

Regulation of α_4 integrin-mediated adhesion of human eosinophils to fibronectin and vascular cell adhesion molecule-1

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Background: Eosinophils selectively accumulate at sites of allergic inflammation. Their recruitment is dependent on both the expression and functional activity of cell adhesion molecules. How the functional activity of cell adhesion molecules on eosinophils is regulated is poorly understood.

Objective: Our objective was to examine the functional activity of α_4 integrins on human eosinophils and its regulation by various agents.

Methods: Function of α_4 integrins on human eosinophils was examined by testing adhesion to immobilized fibronectin and vascular cell adhesion molecule-1 (VCAM-1) in the presence or absence of a monoclonal antibody (mAb) (8A2) that activates β_1 integrin function.

Results: Spontaneous eosinophil adhesion to VCAM-1 was enhanced by 8A2, but adhesion to fibronectin could only be detected in the presence of 8A2. Concentrations of 8A2 that were approximately 100-fold less than saturating induced maximal eosinophil adhesion. Adhesion to VCAM-1 in the presence of 8A2 was effectively inhibited by α_4 and β_1 integrin mAbs; β_7 mAb had partial inhibitory activity. Connecting segment-1 peptide and α_4 mAb blocked 8A2-dependent fibronectin binding; β_1 , β_2 , and β_7 integrin mAbs had partial inhibitory activity. Eosinophils obtained from bronchoalveolar lavage fluids and blood eosinophils stimulated with IL-5, platelet-activating factor, or RANTES displayed increased β_2 integrin-dependent, not α_4 integrin-dependent, attachment. Spontaneous adhesion of eosinophils to VCAM-1 was significantly reduced by the tyrosine kinase inhibitor tyrphostin B46 (inhibitory concentration of 50% $\approx$ 20 μ mol/L); this effect was reversed by 8A2.

Conclusions: The functional activity of integrins on eosinophils can be positively and negatively regulated. Altered integrin avidity may influence eosinophil recruitment in vivo. (J Allergy Clin Immunol 1997;99:648-56.)

Key words: Eosinophil, adhesion, integrin, fibronectin, vascular cell adhesion molecule-1, integrin function

Abbreviations used

BAL:	Bronchoalveolar lavage
BSA:	Bovine serum albumin
CS-1:	Connecting segment-1
IC ₅₀ :	Inhibitory concentration of 50%
mAb:	Monoclonal antibody
MFI:	Mean fluorescence intensity
PAF:	Platelet-activating factor
PBS:	Phosphate-buffered saline
sVCAM:	Soluble vascular cell adhesion molecule-1
VCAM-1:	Vascular cell adhesion molecule-1

Leukocytes express a wide variety of integrins that allow them to interact with other cells and with extracellular matrix proteins.¹⁻³ Although some of these adhesion molecules mediate cell trafficking, many can also modulate cellular functions, including induction of proliferation and changes in morphologic features, which are events referred to as outside-in signaling.³ In particular, eosinophils express $\alpha_4\beta_1$ integrins that are capable of binding to vascular cell adhesion molecule-1 (VCAM-1),⁴⁻⁹ and attachment to VCAM-1 has been shown to affect eosinophil function.¹⁰ VCAM-1 is selectively expressed on endothelial cells after treatment with IL-4 or IL-13¹¹⁻¹³ and is thought to be a crucial adhesion pathway for the specific accumulation of eosinophils and lymphocytes, but not neutrophils, at allergic inflammatory sites, because neutrophils do not express $\alpha_4\beta_1$ integrin.¹⁴

The α_4 integrins have also been shown to bind to the alternatively spliced connecting segment-1 (CS-1) region of fibronectin containing the recognition sequence EILDV, although the affinity of binding is lower compared with that for VCAM-1.¹⁵⁻¹⁷ Despite expression of α_4 integrins on eosinophils, it remains a matter of controversy whether they spontaneously attach to fibronectin. Some studies have found augmentation in eosinophil function after incubation on fibronectin,¹⁸⁻²¹ whereas other studies failed to detect effects on eosinophils or even their attachment.²²⁻²⁴ A confounding issue in these studies is the possibility that eosinophil CD11b engagement is occurring²⁵; this may be responsible for the observed effects on eosinophil function.²⁶

A possible explanation for these inconsistencies may be provided by results of recent studies that suggest that

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the activation state of integrins can dramatically influence cell adhesion and function.^{2, 27-29} For example, ligand binding and certain integrin monoclonal antibodies (mAbs) enhance adhesion, presumably by changing the configuration of the heterodimer,^{30, 31} whereas inhibitors of signal transduction pathways, such as those involving tyrosine kinases, prevent cell adhesion.³² Regarding activation of adhesion in eosinophils, one such antibody, mAb 8A2, has been used to demonstrate enhancement of attachment to matrix proteins; adhesion to VCAM-1 was not tested.²³ In this study, however, blocking mAbs for either α_4 or α_5 integrin were found to inhibit attachment to fibronectin; yet subsequent studies failed to detect α_5 integrins on eosinophils,^{33, 34} raising the possibility of platelet contamination of the eosinophil preparations that can occur.³⁵ In an attempt to clarify the role of β_1 integrin avidity in eosinophil adhesion, additional studies were done to provide a more detailed analysis of α_4 integrin functional activity on eosinophils. Several mechanisms by which the function of α_4 integrins can be regulated on human eosinophils are described.

METHODS

Reagents

Human serum fibronectin was purchased from the New York Blood Center (New York, N.Y.) and kindly provided by Dr. Shaker Mousa (Dupont-Merck Pharmaceuticals, Wilmington, Del.). The CS-1 portion of fibronectin was synthesized and also generously provided by Dr. Mousa. Human soluble recombinant VCAM-1 (sVCAM) was generated as previously described.¹² Bovine serum albumin (BSA), platelet-activating factor (PAF), and phorbol 12-myristate 13-acetate were purchased from Sigma Chemical Co. (St. Louis, Mo.). Human RANTES was provided as a gift (Dr. Tom Schall, DNAX, Palo Alto, Calif.). Human recombinant IL-5 was purchased from R & D Systems (Minneapolis, Minn.). Fragments of fibronectin containing the RGD domain were purchased from Gibco BRL (Grand Island, N.Y.).

mAbs

The β_1 integrin activating mAb 8A2 (mouse IgG1) was generated as previously described.³⁰ The following murine IgG1 mAbs were generously provided: CD18 blocking mAb H52³⁶ (Dr. James Hildreth, Johns Hopkins University School of Medicine); β_1 integrin blocking mAb 33B6³³ (Drs. John Bednarczyk and Bradley McIntyre, University of Texas, Houston); and β_1 integrin activation epitope-detecting mAb 15/7³⁷ (Dr. Ted Yednock, Athena Neurosciences, So. San Francisco, Calif.). Blocking mouse IgG1 mAb recognizing α_4 (HP2/1), α_v (AMF7), and β_3 (SZ.21) integrins (the latter two expressed on platelets but not eosinophils³⁸) were purchased from Immunotech (Westbrook, Maine), a blocking mouse IgG1 mAb recognizing α_5 integrin (PID6) was purchased from Gibco; and an irrelevant mouse IgG1 control antibody was purchased from Coulter Corporation (Hialeah, Fla.). A rat mAb reacting with human β_1 integrin (Fib504) was provided by Dr. Charles Mackay (Leukosite, Inc., Boston, Mass.).

Isolation of human eosinophils

Eosinophils were purified from peripheral blood of donors with allergies by using density gradient centrifugation and

negative selection with immunomagnetic beads as previously described.³⁹ Purity and viability were determined by Diff-Quik staining (American Scientific Products, McGraw Park, Ill.) of cytocentrifuge preparations (Shandon, Pittsburgh, Pa.) and by erythrocin B dye exclusion (Sigma) and were consistently greater than 97% and 99%, respectively ($n = 20$). For some experiments, eosinophils were purified from bronchoalveolar lavage (BAL) fluid obtained 19 hours after segmental challenge of subjects with allergic asthma with allergen⁴⁰ with use of the same purification procedures just described. Purity and viability were 100% and 99%, respectively ($n = 3$).

Culture of Jurkat cells

The Jurkat T lymphocytic cell line, a generous gift of Dr. Vincenzo Casolaro (Johns Hopkins Asthma and Allergy Center), was passaged every 3 to 5 days in RPMI 1640 medium (Gibco BRL) supplemented with 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, Utah), 100 U/ml penicillin G, 100 μ g/ml streptomycin, and 0.25 μ g/ml amphotericin B (Gibco BRL).

Flow cytometric analysis of cell surface integrin expression

Expression of integrins on eosinophils or Jurkat cells was examined by indirect immunofluorescence and flow cytometry as previously described.³³ Briefly, freshly isolated eosinophils or Jurkat cells were incubated (30 minutes, 4° C) in Dulbecco's phosphate-buffered saline (PBS) solution containing 0.1% BSA (Sigma) and 4 mg/ml human IgG (Sigma) with saturating concentrations of mAb or an equivalent concentration of irrelevant IgG1 control mAb. Cells were washed and then incubated (30 minutes at 4° C in PBS containing 0.1% BSA) with 1:20 to 1:50 dilutions of R-phycoerythrin conjugated F(ab')₂ goat anti-mouse IgG antibody (Tago Inc., Burlingame, Calif.). After fixation in 1% paraformaldehyde in PBS, at least 8000 cells were evaluated with use of a Coulter EPICS Profile flow cytometer (Coulter). In some experiments, cells were exposed to various signal transduction inhibitors (see later section) before mAb labeling. Fluorescence intensity was determined on a 3-log scale.

Fibronectin and VCAM-1 adhesion assays

For matrix protein experiments, 96-well microtiter plates (Costar Corp., Cambridge, Mass.) were coated overnight at 4° C with 50 μ l aliquots of fibronectin (0.016 to 500 μ g/ml) diluted in PBS.²⁰ In other experiments, 96-well microtiter plates (NUNC Maxi-sorb Immunoplates, PGC Scientific Corp., Gaithersburg, Md.) were coated overnight at 4° with 50 μ l aliquots of sVCAM (0.4 to 6 μ g/ml) diluted in PBS containing CaCl₂, 130 mg/L, and MgCl₂, 100 mg/L.¹² For both types of plates, the wells were then blocked by incubation with 100 μ l aliquots of PBS containing 3% BSA (heat denatured at 65° C for 1 hour) for at least 1 hour at room temperature to reduce nonspecific adherence to plastic. Control adherence was measured in wells coated with PBS alone and blocked with PBS containing 3% BSA. Wells were washed twice with prewarmed Eagle's minimal essential medium (Gibco) and 50 μ l aliquots of ⁵¹Cr-labeled eosinophils or Jurkat cells (1.25×10^5 total cells per aliquot) were added to wells in triplicate.³³ Cells were allowed to adhere for up to 4 hours (typically 60 minutes, see text) at 37° C after which nonadherent cells were removed by gentle aspiration and rinsing with prewarmed Eagle's medium. Adherent cells were then lysed with NH₄OH, 1 mol/L, for 25 minutes at room temperature and radioactivity of adherent cell

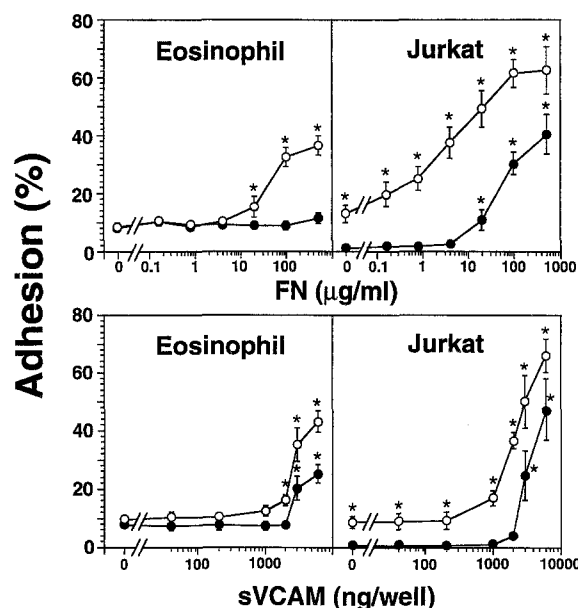


FIG. 1. Comparison of eosinophil and Jurkat cell binding to fibronectin (FN, upper panels) and VCAM-1 (lower panels) at 60 minutes. In the presence of mAb 8A2 (10 μ g/ml, open circles), eosinophil binding to fibronectin was detected, and there was significant enhancement of Jurkat cell binding. For VCAM-1, both eosinophils and Jurkat cells demonstrated spontaneous adhesion that was significantly enhanced by mAb 8A2. Jurkat cell adhesion to BSA was also significantly enhanced by mAb 8A2. Solid circles, Absence of mAb 8A2; $n = 4$ to 13; $*p < 0.05$.

lysates was determined with a gamma counter. Percent adherence was calculated by comparing the radioactivity of adherent cell lysates with that of separate 50 μ l aliquots of cell suspension. In certain experiments, saturating concentrations of blocking or activating mAbs, various concentrations of CS-1 peptide, or various stimuli such as PAF (10^{-7} mol/L), RANTES (100 ng/ml), phorbol 12-myristate 13-acetate (10 ng/ml), or IL-5 (10 ng/ml) were added to the wells simultaneously with cells and allowed to remain throughout the entire adhesion assay.

For experiments designed to examine potential signal transduction pathways regulating cell adhesion, various pharmacologic inhibitors were used. In these experiments, ^{51} Cr-labeled cells were preincubated for 20 minutes at 37°C with up to 50 μ mol/L concentrations of the tyrosine kinase inhibitor tyrphostin B46⁴¹ or the protein kinase C inhibitor staurosporine (up to 50 nmol/L, Sigma) before being added to the wells. In other experiments, cells were preincubated with the G protein inhibitor pertussis toxin (up to 1000 ng/ml, Sigma) for 2 hours and subsequently labeled with ^{51} Cr for adhesion assay as described previously herein.

Statistical analyses

Data are presented as mean plus or minus the standard error of the mean. Statistical significance was determined by *t* test and considered significant at $p < 0.05$.

RESULTS

Eosinophil and Jurkat cell binding to fibronectin and VCAM-1

In initial experiments, eosinophil binding to control BSA-blocked wells was $8.2\% \pm 1.0\%$ and showed no

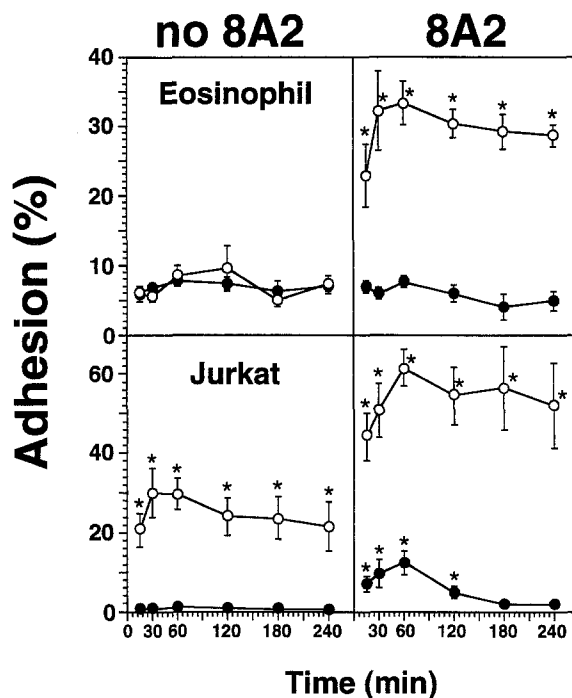


FIG. 2. Kinetics of eosinophil (upper panels) and Jurkat cell (lower panels) binding to fibronectin (100 μ g/ml, open circles) or BSA (closed circles) in the presence or absence of mAb 8A2 (10 μ g/ml). $n = 3$ to 13; $*p < 0.05$.

increase in binding to a wide range of concentrations of immobilized fibronectin (Fig. 1, upper panels). To validate the assay and to confirm adequate coating with fibronectin, Jurkat cells were used. Jurkat cell binding to BSA was very low ($1.1\% \pm 0.3\%$), and significant spontaneous binding to fibronectin was observed at concentrations ≥ 20 μ g/ml (Fig. 1, upper panels). In the presence of saturating concentrations (10 μ g/ml) of the β_1 integrin activating mAb 8A2, eosinophils showed significant binding to fibronectin at concentrations ranging from 20 to 500 μ g/ml. Significant enhancement of Jurkat cell binding was observed at all concentrations of fibronectin tested and with BSA-coated wells.

In contrast to binding to fibronectin, eosinophils showed significant, spontaneous, concentration-dependent binding to VCAM-1 that was maximal at 6 μ g/well sVCAM (Fig. 1, lower panels). Jurkat cells also showed significant spontaneous binding to VCAM-1 at concentrations ≥ 2 μ g/well (maximum $50.3\% \pm 9.4\%$). At each concentration tested, Jurkat cells displayed higher levels of binding than those seen for eosinophils. In the presence of mAb 8A2, eosinophil binding to VCAM-1 (≥ 2 μ g/well) was significantly enhanced. Jurkat cell binding to VCAM-1 was increased at all concentrations of sVCAM tested, as was adhesion to BSA.

Kinetics of eosinophil and Jurkat cell adhesion to fibronectin

To determine whether mAb 8A2 changed the kinetics of cell attachment, eosinophil and Jurkat cell binding to

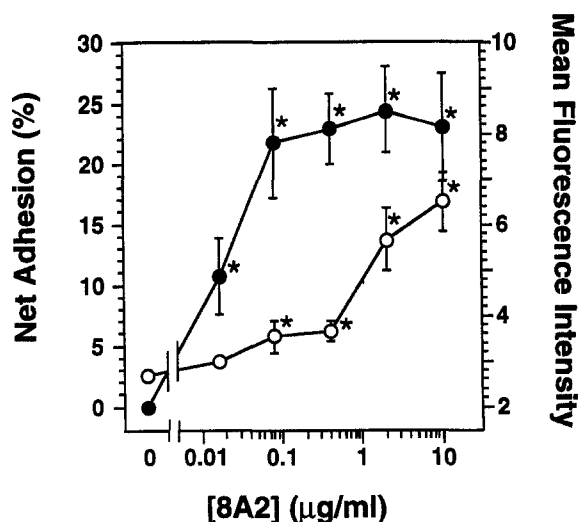


FIG. 3. Titration of binding of mAb 8A2 by indirect immunofluorescence and flow cytometry (open circles) and comparison with its effect on eosinophil adhesion to fibronectin (100 µg/ml, 60 minutes, closed circles). Control fluorescence intensity with IgG1 control mAb was 2.7 ± 0.1 . Values for adhesion to BSA ($13\% \pm 2.6\%$) were subtracted from those for fibronectin adhesion to yield values for net adhesion. $n = 3$ to 4; * $p < 0.05$.

fibronectin (100 µg/ml) and BSA was determined at various times ranging from 15 minutes to 4 hours. Throughout this period, eosinophils failed to bind to fibronectin at levels higher than that seen with BSA alone (Fig. 2, upper panels). When mAb 8A2 was added at the beginning of the adhesion assay, eosinophils showed rapid and sustained increases in binding to fibronectin that were detectable at 15 minutes and maximal at 30 to 60 minutes. Longer incubation times led to a slight decline in adhesion. In contrast to eosinophils, Jurkat cells again bound spontaneously to fibronectin at all times tested (Fig. 2, lower panels). Like eosinophils, Jurkat cell adhesion was maximal at 30 to 60 minutes, then declined slightly at later times. In the presence of mAb 8A2, Jurkat cell binding to fibronectin was augmented but was still maximal at 60 minutes. Augmentation of binding to BSA was also observed at times up to 2 hours.

Titration of mAb 8A2 cell surface binding and effects on adhesion

To determine whether saturating concentrations of mAb 8A2 were needed for optimal augmentation of adhesion, experiments were done in which effects of mAb 8A2 on eosinophil binding to fibronectin were compared with eosinophil surface labeling as measured by indirect immunofluorescence and flow cytometry. As shown in Fig. 3, eosinophil binding to fibronectin in a concentration of 100 µg/ml was significantly enhanced by addition of mAb 8A2 at concentrations as low as 0.0016 µg/ml and was maximally increased at 0.08 µg/ml. This concentration was approximately 2 logs lower than that required for saturable labeling as determined by

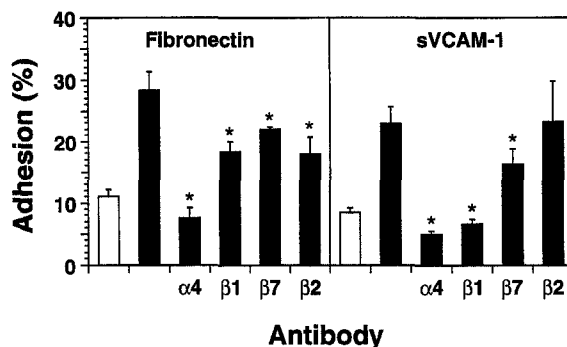


FIG. 4. Effect of integrin blocking mAb on mAb 8A2-induced eosinophil binding to fibronectin and VCAM-1. Values for adhesion to control BSA-coated wells are shown as open bars. $n = 3$ to 8; * $p < 0.05$.

flow cytometry (10 µg/ml). Thus much smaller concentrations of 8A2 mAb were sufficient to induce maximal increases in eosinophil binding to fibronectin.

Effect of integrin function-blocking mAbs on 8A2-induced eosinophil binding to fibronectin and VCAM-1

To examine the specific ligands used by mAb 8A2-treated eosinophils to attach to fibronectin and VCAM-1, adhesion assays were done in the presence and absence of α₄, β₁, β₂, and β₇ integrin blocking mAb. As shown in Fig. 4, eosinophils incubated with mAb 8A2 displayed significant adhesion to both immobilized substrates. Adhesion to VCAM-1 was effectively inhibited by either α₄ or β₁ integrin-specific blocking mAb. Antibody to β₇ also had significant, but less marked, inhibitory activity, whereas β₂ mAb had no effect. Adhesion to fibronectin showed a similar pattern of significant inhibition with α₄, β₁, and β₇ integrin mAb, although the inhibition with β₁ mAb was less complete than that observed in the VCAM-1 adhesion assay. Unlike VCAM-1 adhesion, in the fibronectin adhesion assay the β₂ mAb was as effective as the β₁ mAb in inhibiting adhesion, which suggests that eosinophil attachment was partially β₂ integrin dependent. Indeed, for fibronectin adhesion, a combination of all three β chain mAbs (β₁ + β₂ + β₇) was needed to completely inhibit adhesion ($n = 2$, data not shown), even though the α₄ mAb by itself blocked well. Unlike findings in previous studies,²³ no effect was seen with an α₅ integrin blocking antibody (P1D6, data not shown), consistent with the reported lack of α₅ integrin expression on eosinophils.^{33, 34}

Effect of CS-1 peptide on eosinophil and Jurkat cell adhesion to fibronectin

To determine whether adhesion to fibronectin was primarily to the CS-1 domain of the molecule, binding to fibronectin of mAb 8A2-treated eosinophils and untreated Jurkat cells was examined in the presence and absence of soluble CS-1 fragment. As shown in Fig. 5, binding of both cell types was significantly inhibited at

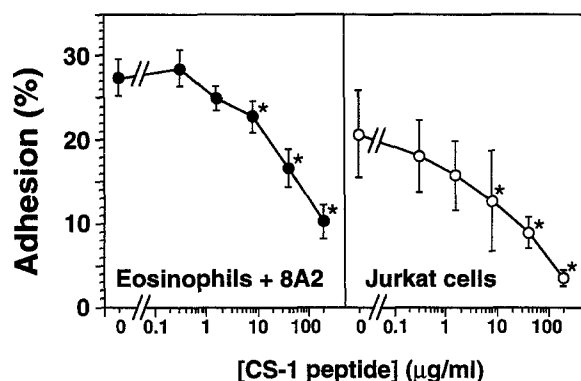


FIG. 5. Effect of CS-1 peptide on mAb 8A2-induced (10 μ g/ml) eosinophils and untreated Jurkat cell binding to fibronectin. Adhesion assays were done for 60 minutes. $n = 3$ to 4; * $p < 0.05$.

concentrations of CS-1 peptide ≥ 40 μ g/ml and approached levels of adhesion seen with BSA. In contrast, addition of similar concentrations of the RGD-containing peptide fragment of fibronectin had no effect on adhesion, nor did CS-1 peptide affect cell binding to VCAM-1 ($n = 2$, data not shown).

Comparison of surface levels of total and activated β_1 integrins and other integrins on eosinophils and Jurkat cells

Because of the higher levels of spontaneous and mAb 8A2-induced adhesion of Jurkat cells compared with that of eosinophils, we determined whether Jurkat cells expressed higher levels of activated β_1 integrins on their surface. To test this hypothesis, both cell types were labeled by indirect immunofluorescence and analyzed by flow cytometry after incubation with mAb recognizing activated β_1 integrin (15/7) or with mAb recognizing β_1 integrins regardless of activation status (33B6).⁴² As shown in Fig. 6, Jurkat cells expressed much higher levels of both total and activated β_1 integrins. In fact, eosinophil labeling with mAb 15/7 could barely be distinguished from that seen with the irrelevant control IgG1 mAb, which is consistent with our previous observation that Jurkat cell adhesion to fibronectin and VCAM-1 was more readily demonstrable than that seen with eosinophils. Platelet attachment to eosinophils was not observed, because no binding of mAb to α_5 , α_v , or β_3 integrins was ever detected (data not shown).

Effect of eosinophil activation in vitro or in vivo on adhesion to fibronectin

Previous studies have shown that adhesion to endothelium and transendothelial migration are enhanced in eosinophils isolated from late phase BAL fluids or after their exposure to PAF, IL-5, or RANTES.⁴³⁻⁴⁷ To determine whether these conditions also resulted in augmented fibronectin binding, purified BAL fluid eosinophils or peripheral blood eosinophils exposed to various stimuli in vitro were tested for adhesion to a range of fibronectin concentrations. BAL fluid eosinophils dis-

Cell count

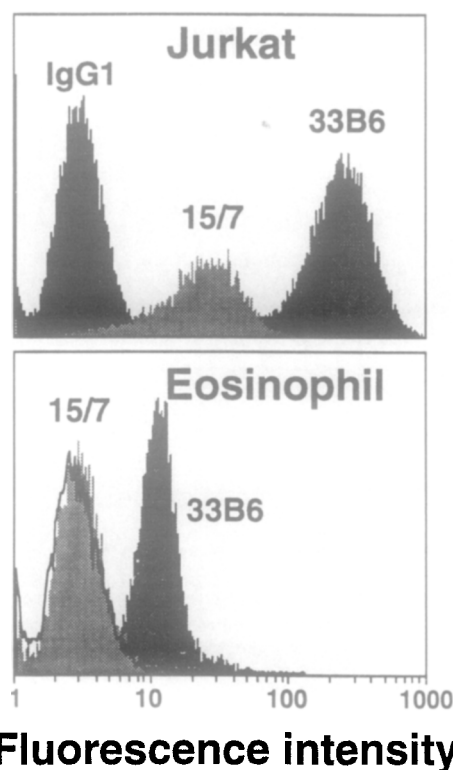


FIG. 6. Expression of β_1 integrin activation epitope detected by mAb 15/7 on eosinophils and Jurkat cells. Eosinophils expressed β_1 integrins (mAb 33B6: mean fluorescence intensity [MFI]: 10.5) with little or no expression of activation epitope detected by mAb 15/7 (MFI: 3.0; IgG control: 3.0). Jurkat cells expressed much higher levels of β_1 integrins (MFI: 120) and the 15/7 activation epitope (MFI: 20). Data are from a single experiment representative of two separate experiments.

played higher than normal levels of control adhesion to BSA alone (Fig. 7); this enhanced adhesion was completely blocked by β_2 integrin mAb (data not shown). Adhesion declined as more fibronectin (and presumably less BSA) was used to coat the wells and, as was seen in Fig. 1, mAb 8A2 was required to demonstrate binding, which was detected over a similar range of fibronectin concentrations. As shown in Fig. 8, stimulation of peripheral blood eosinophils with RANTES in vitro had no effect on adhesion, whereas IL-5, and to a lesser extent PAF, increased control adhesion to BSA. As was seen with BAL fluid eosinophils, adhesion declined as higher concentrations of fibronectin were used. The enhancement of adhesion induced by IL-5 and PAF was completely β_2 integrin dependent (data not shown).

Effect of signal transduction inhibitors on Jurkat cell binding to fibronectin and eosinophil and Jurkat cell binding to VCAM-1

Several studies have implicated tyrosine kinases, such as focal adhesion kinase, and serine-threonine kinases, such as protein kinase C, in integrin signal transduction pathways.³² Therefore studies were done in which phar-

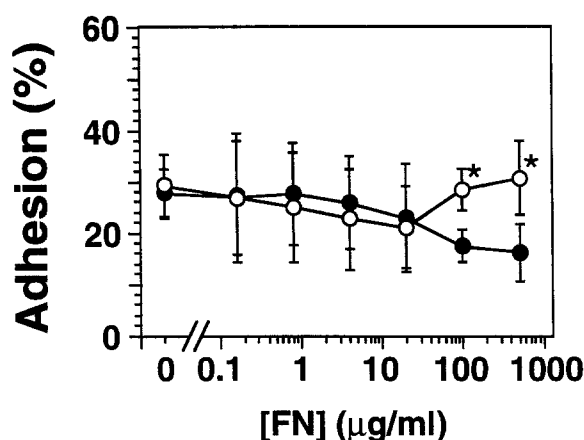


FIG. 7. Adhesion of eosinophils purified from late-phase BAL fluid to fibronectin (FN) in the presence (open circles) or absence (closed circles) of mAb 8A2. $n = 3$ to 5; $*p < 0.05$.

macologic agents that can affect phosphorylation events were tested for their ability to alter adhesion to fibronectin or VCAM-1. Because spontaneous α_4 integrin-dependent adhesion to fibronectin could only be demonstrated with Jurkat cells, the effects of pertussis toxin, staurosporine, and the tyrosine kinase inhibitor tyrphostin B46 were initially examined on these cells. Preincubation with pertussis toxin (up to 1 µg/ml) or staurosporine (up to 50 nmol/L) failed to affect Jurkat cell binding to fibronectin (data not shown). In contrast, Jurkat cell binding to fibronectin was significantly inhibited by tyrphostin B46 in a concentration-dependent manner, with an inhibitory concentration of 50% (IC_{50}) ≈ 20 µmol/L (Fig. 9). This inhibitory effect was completely reversed if mAb 8A2 was present during the adhesion assay. When adhesion of eosinophils and Jurkat cells to VCAM-1 was examined, similar results with tyrphostin B46 were obtained (Fig. 10). Spontaneous eosinophil and Jurkat cell binding to sVCAM (3 µg/well) was significantly inhibited by tyrphostin ($IC_{50} \approx 10$ to 40 µmol/L) and the inhibition was completely reversed by mAb 8A2. During the time of preincubation with tyrphostin and subsequent adhesion (80 minutes total), no effect on eosinophil viability was observed (data not shown), consistent with the ability of 8A2 to completely reverse the effects of tyrphostin.

DISCUSSION

Several mechanisms have been identified by which eosinophils are preferentially recruited into allergic inflammatory tissue sites. Certain cytokines and chemokines can selectively activate eosinophil migratory function (e.g., IL-5, RANTES), whereas others (e.g., IL-4, IL-13) promote endothelial cell expression of the adhesion molecule VCAM-1 that acts as a ligand for α_4 integrins expressed on the eosinophil surface. In addition to these pathways, it is now appreciated that the avidity of integrins, not just the total number of molecules expressed, influences cell adhesion and migra-

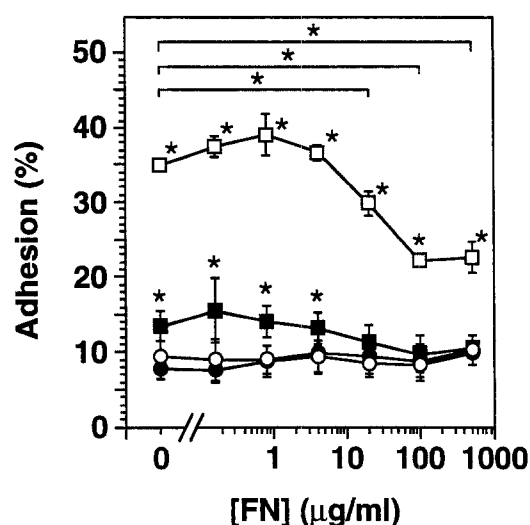


FIG. 8. Effect of IL-5 (10 ng/ml, open squares), PAF (10^{-7} mol/L, closed squares), and RANTES (100 ng/ml, open circles) on eosinophil binding to fibronectin (FN). Adhesion in the absence of stimulus is also shown (closed circles). $n = 3$ to 6; $*p < 0.05$.

tion^{2, 27-29} and that although a particular integrin may have more than one ligand, the avidity for each ligand may differ. It has been known for several years that eosinophils express $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrins, but little is known about how this cell type regulates α_4 integrin function. In one previous report with eosinophils, a β_1 integrin functional activating mAb that enhances adhesion was used to demonstrate that adhesiveness for matrix proteins including fibronectin could be dramatically potentiated.²³ We have used this same mAb in the present studies to extend the work of Kuijpers et al.²³ by examining the kinetics of this effect, by titrating the effects of this mAb, by demonstrating that the effect is via α_4 integrins and not α_5 integrins, by extending the findings to VCAM-1 adhesion, and by demonstrating that the mAb can reverse the inhibitory effects of tyrosine kinase inhibitors on cell adhesion to VCAM-1 and fibronectin. Adhesion of the human Jurkat T cell line was also examined both to further validate the adhesion assays and to compare α_4 integrin function with that of eosinophils.

As expected, eosinophils and Jurkat cells displayed concentration-dependent adhesion to immobilized VCAM-1. The magnitude of adhesion was enhanced by 8A2, but in marked contrast, spontaneous eosinophil adhesion to fibronectin could not be detected, consistent with some,^{23, 24} but not all,¹⁸⁻²¹ previous reports. In the presence of 8A2, however, eosinophil attachment to fibronectin was easily demonstrated (Fig. 1).²³ Jurkat cells demonstrated spontaneous adhesion to both fibronectin and VCAM-1, and adhesion to these substrates was enhanced by 8A2. This was consistent with the finding that Jurkat cells express higher levels of activated β_1 integrins (as detected by labeling with mAb 15/7) than eosinophils (Fig. 6). Once cells were exposed

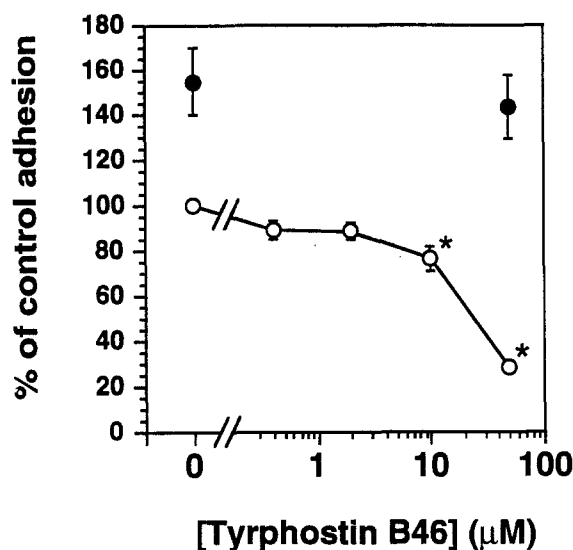


FIG. 9. Effect of tyrphostin B46 on Jurkat cell binding to fibronectin (100 μ g/ml) in the presence (closed circles) or absence (open circles) of mAb 8A2. Control adhesion to fibronectin in absence of mAb 8A2 was $42.4\% \pm 7.1\%$. $n = 5$; $*p < 0.05$.

to 8A2, the maximal levels of adhesion to VCAM-1 and fibronectin became similar, reaching approximately 40% for eosinophils and 70% for Jurkat cells (Fig. 1), which suggests that maximal activation of β_1 integrin function by 8A2 essentially eliminates any constitutive differences in binding avidity for these two ligands. Despite the observation that 8A2 augmented adhesion, several aspects of the adhesion response remained unaltered. In the presence of 8A2 the concentrations of VCAM-1 and fibronectin that yielded maximal adhesion, and the kinetics of adhesion, were unchanged (Figs. 1 and 2). Interestingly, the amounts of 8A2 needed to optimally augment eosinophil adhesion to fibronectin were approximately 2 logs less than the amounts that were found to be saturating by flow cytometry (Fig. 3), which is consistent with the hypothesis that only a small proportion of β_1 integrin molecules need to be activated to initiate firm cellular attachment.^{30, 31, 48}

There are several possible explanations for our inability to detect eosinophil binding to fibronectin in the absence of 8A2. The most plausible explanation is that there simply are too few activated α_4 integrins to mediate binding. Published data with eosinophils that used mAb 15/7 that binds to activated β_1 integrins,⁴² as well as comparative data with eosinophils and Jurkat cells in the present manuscript (Fig. 6), support this concept. It is known that binding of α_4 integrins to fibronectin and VCAM-1 occurs through distinct epitopes and that the affinity for the former is less.^{4, 5, 49-51} We have made extensive efforts to minimize eosinophil activation during purification, to prevent platelet attachment to eosinophils that can occur during cell isolation,³⁵ and to establish adhesion assays wherein any β_2 integrin-dependent adhesion responses to blocking proteins are

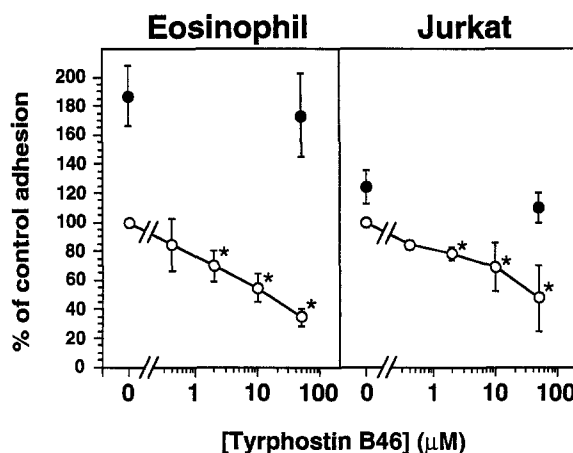


FIG. 10. Effect of tyrphostin B46 on eosinophil (left panel) and Jurkat cell (right panel) binding to VCAM-1 (3 μ g/well) in the presence (closed circles) or absence (open circles) of mAb 8A2. Values for control adhesion to VCAM-1 for eosinophils and Jurkat cells were $20.3\% \pm 4.0\%$ and $24.6\% \pm 8.5\%$, respectively. $n = 5$; $*p < 0.05$.

minimized. Evidence of having achieved this is the finding, illustrated in Fig. 4, that eosinophil adhesion to VCAM-1 in the presence of 8A2 was effectively inhibited by mAbs to α_4 and β_1 integrins and less so by an mAb to β_7 integrin, whereas a blocking β_2 mAb had no inhibitory activity. For adhesion to fibronectin, however, blockade with CS-1 peptide or α_4 mAb was most effective, although β_1 , β_2 , and β_7 integrin mAbs also had partial inhibitory activity, and a combination of all three β chain mAbs ($\beta_1 + \beta_2 + \beta_7$) was needed to completely inhibit adhesion, even though the α_4 mAb by itself blocked well. Our findings regarding the ability of β_7 integrin to serve as a ligand for VCAM-1 and fibronectin are in general agreement with the report of Walsh et al.,⁵² although the reason for the differences in integrin use when binding to VCAM-1 versus fibronectin is not clear. Possible explanations may include one or more of the following: (1) slight differences or overlap in the fibronectin recognition epitopes on β_1 integrin bound by the β_1 activating mAb 8A2, which, if already attached to the cell, prevents the β_1 blocking mAb (33B6) from effectively reaching its fibronectin-binding epitope; (2) the possibility that mAb 8A2 binding to eosinophils activates other integrins for binding to fibronectin; and (3) the possibility that α_4 integrin engagement with fibronectin activates adhesion to fibronectin via other integrins. Leukocyte attachment to fibronectin (or blocking proteins such as BSA) has been reported by some laboratories to occur via CD11b.^{25, 26, 53} Therefore it is also possible that activation of β_2 integrin pathways can, via outside-in signaling, influence leukocyte activation and promote β_1 integrin-mediated attachment responses. However, when eosinophils were obtained from BAL fluids or peripheral blood eosinophils were stimulated in vitro with IL-5, PAF, or RANTES to display increased β_2 integrin-dependent adhesion, α_4 integrin-

dependent attachment could not be demonstrated (Figs. 7 and 8). These results are consistent with a recent report that demonstrated that IL-5 inhibits expression of a β_1 integrin activation epitope on eosinophils and reduces their attachment to VCAM-1.⁴² This report also suggests that conditions that lead to β_2 integrin activation are distinct from those involved in β_1 integrin activation, consistent with a recent report in which C-C chemokines and C5a had differential effects on β_1 and β_2 integrin function in eosinophils.⁵⁴ The precise mechanisms by which β_1 and β_2 integrin function might have been reciprocally altered was not the focus of the current studies and will require additional investigation.

Data presented in Figs. 9 and 10 show that spontaneous adhesion of eosinophils to VCAM-1 and of Jurkat cells to both VCAM-1 and fibronectin was significantly reduced by the tyrosine kinase inhibitor tyrphostin B46 ($IC_{50} \approx 20 \mu\text{mol/L}$). The concentrations needed for activity were more than 1 log higher than that reported for inhibition of epidermal growth factor receptor kinase activity ($IC_{50} = 0.7 \mu\text{mol/L}$), but were similar to that reported for inhibition of epidermal growth factor-dependent cell growth ($IC_{50} = 25 \mu\text{mol/L}$).⁴¹ These results are consistent with the emerging concept that integrin-mediated signaling events are associated with tyrosine phosphorylation and formation of focal adhesions.³² The effect of tyrphostin B46 on eosinophil and Jurkat cell adhesion was reversed by 8A2, which demonstrates that even in the presence of a tyrosine kinase inhibitor, 8A2 has full activity, supporting the hypothesis that the effect of this β_1 integrin-activating antibody is a result of changes in the conformation of the extracellular portion of the molecule. Recently it was reported that a different tyrosine kinase inhibitor, genistein, blocks augmented superoxide anion production that occurs when eosinophils are allowed to attach to immobilized VCAM-1, although this compound failed to affect eosinophil adhesion to VCAM-1.¹⁰ This was confirmed in our hands as well (data not shown). Although other kinases, including the serine-threonine kinase protein kinase C, have also been implicated in integrin signaling, staurosporine did not affect eosinophil attachment. Because both tyrphostins and genistein are often used as tyrosine kinase inhibitors and these compounds have disparate effects on eosinophils, these data suggest the activity of different tyrosine kinases or other unidentified pharmacologic effects are occurring as a result of drug treatment.

Taken together, our results extend those of previous studies by demonstrating several mechanisms that regulate eosinophil α_4 integrin function. Whether a cell increases or decreases β_1 or β_2 integrin function in vivo will likely determine whether the cell will undergo firm endothelial cell attachment, transendothelial migration, strong binding to matrix proteins, or continue to migrate through the tissue. For airway inflammation, it also seems likely that the mechanisms by which eosinophils undergo transepithelial migration to enter the airway lumen will be found to be influenced by the relative

avidity of integrins. Data presented so far have identified stimulus-induced downregulation of β_1 integrin function that occurs along with upregulation of β_2 integrin activity. However, whether there exists an opposite pathway by which a physiologic stimulus enhances β_1 integrin function while reducing β_2 integrin activity remains to be elucidated.

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