Brief Definitive Report

Eosinophil Adhesion to Nasal Polyp Endothelium Is P-Selectin-dependent

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Summary

Tissue eosinophilia is a characteristic feature of a number of inflammatory diseases including asthma and nasal polyposis. Eosinophil migration into tissues is controlled in part by interactions between eosinophil adhesion receptors and counter-structures on the vascular endothelium. To determine the receptors used by eosinophils to adhere to vascular endothelium in allergic inflammation we have adapted the Stamper-Woodruff frozen section assay (FSA) to study eosinophil adhesion to nasal polyp endothelium. Immunohistology indicated that intercellular adhesion molecule 1 (ICAM-1), E-selectin and P-selectin were well expressed by nasal polyp endothelium, whereas expression of vascular cell adhesion molecule 1 (VCAM-1) was weak or absent. Unstimulated human peripheral blood eosinophils adhered specifically to nasal polyp endothelium. Adherence was temperature and divalent cation–dependent and saturable at cell densities $>5 \times 10^6$ cells/ml. Eosinophil adhesion was almost completely inhibited by a monoclonal antibody (mAb) against P-selectin and by a chimeric molecule consisting of the F_c portion of human IgG and the lectin binding domain of P-selectin, which binds to the P-selectin ligand on leucocytes. Anti-Mac-1 mAb partially inhibited eosinophil adhesion whereas mAb against E-selectin, L-selectin, ICAM-1, VCAM-1, very late activation antigen 4, and lymphocyte function-associated antigen 1 had no effect. P-selectin is stored in intracellular granules within the endothelial cell and in vitro is only transiently expressed. To determine if P-selectin was expressed on the membrane of the nasal polyp endothelium we compared P-selectin expression in normal skin and nasal polyps after acetone fixation, which permeabilizes cells, and paraformaldehyde, which only allows staining of membrane expressed receptors. In the skin, good expression was seen with acetone fixation but no expression was seen after paraformaldehyde treatment, whereas in nasal polyps, similar expression was observed with both fixatives. In addition immunofluorescence with confocal microscopy demonstrated lumenal staining of nasal polyp endothelium indicating that P-selectin was located on the surface of endothelial cells while in skin only an intracellular granular distribution was apparent. Lastly, whereas eosinophils bound consistently to nasal polyp endothelium, no binding was observed to blood vessels in normal skin further supporting the idea that eosinophils were binding to membrane expressed and not intracellular P-selectin. The importance of P-selectin in eosinophil adhesion to nasal polyp endothelium suggests that P-selectin antagonists may be effective at inhibiting eosinophil accumulation at sites of allergic inflammation.

A consistent feature of asthma and related diseases such as allergic rhinitis and atopic dermatitis is infiltration of the tissue by eosinophils (1, 2). Eosinophil accumulation in these diseases is relatively selective in that it generally occurs without an increase in neutrophils, although increased numbers of T cells and monocytes are often seen. One explanation for the specific tissue migration of eosinophils in allergic inflammation is a selective pathway of eosinophil adhesion and transmigration. One such potential pathway uses very late activation antigen 4 (VLA-4), a receptor expressed by eosinophils but not neutrophils, binding to vascular cell adhesion molecule 1 (VCAM-1) on endothelium (3–6). Evidence for the importance of this pathway in eosinophil migration was provided by the selective increase in VCAM-1 expression on cultured human umbilical vein endothelial cells (HUVEC) induced by IL-4 (7). IL-4, which is generated in asthmatic airways, also mediated eosinophil transmigration through HUVEC in a VLA-4/VCAM-1–dependent fashion and IL-4 transgenic mice had an eosinophilic conjunctivitis (8, 9). In contrast VCAM-1 was weakly expressed in chronic

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asthmatic airways with no evidence of a difference in expression between normal and diseased tissue (10). Further studies have revealed few other major differences in the profile of adhesion receptors expressed by eosinophils compared with other leukocytes. They express L-selectin, which is shed on cell activation, and functional ligands for E-selectin and P-selectin (11–13). Their expression of leukocyte integrins is similar to that of neutrophils (14), and like neutrophils, eosinophil adhesion to HUVEC can be inhibited by blocking antibodies against LFA-1, Mac-1, and intercellular adhesion molecule 1 (ICAM-1) (15). Despite the relative lack of selectivity in the expression of adhesion receptors on eosinophils compared with other leukocytes it remains possible that there are functional differences in adhesion receptor usage. In addition, the pattern of adhesion receptor expression on HUVEC may not reflect the expression of adhesion receptors in vivo in allergic inflammation. To determine the adhesion receptors used by eosinophils to adhere to airway endothelium we have adapted the frozen section assay (FSA), first used by Stamper and Woodruff (16) to study lymphocyte homing to lymph nodes, to investigate eosinophil adhesion to nasal polyp endothelium. We have shown that adhesion of unstimulated eosinophils to nasal polyp endothelium is primarily mediated by P-selectin.

Materials and Methods

Reagents. Control mouse myeloma proteins MOPC (IgG1, IgG2a, and IgG2b, mixed in equal proportions), human IgG, and FITC-labeled goat anti-mouse IgG Fc $F(ab)_2$ fragments, (Sigma Chemical Co., Poole, UK). Micromagnetic beads bound to anti-CD16 mAb and magnetic-activated cell sorter (MACS) columns, (Eurogenetics, Teddington, UK). The P-selectin–IgG chimeric molecule consists of the lectin and epidermal growth factor–binding domains and one complement binding protein-like domain of human P-selectin engineered to the human IgG1 Fc portion. The chimera is known to bind to the human P-selectin ligand (17) and was found to bind to human eosinophils by FACS® analysis (Becton Dickinson & Co., Oxford, UK). Binding was cation dependent and independent of the activation status of the eosinophils (data not shown).

Antibodies. The mouse mAb listed below were used in adhesion blockade studies and were received as kind gifts from the following groups: IF-11 (anti-E-selectin,), 11C8-I (anti-ICAM-1), and 4B2 (anti-VCAM-1) from Dr. L. Needham (British Biotechnology, Oxford, UK); HP1/2 (anti-VLA-4) from Dr. F. Sanchez-Madrid (Hospital de la Princesa, Madrid, Spain); TSI/22 (anti-LFA-1) from Dr. T. Springer (Dana Farber Cancer Institute, Boston, MA); G1 (anti-P-selectin) from Dr. R. McEver (Oklahoma Medical Research Foundation, Oklahoma City, OK); Dreg 56 (anti-L-selectin) from Dr. K. Kishimoto (Boehringer Ingelheim, Ridgefield, CT). The anti-Mac-1 mAb was purchased from Dako (M741 Clone 2LPM19C; High Wycombe, UK); anti-CD18 mAb from British Biotechnology (Oxford, UK, BCA5 Clone MEM48) and Leu 8 (anti-L-selectin) was purchased from Becton Dickinson & Co. Leu 8 was used to confirm expression of L-selectin by eosinophils and in initial inhibition experiments. As the manufacturer could not confirm the ability of Leu 8 to block L-selectin activity we changed to Dreg 56, a known blocking mAb. No inhibition of adhesion was seen using either antibody. For immunohistochemical staining of tissue sections: we used mAbs RUU-SP1.18 (anti-P-selectin) from Dr. H. K. Nieuwenhuis and Dr. M. J. Metzelaar (University Hospital Utrecht, Utrecht, Netherlands); 140-13 (anti-CD41, a platelet specific marker) from Dr. R. Vilella (Hospital Clinic, Barcelona, Spain); BMK-13 (anti–human eosinophil major basic protein) from Dr. R. Moqbel (National Heart and Lung Institute, London, UK). BBA-1 (anti-E-selectin), BBA-3 (anti-ICAM-1) and BBA-5 (anti-VCAM-1) were purchased from British Biotechnology and EN4 (antiendothelial cell) was obtained from Bradsure Biologicals (Loughborough, UK). All mAbs were used as whole molecules rather than $F(ab)_2$ fragments as freshly isolated eosinophils do not express high affinity Fc receptors and previous studies have indicated no interference by Ig Fc in eosinophil–endothelial cell interactions (6, 18).

Tissue. Nasal polyps (n = 8) were obtained after routine surgery in the Ear, Nose, and Throat Department (Leicester Royal Infirmary). Patients were aged 19–84. Two patients were taking nasal steroids. Nasal polyps were received within 0.5 h of removal, washed briefly in PBS, dissected, and snap frozen in liquid N_2. The highly eosinophilic nature of the polyps was confirmed by immunostaining for eosinophil major basic protein (MBP). Skin tissue from breast, removed during mastectomy (n = 2), and forearm, isolated by punch biopsy (n = 1), were snap frozen immediately upon receipt. All tissue samples were stored in vapor phase liquid N_2 until required.

Eosinophil Purification. Eosinophils were isolated from 100 ml of blood from healthy volunteers with no clinical evidence of allergic symptoms at the time of venesection and eosinophil counts of $<0.4 \times 10^6$/ml. After dextran sedimentation leukocytes were subjected to a slow spin (200 g) to remove platelets before centrifugation (Histopaque 1083; Sigma Chemical Co.) (400 g, for 25 min, at room temperature [RT]). Eosinophils were isolated by negative immunomagnetic selection using CD16 Microbeads and the MACS system (19). Isolates consisting of >99% eosinophils, with viabilities of >95%, as judged by trypan blue exclusion, were routinely obtained. Eosinophils were occasionally used after storage overnight at 4°C, in assay medium (medium 199 with Earle's salts, L-Gln, Hepes plus 2% FCS) and EGTA. No difference in adherence was found between stored and fresh cells. Eosinophils did not express the platelet adhesion receptor IIb/IIIa demonstrating that there was no platelet contamination.

FSA. The frozen section assay was adapted from the method of Stamper and Woodruff (16). Briefly, 100 μl of eosinophils (5×10^6 cells/ml) in assay medium ± EGTA, were pipetted over 8 μm nasal polyp sections, on silane coated microscope slides. Slides were rotated at 70 rpm for 30 min at RT on a rotating platform. Unbound cells were gently tipped off before fixing in 2% glutaraldehyde in PBS for 30 min. Slides were washed for 10 min in PBS, with rotation, before staining using May Grunwald/Giemsa. Eosinophil adhesion was assessed visually, on blinded slides, by counting the number of blood vessels that bound two or more eosinophils from 100 vessels located randomly within each section and expressed as the percent of blood vessels which bound eosinophils. Each assay was carried out at least in duplicate. The intraassay coefficient of variation was 9.2%.

Immunohistology. Nasal polyps (8 patients) and skin (n = 3) were examined. 6-μm sections were air dried, fixed in either acetone (100%, 10 min at RT) or paraformaldehyde (4% freshly prepared, ice cold paraformaldehyde, 5 min at RT), and then immunostained using the streptavidin-biotin (ABC), alkaline phosphatase method, as recommended by the manufacturer (Dako). The number of blood vessels staining positive for each adhesion receptor mAb was expressed as the percent of total blood vessels, as estimated by positive staining with the endothelial-specific mAb, EN4.

Immunofluorescence. 8-μm frozen sections were fixed in acetone before indirect immunofluorescence staining for P-selectin (RUU-

SP1.18 mAb) using a FITC-labeled second Ab. Sections were analyzed using a confocal microscope (model MRC 600; Bio-Rad Laboratories, Richmond, CA).

Statistics. Significance was determined using an unpaired student's t test. Data is quoted as the mean ± SEM, with $p \leq 0.05$ being considered significant.

Results and Discussion

Under the conditions of the FSA eosinophils adhered specifically to nasal polyp endothelium with minimal adhesion to other structures within the section (Fig. 1 *A*). Although two adherent eosinophils were taken as the minimum to record a positive blood vessel, most positive vessels had considerably more bound cells. Adhesion was almost completely abolished by treating either the section or the eosinophils with EGTA, indicating cation dependence and suggesting that adhesion was mediated by specific receptors (Fig. 1 *B*).

Adhesion was dependent on eosinophil concentration, being undetectable below 10^5 cells/ml and maximal (48.9 ± 5.3% positive blood vessels) at 0.5–1 × 10^7 cells/ml (n = 3). Above 10^7 cells/ml, marked adhesion throughout the section was observed. Adhesion was temperature dependent, increasing from 26.6 ± 1.6% positive blood vessels at 4°C to an optimum of 43.3 ± 3.5% at 24°C, and falling back to 32.7 ± 3.1% at 37°C (n = 3). All further experiments were therefore carried out at RT with a cell concentration of 5 × 10^6 cells/ml.

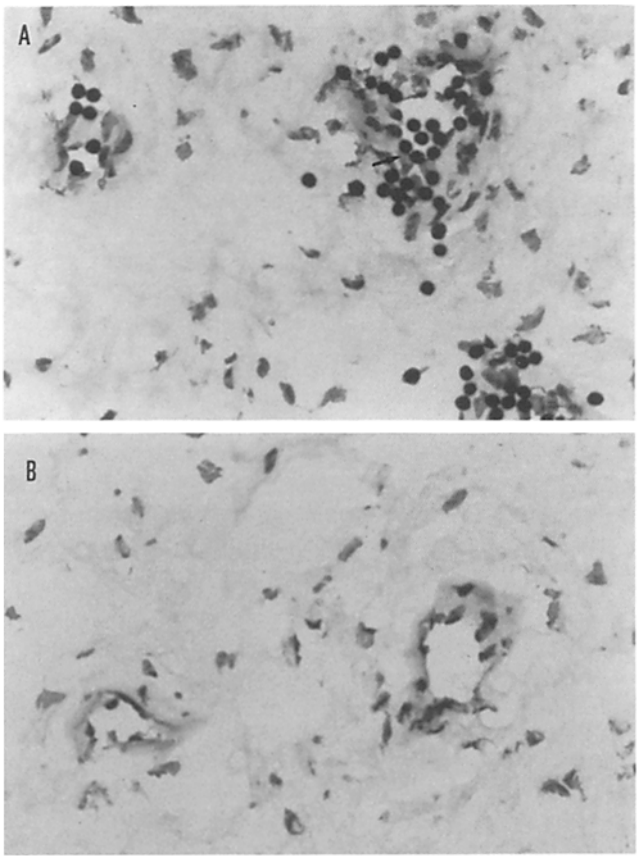

Figure 1. Eosinophil adhesion to nasal polyp endothelium in the absence (*A*) or presence (*B*) of EGTA. Eosinophils (5 × 10^6 cells/ml) were washed and resuspended in assay medium ± EGTA before layering over nasal polyp sections in the FSA. Adherent eosinophils are indicated by the arrows.

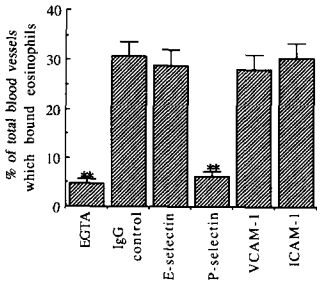

Figure 2. Inhibition of eosinophil adhesion to nasal polyp endothelium by mAbs against endothelial adhesion molecules. Nasal polyp sections were incubated for 30 min at RT with mAb against various endothelial adhesion molecules before the FSA. Eosinophil adhesion was inhibited either by the anti-P-selectin mAb, G1, or treatment of eosinophils with EGTA, (p <0.0005), compared with an isotype matched IgG control, but not by mAbs against E-selectin (IF-11), ICAM-1 (11C8-I), or VCAM-1 (4B2) (n = 9). Experiments were carried out on nasal polyps from three patients.

To determine which receptors were mediating eosinophil adhesion we first investigated the expression of adhesion molecules by nasal polyp endothelium. ICAM-1 (40%), E-selectin (29%), and P-selectin (45%) were well expressed in all the polyps studied. Figures in brackets refer to the mean percentage of total blood vessels that stained positive in sections from eight polyps. P- and E-selectin staining was confined to the endothelium whereas ICAM-1 was also expressed on tissue leukocytes and the epithelium. In contrast, expression of VCAM-1 (5%) was either absent or very weak.

We than investigated the inhibitory effects of blocking mAbs against both endothelial and eosinophil adhesion receptors. Of the endothelial receptors a mAb against P-selectin (G1) almost completely inhibited eosinophil adhesion to nasal polyp endothelium (p <0.0005). Blocking antibodies against E-selectin, ICAM-1 and VCAM-1 had no effect (Fig. 2). Of the antibodies directed against eosinophil adhesion receptors only a mAb directed against the α chain of Mac-1 caused significant inhibition and this was modest in degree. Antibodies against L-selectin, the α and β chains of LFA-1, and the α chain of VLA-4 had no effect. In contrast, a P-selectin chimeric molecule almost completely inhibited eosinophil adhesion, whereas control human IgG had no effect (Fig. 3).

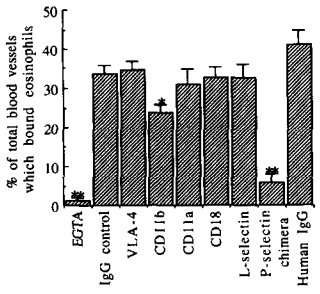

Figure 3. Inhibition of eosinophil adhesion to nasal polyp endothelium by mAbs against eosinophil adhesion molecules. Eosinophils were incubated at RT for 10 min with mAb against eosinophil adhesion molecules, isotype matched IgG controls or EGTA-treated assay medium, before FSA. Eosinophil adhesion was inhibited by the P-selectin chimera, which binds the P-selectin ligand, EGTA (p <0.0005) and by an anti-CD11b mAb (M741) (p <0.005). Antibodies against VLA-4 (HP1/2), CD11a (TSI/22), CD18 (BCA5), and L-selectin (Leu 8 and Dreg 56) had no effect (n = 7). Experiments were carried out on nasal polyps from four patients.

P-selectin is stored within the Weibel-Palade bodies of endothelial cells (20). To exclude the possibility that eosinophils were binding to intracellular P-selectin we compared P-selectin expression and eosinophil binding to the endothelium of nasal polyps and normal skin. In acetone-fixed tissue the percentage of blood vessels that expressed P-selectin was similar in skin and nasal polyps. However eosinophils bound avidly to nasal polyp endothelium, whereas virtually no binding to skin blood vessels was observed, suggesting that in skin, P-selectin was intracellular and not available for interaction with eosinophils (Table 1). When paraformaldehyde, which does not permeabilize cell membranes, was substituted for acetone, no P-selectin expression was seen in skin (Fig. 4, *A* and *B*), whereas good expression was seen in nasal polyps with both fixatives (Fig. 4, *C* and *D*). This further suggests that endothelial P-selectin in skin is intracellular, whereas in nasal polyps it is also expressed on the cell surface. The pattern observed with immunofluorescence staining and confocal microscopy also supported this idea in that membrane staining could be clearly seen on nasal polyp endothelium (Fig. 4 *E*) whereas in the skin a more granular pattern was observed consistent with staining of intracellular P-selectin stored in Weibel-Palade bodies (Fig. 4 *F*).

There is good evidence that migration of granulocytes through endothelium is a staged process involving an initial, selectin-mediated stage, which occurs under flow conditions, followed by cell activation, which allows transmigration mediated by integrin/immunoglobulin family adhesion receptor interactions (21). The conditions of our assay, using unstimulated eosinophils from normal donors, at RT, and rotating the eosinophils over the frozen sections to create a degree of shear stress, favors a selectin-mediated interaction. This may explain why antibodies against ICAM-1 and the leukocyte integrins were largely ineffective despite these molecules playing a part in eosinophil migration through cultured HUVEC. We did see a small but significant inhibitory effect with a mAb against the α, but not the β chain of Mac-1. The reason for this discrepancy is not clear as both mAbs block Mac-1–dependent functions. Mac-1 can bind ICAM-1 through a site distinct from LFA-1 (22) and it is possible that the antibody we used does not block Mac-1/ICAM-1 interactions. Alternatively there may be another, as yet unidentified, ligand for Mac-1 expressed on nasal polyp endothelium. An antibody against VLA-4 was also ineffective presumably because of the lack of expression of its endothelial ligand VCAM-1, which does not appear to have a major role to play in established allergic inflammation.

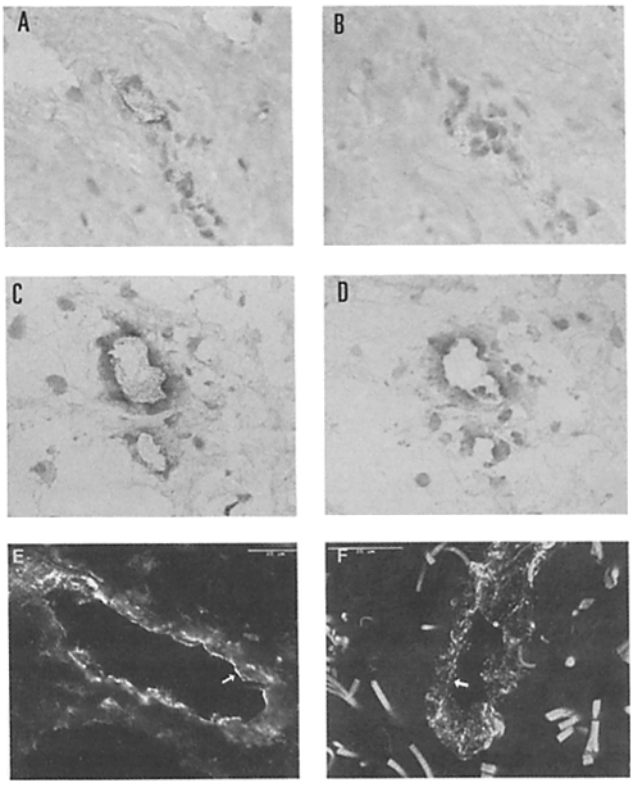

Figure 4. Cellular location of P-selectin in nasal polyp and skin endothelial cells. Immunohistochemical staining of tissue endothelium using the mAb RUU-SP1.18, on acetone fixed skin (*A*) and nasal polyp (*C*) and paraformaldehyde-fixed skin (*B*) and nasal polyp (*D*) tissue sections. Tissue sections were sequential so that the same blood vessels could be observed with each fixative. P-selectin staining was apparent on both acetone fixed nasal polyp and skin endothelium but only on nasal polyp blood vessels when sections were fixed using paraformaldehyde. Immunofluorescence staining of tissue endothelium for P-selectin and confocal microscopy, as indicated in Materials and Methods, showed lumenal staining of nasal polyp endothelium (*arrow*) (*E*) and granular staining of skin endothelium (*arrow*) (*F*).

Table 1. *Comparison of P-selectin Expression and Eosinophil Adherence to Skin and Nasal Polyp Tissue*

Tissue	Percent of total blood vessels that are P-selectin positive	Percent of total blood vessels that bound eosinophils
Skin	50.0 ± 4.82	1.80 ± 0.69
Nasal polyp	58.9 ± 4.39	25.1 ± 4.37

Results were from a single experiment on four consecutive sections and are representative of two other experiments using tissue from different donors.

In vitro studies have demonstrated that eosinophils can potentially use all three selectins; however, our study raises the possibility that in vivo P-selectin plays a dominant role in mediating the selectin stage of eosinophil adhesion. This would also suggest that in the case of eosinophils P-selectin is not a physiological ligand for L-selectin (23). Neutrophil adhesion both in vitro and in vivo has been shown to be inhibited by antibodies against all three selectins (24–27). In the case of E-selectin a blocking mAb inhibited the influx of neutrophils, but not eosinophils, into the airways of cyanomologous monkeys after challenge with ascaris antigen (28). This raises the exciting possibility that P-selectin antagonists may abolish eosinophil migration into tissue without

completely blocking neutrophil migration and therefore act as a relatively selective inhibitor of eosinophil migration.

In vitro evidence with cultured HUVEC has demonstrated that P-selectin is rapidly and transiently mobilized to the cell surface over the course of ~0.5 h (29). It has therefore been generally considered that P-selectin's major role is restricted to directing the initial influx of neutrophils during the acute inflammatory response. We were therefore concerned either that eosinophils were binding to intracellular P-selectin or that P-selectin was mobilized to the cell surface during the processing of the nasal polyps. The pattern of staining of P-selectin on nasal polyp endothelium compared with skin strongly suggested that, in normal skin P-selectin was intracellular, while in polyps it was expressed on the cell membrane. Moreover, P-selectin on nasal polyp endothelium was capable of supporting eosinophil adhesion, whereas P-selectin in skin did not, suggesting both that P-selectin is not mobilized to the cell surface during procedures such as polypectomy or skin removal (both equally traumatic and involving a general anaesthetic) and that intracellular P-selectin does not support eosinophil adhesion in this assay. It is possible that eosinophils adhered to nasal polyps but not skin because an additional ligand was involved which was not expressed in skin. We feel this unlikely as antibodies against the major known eosinophil adhesion receptors had no effect and eosinophils can bind readily to P-selectin–coated plates (14). Independent of our study, although using a similar strategy, Grober et al. recently reported that P-selectin was constitutively expressed on the membrane of the synovial vascular endothelium in rheumatoid arthritis and that monocyte adhesion to synovium had a large P-selectin component (30). P-selectin is expressed by platelets and a further concern was that platelets either in the tissue or adhering to eosinophils could be involved in some way. However our eosinophil separation technique avoids platelet contamination and virtually no platelets were seen in the nasal polyp section either by routine histology or after immunostaining using mAb 140-13 directed against platelet gpIIb/IIIa (data not shown).

Thus, in summary, using a modification of the Stamper-Woodruff FSA, we have demonstrated that unstimulated peripheral blood eosinophils adhered specifically to nasal polyp endothelium, primarily using the endothelial adhesion receptor P-selectin, binding to an as yet unidentified counter-receptor on eosinophils. This suggests that P-selectin, possibly induced by mast cell and basophil derived mediators such as histamine or Th2-derived cytokines, has an important role to play in directing eosinophil migration to sites of chronic eosinophilic inflammation and that P-selectin antagonists may be effective in the treatment of allergic disease.

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