt is dat de $\alpha_4\beta_1$ integrine zich in een intermediaire aktivatie staat bevindt op een rustende (=niet geaktiveerde) eo en daardoor aan een hechtingsmolecuul op het endotheel kan binden.
In hoofdstuk 3 worden de hechtingsmechanismen van eo's van astma patiënten aan endotheelcellen onder stromingscondities beschreven. De hechting verliep bij deze eo's net als bij eo's van gezonde donoren via E-selectine en $\alpha_4\beta_1$ integrine. Maar in tegenstelling tot de situatie bij eo's van gezonde donoren (gebruikt in de studies beschreven in hoofdstuk 2) kon de rolling en hechting van eo's van astma patiënten niet

totaal worden verhinderd door het blokkeren van E-selectine en $\alpha_4\beta_1$ integrine. Het resterende hechtende deel deed dat volgens een bepaald patroon namelijk in groepjes; de eo's klusterden op het endotheel. Dit is een mechanisme wat cellen vaker gebruiken. Het werkt doordat een eo (eo 1) die aan komt stromen hecht aan een eo (eo 2) die al vast aan het endotheel zit. Eo 1 komt vervolgens vlak achter eo 2 terecht. Als dit proces vaker optreedt komen de cellen heel dicht bij elkaar in groepjes te liggen. Het opmerkelijke aan de eo's van allergische patiënten was dat er bloedplaatjes aan de eo's bonden. Deze bloedplaatjes zorgden voor de klustervorming van eo's omdat de klustervorming voorkomen kon worden door een rollingsmolecuul (P-selectine), dat in het testsysteem alleen op plaatjes voorkomt, te blokkeren. Het idee is dat bloedplaatjes een brug tussen eo's kunnen slaan en daardoor klustering kunnen bevorderen. Tevens werden er aanwijzingen gevonden dat β_2 integrines op eo's van astmatische patiënten een beetje meer geaktiveerd zijn dan β_2 integrines op eo's van gezonde donoren.

In hoofstuk 4 wordt de rol van de aktivator IL-8 op de hechtingsmechanismen van de eo beschreven. IL-8 is oorspronkelijk gekarakteriseerd als een zeer goede aktivator van neutrofiele granulocyten (het broertje van de eo) maar werd lang als een slechte aktivator van eo's beschouwd. Experimenten in hoofdstuk 4 tonen aan dat IL-8 rollende eo's stil liet zitten. Maar de eo's bleven niet lang stilzitten en begonnen weer te rollen na ongeveer een minuut. Deze observatie wordt "transiënte hechting" genoemd. Als eotaxine, een specifieke eo aktivator, werd toegevoegd aan rollende eo's gingen de eo's stilzitten en platten ze tevens af. Eotaxine is dus een sterkere aktivator voor eo's dan IL-8. Maar IL-8 kan wel degelijk eo's aktiveren. Dit wordt mede onderbouwd door het feit dat IL-8 naast het hechtingssignaal ook een ander signaal gaf namelijk het verhogen van de concentratie calcium ionen in de cel. IL-8 kon dit alleen als eo's gehecht waren op een oppervlak en niet wanneer de cellen in een vloeistof dreven. Daarom is de hypothese dat 1) IL-8 eerst leidt tot aktivatie van de integrines, 2) dat de integrines door de stevige hechting verder worden geaktiveerd, 3) dat de integrines vervolgens signalen naar de binnenkant van de eo geven wat leidt tot verhoging van de calcium ionen in de cel. Kortom, IL-8 leidt niet direct tot een verhoging van calcium ionen maar doet dit via de integrines.

In hoofdstuk 5 zijn de aktivatoren IL-8, eotaxine en C5a vergeleken. Het bleek dat de verschillende aktivatoren verschillende routes binnen de cel gebruikten om de integrines aan te schakelen. Het enzym (een eiwit dat kan knippen) phopholipase C leek een grote rol te spelen in deze regulatie want het blokkeren van dit enzym bewerkstelligde dat de aktivatoren de integrines niet of nauwelijks nog konden aktiveren. En dus konden de eo's niet meer hechten aan de endotheelcellen.

In hoofdstuk 6 worden proeven beschreven waarin de stap werd onderzocht die volgt na het rollen en het stilzitten namelijk het kruipen van de eo. Het is bekend dat als cellen kruipen over een oppervlak ze een typerende vorm aannemen. Het voorste deel

(lamellipodium) is heel breed en het achterste deel (uropod) van de cel is smal (zie figuur 4 van hoofdstuk 1). Het voorste deel schuift over het oppervlak en het achterste deel wordt bijgetrokken. De proeven in hoofstuk 6 laten zien dat de eo problemen heeft met het bijtrekken van haar achterste. Alleen als de eo de goede stimulator “ruikt” dan kan hij haar achterste bijtrekken en verder vooruit komen. Serum (=bloed met alle cellen en stollingsprodukten daaruit verwijderd) bleek een goede stimulator te zijn. Serum kon zowel de voorkant naar voren laten gaan en de achterkant bij laten trekken. Eotaxine daartegenover kon wel de voorkant van de eo in beweging zetten maar kon niet de achterkant laten bijtrekken wat resulteerde in een langgerekte cel die niet vooruit kwam. Vervolgens is laten zien dat een molecuul in de cel, RhoA genoemd, belangrijk is voor het bijtrekken van de achterkant. Inderdaad, serum kon RhoA langdurig aktiveren maar eotaxine kon het slechts kort aktiveren wat overeenkomt met de discrepantie tussen serum en eotaxine in het kruipgedrag. Additioneel bewijs dat RhoA belangrijk is voor het bijtrekken van de achterkant komt uit de proeven waarin een aktief RhoA eiwit in de eo werd gebracht. Nu konden eo’s opeens wel hun achterste bijtrekken in de aanwezigheid van eotaxine.

Conclusies:

Eo’s rollen over E-selectine en kunnen ook hun α_4 integrine gebruiken voor de eerste hechting aan endotheelcellen.
Eo’s van astma patiënten hebben een additief mechanisme voor het hechten namelijk het gebruik van bloedplaatjes.
Eo’s kunnen worden gestimuleerd door IL-8 wat resulteert in hechting van de eo’s en in het verhogen van de calcium ion concentratie in de cellen.
Verschillende aktivatoren kunnen de integrines op een eo via verschillende wegen aktiveren.
RhoA is belangrijk voor het bijtrekken van het achterste deel van de cel.

Interfereren met hechting: een middel om astma te verhelpen?

De blokkade van hechtingsmoleculen op eo’s door antilichamen wordt als een mogelijke nieuwe therapie beschouwd. De grootste vraag is: Welk hechtingsmolecuul kun je het beste blokkeren? Studies (onder andere dit proefschrift) tonen aan dat meerdere hechtingsmoleculen een rol spelen. Kandidaten zijn selectines, β_2 integrines en α_4 integrines. Het blokkeren van selectines lijkt een optie maar er bestaat een ziekte

(Leukocyte Adhesion Deficiency type II, LAD II) waarbij de cellen van de patiënt geen goede receptoren voor de selectines hebben. Deze patiënten zijn erg ziek omdat witte bloedcellen (vooral neutrofiele granulocyten) niet meer naar de plaats kunnen komen waar b.v. een bacterie is binnen gedrongen. Het ontbreken van de selectines zelf zou tot dezelfde verschijnselen kunnen leiden. Dit kan een reden zijn om de selectines niet tot doel van antilichaam-therapie te maken. Het blokkeren van β_2 integrines lijkt ook een mogelijkheid. Maar ook hiervan zijn natuurlijke voorbeelden bekend dat dit niet ongestraft gaat. Kinderen die geboren worden met Leukocyte Adhesion Deficiency type I hebben op geen enkele cel β_2 integrines zitten. Dit leidt eveneens tot infecties van allerlei organen net als bij LAD II. Blokkeren van β_2 integrines in astma patiënten zou dus kunnen leiden tot grotere neveneffecten dan het eigenlijke verhelpen van de ziekte. Waarschijnlijk zal de beste kandidaat de α_4 integrine zijn. Dan blijven er voldoende adhesiemoleculen over om het dagelijkse werk te doen: het bewaken van het lichaam en het opruimen van bacteriën. Daarnaast wordt niet alleen de eo verhinderd in de hechting aan endotheel maar ook de lymfocyte (weer een ander type witte bloedcel). De lymfocyte speelt namelijk ook een belangrijke rol in het ontstekingsproces van astma. Of het blokkeren van α_4 genoeg is om de eo's buiten het longweefsel te houden, is een interessant vraagstuk dat in de toekomst opgelost zal worden.

Publications

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Curriculum Vitae

De schrijfster van dit proefschrift werd geboren op 8 februari 1974 te Holten. Na het behalen van haar Atheneum diploma aan de Scholengemeenschap Holten werd in 1992 begonnen met de studie Biologie aan de Rijks Universiteit Groningen. In augustus 1997 haalde zij haar doctoraal examen met als hoofdvakken Immunologie (onder begeleiding van Dr. G. Molema en Prof. Dr. L.F.M.H. de Leij, faculteit Geneeskunde, Rijks Universiteit Groningen) en Experimentele Immunologie (onder begeleiding van Dr. U.H. von Andrian, Center for Blood Research, Harvard Medical School, Boston). In september 1997 begon zij aan haar onderzoeksproject als assistent in opleiding bij de afdeling Experimentele Longziekten van het Universitair Medisch Centrum Utrecht onder begeleiding van Prof. Dr. Leo Koenderman, Prof. Dr. Jan-Willem J. Lammers en Dr. Jaap-Jan Zwaginga. Sinds september 2001 is ze aangesteld als postdoctoraal onderzoeker in dezelfde groep.

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