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Adhesion to fibronectin primes eosinophils via $\alpha_4\beta_1$ (VLA-4)

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SUMMARY

Human peripheral blood eosinophils adhered specifically to microtitre plates coated with plasma fibronectin (Fn) in a dose- and time-dependent fashion. Adhesion was optimal at 60 min at a concentration of 100 μg/ml. Adherence to Fn was up-regulated by platelet-activating factor (PAF; optimum concentration of 10^{-6} M) and was significantly inhibited by a polyclonal anti-Fn antibody ($P < 0{\cdot}05$). The following evidence suggested that eosinophil adhesion to Fn was mediated by $\alpha_4\beta_1$: (1) eosinophil adherence to Fn was not inhibited by an Arg-Gly-Asp-Ser (RGDS) synthetic peptide; (2) there was a dose-dependent adherence of eosinophils to microtitre plates coated with the 40,000 MW proteolytic fragment of Fn that contains the CS-1 $\alpha_4\beta_1$ binding region, whereas adherence to the 120,000 MW chymotryptic fragment of Fn, which contains the RGD-dependent binding site, was weak and only observed at high concentrations (>250 μg/ml); (3) significant inhibition of eosinophil adherence to Fn was achieved by monoclonal antibodies (mAb) against the α chain of VLA-4 but not by a mAb against CD45 or a mouse myeloma antibody as negative controls. After adhesion to Fn, eosinophils were investigated for their capacity to release leukotriene C_4 in response to stimulation with a suboptimal concentration of calcium ionophore (2×10^{-6} M). Significant enhancement of release was detected with Fn-coated plates but not with the control bovine serum albumin (BSA) ($P < 0{\cdot}01$). Furthermore, this enhancement was significantly inhibited by the $\alpha_4\beta_1$ mAb HP2/1 ($P < 0{\cdot}05$) but not by an anti-CD45 mAb. From these studies we conclude that (1) $\alpha_4\beta_1$ (VLA-4) integrin is a major receptor for Fn on human eosinophils and (2) adhesion to Fn may prime eosinophils for mediator release during allergic inflammation.

INTRODUCTION

Human eosinophils are thought to be important proinflammatory cells in asthma and allergic inflammation.[1,2] The sequence of events that leads to the activation and degranulation of eosinophils at sites of inflammation is poorly understood. Following their migration from the circulation into tissues, eosinophils make contact with various extracellular matrix (ECM) proteins, including fibronectin (Fn). Interaction between eosinophils and matrix proteins may be important in modulating cellular localization and in enhancing eosinophil effector function in allergic and parasitic diseases.

The glycoprotein Fn is a major constituent of the extracellular matrix and is also present in plasma. It comprises several structural domains that contain binding sites for a number of ligands, including cell-surface receptors.[3] Fn has two cell binding regions. One region, contained within a 120,000 MW N terminal chymotrypsin fragment, mediates Arg-Gly-Asp (RGD)-dependent adhesion.[4,5] The second site, which was shown to mediate Fn adhesion to murine melanoma and avian neural crest cells,[6,7] is located in an alternatively spliced region of Fn termed the III CS region, which in turn is contained within a C terminal 40,000 MW heparin-binding fragment. The most active binding site of the III CS region was identified as the segment encompassed by the first 25 amino acid residues, i.e. CS-1 (also called V25).[6,8]

Most of the receptors that mediate adhesion to ECM belong to a family of adhesion molecules known as the integrins.[5,9,10] The integrins are a gene superfamily of $\alpha\beta$ heterodimeric, glycosylated transmembrane proteins non-covalently bound on the cell surface. They were initially divided into three subfamilies based on a common β chain combining with a number of α chains. Thus, the β_1 integrin family consisted of a single β chain (CD29) combining with 6α chains (CD49a–f, $\alpha_{1-6}\beta_1$) to form the very late activation (VLA) family.[11] The β_2 integrin family (leucocyte integrins: CD18/CD11a–c; LFA-1, Mac-1, p150, 95; $\alpha_L\beta_2$, $\alpha_M\beta_2$, $\alpha_X\beta_2$) is a family of three receptors whose expression is restricted to leucocytes.[12] The β_3 family constitutes the cytoadhesions

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(CD41/CD61, CD51/CD61; gpIIb/IIIa, vitronectin receptor; $\alpha_{IIb}\beta_3$, $\alpha_V\beta_3$). Since that original classification, it has become apparent that the association between α and β chains is not as restricted as once thought. In addition, a number of new α and β chains have been characterized, giving the integrin family another level of complexity.[10] Some integrin receptors bind several different matrix proteins via a common recognition motif Arg-Gly-Asp (RGD) found in a number of matrix proteins. The β_1 integrin (VLA) subfamily is widely expressed in both haemopoietic and non-haemopoietic cells. Within the members of the β_1 subfamily there are at least three receptors capable of binding to Fn, i.e. $\alpha_3\beta_1$, $\alpha_4\beta_1$ and $\alpha_5\beta_1$.[13–16] The $\alpha_5\beta_1$ binds to the RGD sequence.[15] While $\alpha_4\beta_1$ mediates cell adhesion to Fn by binding to the CS-1 segment,[13] $\alpha_4\beta_1$ is a receptor for vascular cell adhesion molecule-1 (VCAM-1) which is induced on endothelial cells in response to stimulation by inflammatory cytokines interleukin-1 (IL-1) and tumour necrosis factor-α (TNF-α).[17,18] Recently, Walsh *et al.*[19] and others[20–22] have demonstrated that human eosinophils constitutively express and utilize $\alpha_4\beta_1$ to bind to VCAM-1 expressed on IL-1-stimulated human umbilical vascular endothelial cells. These findings led us to investigate the possible interaction between human eosinophils and Fn. In this study peripheral blood eosinophils were tested for their ability to adhere to Fn-coated microtitre plates. We demonstrated that (1) eosinophils adhered specifically to Fn; (2) $\alpha_4\beta_1$ integrin was a major receptor mediating eosinophil adhesion to Fn; and (3) adherence to Fn primed eosinophils for enhanced release of leukotriene C_4 (LTC_4).

MATERIALS AND METHODS

Reagents

Reagents were obtained as follows. Bovine serum albumin (BSA) grade V, human plasma Fn and the synthetic peptides Arg-Gly-Asp-Ser (RGDS) and Arg-Phe-Asp-Ser (RPDS) were from Sigma Chemical Co. (Poole, U.K.). The Fn proteolytic fragments 40,000 and 120,000 MW were from Telios Pharmaceuticals Inc. (San Diego, CA). Dextran 110 was from Fluka Chemicals Ltd (Glossop, U.K.); metrizamide from Nyegaard Ltd (Birmingham, U.K.); and RPMI-1640 from Flow Laboratories (Rickmansworth, U.K.).

Antibodies

Monoclonal antibodies (mAb) HP2/1 and HP1/3 (anti-CD49d, VLA a4) were kind gifts from Dr F. Sanchez-Madrid (Hospital de La Princesa, Madrid, Spain). Monoclonal antibodies against CD45 were purchased from Becton Dickinson (U.K.) Ltd (Oxford, U.K.). All of these mAb were IgG and of murine origin. Control mouse myeloma proteins MOPC (IgG1) and UPC10 (IgG2a) were purchased from Sigma. Polyclonal rabbit anti-human Fn was purchased from Cooper Biomedical Inc. (West Chester, PA).

Leucocyte separation

Heparinized venous blood (10 U/ml) was obtained from donors with mild eosinophilia (5–13%) associated with allergic rhinitis or bronchial asthma. Some subjects were on intermittent inhaled β_2 agonists but no other medication. Eosinophils were isolated by dextran sedimentation and discontinuous metrizamide gradient centrifugation, as previously described.[23] In all experiments described here the eosinophil purity was >90% and >97% viability. For the experiments involving mononuclear cells these cells were obtained from the upper two layers of the metrizamide gradient.

Preparation of protein-coated microtitre plates

One-hundred microlitres of the appropriate concentration of human plasma Fn, Fn fragments (120,000 and 40,000 MW) or BSA was added to each well in 96-well flat-bottomed non-tissue culture-treated microtitre plates (Becton Dickinson & Co., Lincoln Park, NJ). After incubation for 1 hr at 37°, the plates were washed three times with phosphate-buffered saline (PBS) to remove the unbound protein. To block the non-specific protein-binding sites, 200 μl of 1% heat-denatured BSA in PBS was added to each well and the plates were incubated for 2 hr at room temperature. Before cells were added the plates were washed twice with RPMI/1% BSA.

Adherence assay

Purified eosinophils were labelled with ^{51}Cr (100 μCi/10^6 cells) for 1·5 hr at 37°, washed twice and adjusted to 1×10^6/ml in RPMI-1640/1% BSA containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (adhesion medium). One hundred microlitres of this cell suspension (1×10^5 cells) was then added in duplicate to the protein-coated wells and the plates were incubated for 1 hr at 37° in a 5% CO_2 humidified incubator. The non-adherent cells were then detached by gentle washing and the adherent cells were lysed by 1% Triton X-100. The cell lysates were counted in a γ-counter and the percentage of adherent cells was calculated as follows:

$$\% \text{ adherence} = \frac{^{51}\text{Cr c.p.m. test well}}{^{51}\text{Cr c.p.m. total cells added}} \times 100.$$

In the platelet-activating factor (PAF) stimulation experiments, eosinophils were preincubated with PAF for 15 min before they were added, without washing, to Fn-coated wells (50 μg/ml) for 1 hr.

For inhibition of eosinophil adherence, ^{51}Cr-labelled cells were incubated in a rotary mixer with various concentrations of the synthetic peptides RGDS or RPDS at room temperature for 45 min, washed once and adjusted to 1×10^6/ml in the adhesion medium before they were added to duplicate wells and the adhesion assay performed as above.

For mAb inhibition of eosinophil adherence, labelled cells were preincubated with the appropriate mAb (or the isotype irrelevant control) for 30 min at 4° before they were added to the coated wells without washing.

For inhibition of adherence by the anti-Fn antibody, the Fn-coated wells were incubated with 1:20 dilution of the antibody (or the control normal rabbit serum) for 1 hr at room temperature. The wells were then washed twice with the RPMI-1640/1% BSA before the cells were added to the wells and the adhesion assay was continued as described above.

LTC_4 release

Preliminary experiments established an optimum cell concentration of 3×10^5 cells/well which was used in all subsequent experiments. The cells were added to the Fn (or control BSA)-coated wells and allowed to adhere for 1 hr at 37°. No cells were washed out of either the BSA- or Fn-coated wells at any stage

of the experiment, so that all the cells added were exposed to the calcium ionophore A23187 (Sigma), which was added to a final concentration of 2×10^{-6} M, and the plates were reincubated for 15 min at 37°. The plates were then centrifuged at 250 *g* for 10 min at 4° and cell-free supernatants collected from each well and stored at −80° until they were assayed.

LTC_4 assay

LTC_4 in the supernatants was measured in duplicate with a double antibody radioimmunoassay using a rabbit anti-LTC_4, as described previously.[24,25]

Statistical analysis

The results of all experiments described here are expressed as mean ± SEM and significance was assessed by Students' *t*-test.

RESULTS

Dose–response and kinetics of eosinophil adhesion to Fn

Eosinophil adhesion to Fn increased in a concentration- and time-dependent manner. Adhesion appeared to plateau at a concentration of 100 μg/ml (Fig. 1a), with an optimal time of 1 hr (which was used in subsequent experiments) (Fig. 1b). There was background adhesion of about 13% to BSA-coated wells which was independent of time or BSA concentration.

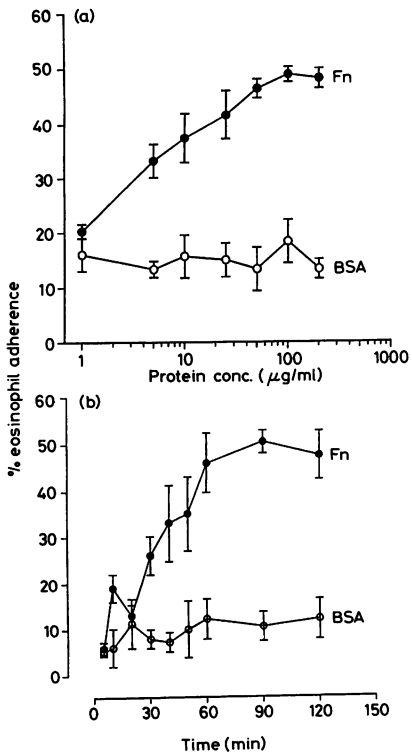

Figure 1. (a) The effect of increasing protein (Fn or BSA) concentration on the adhesion of human eosinophils. Eosinophils were added to protein-coated wells and allowed to adhere for 1 hr before being removed by gentle washing. The points represent the mean ± SEM ($n = 5$). (b) Time–course of eosinophil adhesion to Fn. Eosinophils were allowed to adhere to Fn (100 μg/ml) or BSA (1%)-coated wells for a variable time before non-adherent cells were removed by gentle washing. Each point represents the mean ± SEM ($n = 4$).

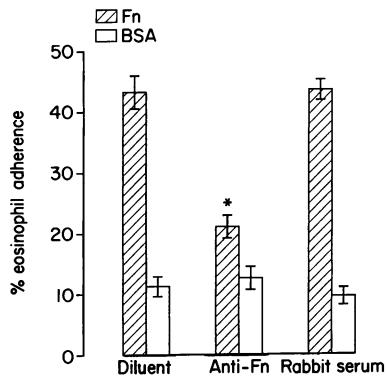

Figure 2. The inhibition of eosinophil adhesion to Fn by rabbit anti-human Fn. Fn- or BSA-coated wells (100 μg/ml–1%) were incubated with the polyclonal antiserum or normal rabbit serum as described in the Materials and Methods and eosinophils were then allowed to adhere for 1 hr. The anti-Fn antiserum significantly inhibited eosinophil adhesion to Fn. The results represent the mean ± SEM ($n = 5$). (*$P < 0.05$).

The effect of Fn antibody on adhesion

To establish the specificity of the adherence reaction between human eosinophils and Fn, we incubated Fn-coated plates with a polyclonal rabbit anti-human Fn antibody before the cells were added. This antibody resulted in a significant ($P < 0.05$) inhibition of eosinophil adherence which was not observed with a control normal rabbit serum (Fig. 2).

Up-regulation of adhesion to Fn by PAF

Adhesion of eosinophils to Fn was significantly enhanced by short-term priming with PAF, which was optimal at a concentration of 10^{-6} M (Fig. 3).

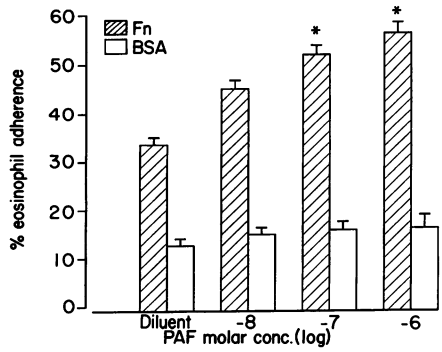

Figure 3. The effect of PAF (10^{-8}–10^{-6} M) on eosinophil adhesion to Fn. Eosinophils were preincubated with PAF for 15 min and then added without washing to Fn-(50 μg/ml) or BSA-coated wells for 1 hr. PAF significantly enhanced eosinophil adhesion to Fn at 10^{-6} and 10^{-7} M. The results represent the mean ± SEM ($n = 5$). (*$P < 0.05$).

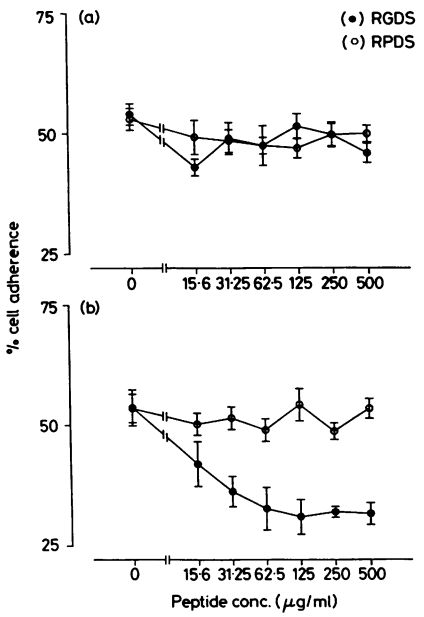

Figure 4. The effect of the synthetic peptides RGDS and RPDS on the adhesion of human eosinophils (a) and mononuclear cells (b) to Fn. Cells were preincubated with various concentrations of the oligopeptides as described in the Materials and Methods and then added to Fn (100 μg/ml)-coated wells for 1 hr. The points represent the mean ± SEM ($n = 4$).

The role of RGDS and RPDS synthetic peptides and the Fn proteolytic fragments

Eosinophils and mononuclear cells were preincubated with increasing concentrations of the synthetic peptides RGDS or RPDS as a control. The cells were then allowed to adhere to Fn-coated plates. There was no inhibition of eosinophil adherence to Fn after preincubation with either of the two synthetic peptides (Fig. 4a). However, there was a dose-dependent inhibition of mononuclear cell adhesion to Fn by the RGDS peptide but not with the control peptide RPDS (Fig. 4b). This suggested that eosinophil adhesion to Fn was mediated through a non-RGD-dependent mechanism. To study this further, adhesion of eosinophils to two Fn proteolytic fragments (i.e. the 40,000 MW fragment which contains the CS-1 $\alpha_4\beta_1$-binding site and the 120,000 MW fragment which contains the RGD sequence) was investigated. Eosinophils added to increasing concentrations of the 40,000 MW fragment coated on microtitre plates exhibited a dose-dependent adherence that was similar to that observed with intact Fn (Fig. 5). In contrast, adhesion of eosinophils to the 120,000 MW fragment was weak and only became significantly higher than the BSA control when relatively high concentrations (250 and 500 μg/ml) of the Fn 120,000 MW fragment were used.

The role of the $\alpha_4\beta_1$ (VLA-4) integrin in adhesion to Fn

To investigate the possible role of $\alpha_4\beta_1$ in mediating eosinophil adhesion to Fn, two anti-$\alpha_4\beta_1$ mAb, HP2/1 and HP1/3, were tested for their ability to block eosinophil adhesion. HP2/1 significantly inhibited eosinophil adhesion when compared to

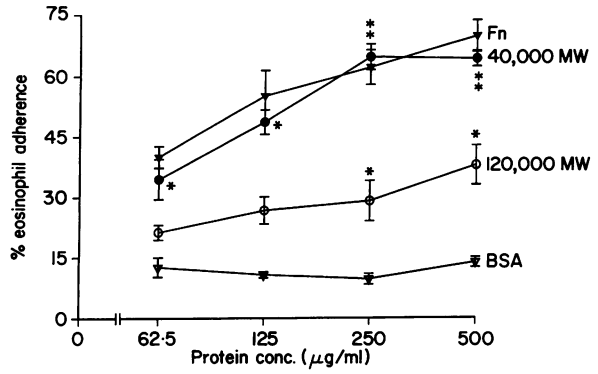

Figure 5. The adhesion of human eosinophils to increasing concentrations of Fn and the 40,000 and 120,000 MW Fn proteolytic fragments. Eosinophils adhered to various concentrations of protein for 1 hr. The points represent the mean ± SEM ($n = 3$). (*$P < 0.05$; **$P < 0.01$).

the control mouse IgG1, IgG2a and the anti-CD45 mAb (Fig. 6). In contrast, the second anti-$\alpha_4\beta_1$ mAb (HP1/3) had no inhibitory effect.

LTC_4 release by eosinophils following Fn adhesion

To investigate the effect of adhesion to Fn on eosinophil function we determined the generation of LTC_4 by eosinophils adherent to Fn or BSA after culture in medium or calcium ionophore for 1 hr. There was a small, non-significant increase in LTC_4 release by eosinophils adhering to Fn compared with BSA in the absence of calcium ionophore (Fig. 7). After stimulation with calcium ionophore there was an approximately twofold increase in the secretion of LTC_4 from eosinophils adhering to Fn-coated wells as opposed to BSA-coated wells ($P < 0.01$). This increase in LTC_4 release in the presence of Fn was significantly inhibited by an anti-$\alpha_4\beta_1$ mAb (HP2/1) ($P < 0.05$), but not by a mAb against CD45. The difference in LTC_4 release between cells treated with anti-CD45 and anti-VLA-4 was also significant ($P < 0.05$).

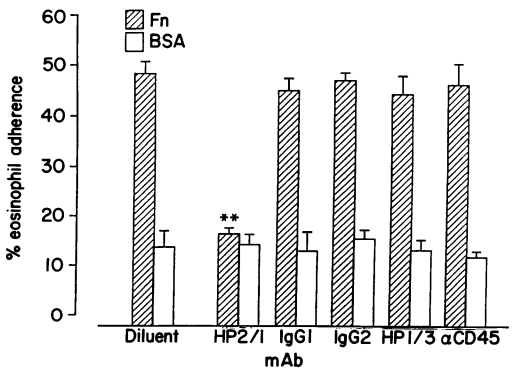

Figure 6. Inhibition of eosinophil adhesion to Fn by anti-$\alpha_4\beta_1$ mAb. Eosinophils were preincubated with various mAb as described in the Materials and Methods before being allowed to adhere to Fn (100 μg/ml) and BSA-coated wells for 1 hr. Significant inhibition was observed with one mAb against the α chain of $\alpha_4\beta_1$ (HP2/1) but not with a second mAb against $\alpha_4\beta_1$ (HP1/3) or a mAb against CD45 or isotype control mouse antibodies. The figures represent the mean ± SEM ($n = 5$). (**$P < 0.01$).

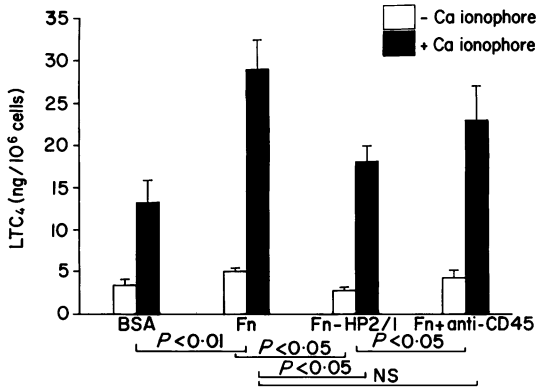

Figure 7. The release of LTC_4 by human eosinophils after adhesion to Fn (100 μg/ml). 3×10^5 cells were allowed to adhere to Fn (or BSA)-coated plates for 1 hr at 37°, in some cases after preincubation with mAb as indicated, before calcium ionophore A23187 (2×10^{-6} M) was added. No cells were removed from either the BSA- or Fn-coated wells throughout the experiment. The cells were then reincubated for 15 min and the plates were centrifuged at 250 *g* and the cell-free supernatants were collected for LTC_4 measurements. Results were expressed as the amount of LTC_4 released/10^6 cells. The significant enhancement of LTC_4 release by cells in contact with Fn compared with BSA was inhibited by a mAb against $\alpha_4\beta_1$ (HP2/1). The figures represent the mean ± SEM ($n = 5$).

DISCUSSION

We have demonstrated that unstimulated peripheral blood human eosinophils adhere specifically to Fn-coated microtitre plates. Adhesion was mediated by the cell-surface integrin $\alpha_4\beta_1$ (VLA-4), presumably recognizing the CS-1 binding site of Fn. The suggestion that $\alpha_4\beta_1$ is a major receptor for Fn on eosinophils is supported by several lines of evidence. Firstly, unlike mononuclear cells, eosinophil adhesion was not inhibited by the synthetic peptide RGDS; secondly, eosinophils showed significant adhesion to the 40,000 MW Fn fragment, containing the CS-1 region, but only very weak adhesion to the 120,000 MW fragment (containing the RGD sequence); thirdly, eosinophil adhesion to Fn was significantly blocked by a mAb against the α chain of $\alpha_4\beta_1$. This suggests that eosinophils do not express other Fn receptors, in particular $\alpha_5\beta_1$. However, $\alpha_5\beta_1$, or other Fn receptors, might be expressed but remain inactive unless the eosinophil is stimulated. The C terminal end of fibronectin contains up to three binding sites for VLA-4: a region in the heparin II domain which is not yet fully characterized and two parts of the alternatively spliced 111CS region termed CS-1 and CS-5. The minimal recognition sequence in CS-1 is the tripeptide LDV. The ability of leucocytes to recognize the LDV region depends on the activation state of VLA-4 on the cell in question.[26] The interaction between VLA-4 and fibronectin is therefore extremely complex. Studies are currently underway in our laboratory to examine the exact binding sites in the C terminal region of fibronectin important for eosinophil adhesion and mediator release.

Two mAb (HP2/1 and HP1/3) were used which recognize the α chain of $\alpha_4\beta_1$. Inhibition of adhesion was observed only with HP2/1. This is consistent with the observation that HP1/3 only inhibits $\alpha_4\beta_1$-dependent adhesion to VCAM-1 and not to fibronectin, whereas HP1/2 inhibits adhesion to both ligands.[27]

Eosinophil adhesion to fibronectin was observed at concentrations as low as 5 μg/ml. These increased in a dose-dependent manner up to 100 μg/ml, where the effect plateaued. However, in subsequent experiments using the chymotryptic fragments a greater degree of adhesion was observed at concentrations up to 500 μg/ml. The kinetic studies demonstrated increases in adherent eosinophils, peaking at 1 hr. This time-course is slower than that observed for eosinophil adhesion to human umbilical vein endothelial cells (HUVEC), where adhesion is optimal within 30 min. Stimulation of the eosinophils by PAF resulted in enhanced adhesion. In addition, adhesion to Fn itself primed the eosinophils for enhanced LTC_4 release. Therefore, one explanation for the kinetics of adhesion to Fn is that as the cells adhere they become primed and this further increases their capacity to bind to Fn.

The observation that PAF enhanced eosinophil adhesion to Fn is in agreement with previous reports on leucocyte adhesion to matrix proteins. Neutrophils showed a marked up-regulation of adhesion to both laminin and Fn after treatment with PMA, calcium ionophore and *N*-formyl-methonyl-leucyl-phenylalanine (FMLP), respectively.[28,29] Also adhesion of natural killer (NK) cells and T lymphocytes to Fn could be rapidly up-regulated by the use of phorbol esters.[30,31] In the present study it is unlikely that PAF-induced enhancement of adhesion was due to the expression of new Fn receptors on the eosinophil surface membrane, since $\alpha_4\beta_1$ is constitutively expressed by eosinophils and its expression cannot be enhanced by PAF.[19] PAF stimulation may promote a conformational change in $\alpha_4\beta_1$ resulting in increased affinity for Fn, or alternatively lead to clustering of receptors on the cell membrane and thus to higher affinity binding.

In this study we used eosinophils from subjects with mild allergic disease who had higher than normal blood eosinophil counts. There is evidence that eosinophils from such individuals have greater chemotactic responsiveness to mediators such as PAF and LTB_4 compared with eosinophils from subjects with a normal eosinophil count, possibly as a result of *in vivo* priming by cytokines.[32] $\alpha_4\beta_1$ is constitutively expressed by eosinophils and can mediate adhesion to VCAM-1 without the need for cell activation. As discussed above, expression of $\alpha_4\beta_1$ by eosinophils is not affected by either *in vivo* or *in vitro* cell activation. It is therefore unlikely that there are major differences between eosinophils from mildly eosinophilic donors and eosinophils from normal donors in their interaction with Fn.

There is increasing evidence that integrins can act as signalling receptors which, when engaged by ligand, lead to cell activation and triggering of mediator release.[10] Adhesion to various matrix protein surfaces, including Fn, has previously been reported to enhance superoxide production by eosinophils stimulated with FMLP.[33] We have recently demonstrated that peripheral blood eosinophils when cultured on fibronectin-coated wells have increased survival compared with eosinophils cultured on plastic or BSA-coated wells. This appeared to be due to autocrine generation of granulocyte–macrophage colony-stimulating factor (GM-CSF) by eosinophils as a result of triggering through VLA-4.[34] In the present paper we have extended that work to show that eosinophils are also primed for the release of lipid mediators by short-term adherence to fibronectin. There was a slight increase in LTC_4 generation from eosinophils adherent to Fn compared to BSA,

with a significant inhibition of direct LTC_4 release by anti-$\alpha_4\beta_1$ mAb, but the amounts detected were very low and of doubtful biological significance. However, after stimulation with calcium ionophore eosinophils in contact with Fn had a twofold greater release of LTC_4 than cells in contact with BSA. No cells had been removed from any of the wells; therefore, the same number of eosinophils was present in both the Fn wells and the BSA wells. If we had washed out the non-adherent cells from the wells we would have been left with unequal numbers of cells in the BSA and Fn wells which would have made the data impossible to interpret. However, in this context it is inappropriate to consider the eosinophils as either adherent or non-adherent. The distinction between adherent cells and non-adherent cells in an *in vitro* adherence assay is in a sense artificial as the number of adherent cells will depend on the vigour of the washing procedure, the conditions of the system and affinity of the interaction. The eosinophils in the LTC_4 release experiments formed a monolayer in the well and were therefore all adherent to the BSA or Fn and all therefore able to interact with the protein with greater or less avidity. Our data demonstrate that those cells in contact with Fn produced more LTC_4 than the same number of cells in contact with BSA. It is likely that there was a continuum in the avidity of binding between the eosinophils and the Fn, with some eosinophils binding very tightly and some loosely. It is also likely that the degree of enhancement of LTC_4 release was related to the avidity of binding but we have not addressed this question in these experiments. Inhibition of enhanced release by HP2/1 suggested that this effect was dependent on interaction between $\alpha_4\beta_1$ and Fn. Whether the enhanced release was due to alterations in the cytoskeletal framework of the eosinophil as a result of the integrin-mediated adhesion process or whether perturbation of $\alpha_4\beta_1$ resulted in priming through activation of secondary intracellular signalling pathways remains to be determined. The mAb against CD45 did result in some inhibition of LTC_4 release. However, this was inconsistent and non-significant. Anti-CD45 was used as a control antibody as CD45 is not thought to be an adhesion receptor, but is well expressed by eosinophils.

In conclusion, the results of the present study indicate that the adhesion of human eosinophils to Fn through the $\alpha_4\beta_1$ integrin/CS-1 pathway may represent an important mechanism in selective localization of these cells at sites of allergic inflammation. Furthermore, the enhancement of LTC_4 release by eosinophils following adhesion to Fn may suggest that the interaction between these cells and extracellular matrix proteins may lead to the up-regulation of their proinflammatory effector potential at the sites of allergic inflammation.

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REFERENCES

1. Gleich G.J. (1990) The eosinophil and bronchial asthma: current understanding. *J. Allergy clin. Immunol.* **85,** 422.
2. Wardlaw A.J. & Kay A.B. (1987) Role of eosinophils in the pathogenesis of asthma. *Allergy*, **42,** 321.
3. Yamada K.M. (1989) Fibronectin domains and receptors. In: *Fibronectin* (ed. D. F. Mosher), p. 47. Academic Press, New York.
4. Pierschbacher M.D. & Ruoslahti E. (1984) Cell attachment activity of fibronectin can be duplicated small synthetic fragments of the molecule. *Nature*, **309,** 30.
5. Ruoslahti E. & Pierschbacher M.D. (1986) Arg-Gly-Asp: a versatile cell recognition signal. *Cell*, **44,** 517.
6. Humphries M.J., Komoriya A., Akiyama S.K., Olden K. & Yamada K.M. (1987) Identification of two distinct regions of the type III connecting segment of human plasma fibronectin that promote cell type-specific adhesion. *J. biol. Chem.* **262,** 6886.
7. Dufour S., Duband J.L., Humphries M.J., Obara M., Yamada K.M. & Thiery J.P. (1988) Attachment, spreading and locomotion of avian neural crest cells are mediated by multiple adhesion sites on fibronectin molecules. *EMBO J.* **7,** 2661.
8. Humphries M.J., Akiyama S.K., Komiriya A., Olden K. & Yamada K.M. (1986) Identification of an alternatively spliced site in human plasma fibronectin that mediates cell type-specific adhesion. *J. Cell Biol.* **103,** 2627.
9. Hynes R.O. (1987) Integrins: a family of cell surface receptors. *Cell*, **48,** 549.
10. Hynes R.O. (1992) Integrins: versatility, modulation and signaling in cell activation. *Cell*, **69,** 11.
11. Hemler M.E. (1990) VLA proteins in the integrin family: structures, functions and their role on leukocytes. *Annu. Rev. Immunol.* **8,** 365.
12. Larson R.S. & Springer T.A. (1990) Structure and function of leucocyte integrins. *Immunol. Rev.* **114,** 181.
13. Wayner E.A., Garcia-Pardo A., Humphries M.J., McDonald J.A. & Carter W.G. (1989) Identification and characterization of the lymphocyte adhesion receptor for an alternative cell attachment domain (CS-1) in plasma fibronectin. *J. Cell Biol.* **109,** 1321.
14. Mould A.P., Wheldon L.A., Komoriya A., Wayner E.A., Yamada K.M. & Humphries M.J. (1990) Affinity chromatographic isolation of the melanoma adhesion receptor for the III CS region of fibronectin and its identification as the integrin $\alpha1/\beta1$. *J. biol. Chem.* **265,** 4020.
15. Takada Y., Huang C. & Hemler M.E. (1987) Fibronectin receptor structures in the VLA family of heterodimers. *Nature*, **326,** 607.
16. Takada Y., Wayner E.A., Carter W.G. & Hemler M.E. (1988) Extracellular matrix receptors, ECMRII and ECMRI, for collagen and fibronectin correspond to VLA-2 and VLA-3 in the VLA family of heterodimers. *J. Cell Biochem.* **37,** 385.
17. Elices M.J., Osborn L., Takada Y., Crouse C., Luhowskyj S., Hemler M.E. & Lobb R.R. (1990) VCAM-1 on activated endothelium interacts with the leucocyte integrin VLA-4 at a binding site distinct from the VLA-4/fibronectin binding site. *Cell*, **60,** 577.
18. Schwartz B.R., Wayner E.A., Carlos T.M., Ochs H.D. & Harlan J.M. (1990) Identification of surface proteins mediating adherence of CD11/18-deficient lymphoblastoid cells to cultured endothelium. *J. clin. Invest.* **85,** 2019.
19. Walsh G.M., Mermod J., Hartnell A., Kay A.B. & Wardlaw A.J. (1991) Human eosinophil, but not neutrophil, adherence to IL-1 stimulated human umbilical vascular endothelial cells is $\alpha_4\beta_1$ (very late antigen-4) dependent. *J. Immunol.* **146,** 3419.
20. Bochner B.S., Luscinskas F.W., Gimbrone M.A., Newman W., Sterbinsky S.A., Derse-Anthony C., Klung D. & Schleimer R.P. (1991) Adhesion of human basophils, eosinophils, and neutrophils to IL-1-activated human vascular endothelial cells: contributions of endothelial cell molecules. *J. exp. Med.* **173,** 1553.
21. Weller P.F., Rand T.H., Goelz S.E., Chi-Rosso G. & Lobb R.R. (1991) Human eosinophil adherence to vascular endothelium

mediated by binding to vascular cell adhesion molecule 1 and endothelial leukocyte adhesion molecule 1. *Proc. natl. Acad. Sci. U.S.A.* **88,** 7430.

22. Dobrina A., Menegazzi R., Carlos T.M., Nardon E., Cramer R., Zacchi T., Harlan J.M. & Patriarca P. (1991) Mechanisms of eosinophil adherence to cultured vascular endothelial cells: eosinophils bind to the cytokine-induced endothelial ligand vascular cell adhesion molecule-1 via the very late activation antigen-4 integrin receptor. *J. clin. Invest.* **88,** 20.
23. Vadas M.A., David J.R., Butterworth A.E., Pisani N.T. & Siongok T.A. (1979) A new method for the purification of human eosinophils and neutrophils and a comparison of the abiilty of these cells to damage schistosomula of *Schistosoma mansoni*. *J. Immunol.* **122,** 1228.
24. Hayes E.C., Lombardo D.L., Girard Y., Maycock A.L., Rokach J., Rosenthal A.S., Young R.N., Egan R.W. & Zweerink H.J. (1983) Measuring leukotrienes of slow reacting substance of anaphylaxis: development of a specific radioimmunoassay. *J. Immunol.* **131,** 429.
25. Cromwell O., Shaw R.J., Walsh G.M., Mallet A.I. & Kay A.B. (1985) Inhibition of leukotriene C_4 and B_4 generation by human eosinophils and neutrophils with lipoxygenase pathway inhibitors U-60, 257 and BW755C. *Int. J. Immunopharmacol.* **7,** 775.
26. Wayner E.A. & Kovach N.L. (1992) Activation-dependent recognition by haemopoetic cells of the LDV sequence in the V region of fibronectin. *J. Cell Biol.* **16,** 489.
27. Elices M.J. & Hemler M.E. (1989) The human integrin VLA-2 is a collagen receptor on some cells and a collagen/laminin receptor on others. *Proc. natl. Acad. Sci. U.S.A.* **89,** 9906.
28. Bohnsack J.F., Akiyama S.K., Damsky C.H., Knape W.A. & Zimmerman G.A. (1990) Human neutrophil adherence to laminin *in vitro*: evidence for a distinct neutrophil integrin receptor for laminin. *J. exp. Med.* **171,** 1221.
29. Suchard S.J., Burton M.J., Dixit V.M. & Boxer L.A. (1991) Human neutrophil adherence to thrombospondin occurs through a CD11/CD18-dependent mechanism. *J. Immunol.* **146,** 3945.
30. Gismondi A., Morrone S., Humphries M.J., Piccoli M., Frati L. & Santonia A. (1991) Human natural killer cells express VLA-4 and VLA-5, which mediate their adhesion to fibronectin. *J. Immunol.* **146,** 384.
31. Shimizu Y., Van Seventer G.A., Horgan K.J. & Shaw S. (1990) Regulated expression and binding of three VLA (β1) integrin receptors on T cells. *Nature*, **345,** 250.
32. Sehmi R., Wardlaw A.J., Cromwell O., Kurihara K., Waltman P. & Kay A.B. (1992) Interleukin-5 selectively enhances the chemotactic response of eosinophils obtained from normal but not eosinophilic subjects. *Blood*, **79,** 2952.
33. Dri P., Cramer R., Spessotto P., Romano M. & Patriarca P. (1990) Eosinophil activation on biological surfaces: Production of O_2^- in response to physiologic soluble stimuli is differentially modulated by extracellular matrix components and endothelial cells. *J. Immunol.* **147,** 613.
34. Anwar A.R.E., Moqbel R., Walsh G.M., Kay A.B. & Wardlaw A.J. (1993) Adhesion to fibronectin prolongs eosinophil survival. *J. exp. Med.* **177,** 839.