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# Differential regulation of β1 and β2 integrin avidity by chemoattractants in eosinophils

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**ABSTRACT** **The CC chemokines *r*egulated on *a*ctivation *n*ormal *T e*xpressed and *s*ecreted (RANTES) and monocyte chemotactic protein 3 (MCP-3), and the anaphylatoxin C5a, induce activation, degranulation, chemotaxis, and transendothelial migration of eosinophils. Adhesion assays on purified ligands showed differential regulation of β1 and β2 integrin avidity in eosinophils. Adhesiveness of VLA-4 (α4β1, CD29/CD49d) for vascular cell adhesion molecule 1 or fibronectin was rapidly increased but subsequently reduced by RANTES, MCP-3, or C5a. The deactivation of VLA-4 lead to cell detachment, whereas phorbol 12-myristate 13-acetate induced sustained activation of VLA-4. In contrast, chemoattractants stimulated a prolonged increase in the adhesiveness of Mac-1 (αMβ2, CD11b/CD18) for intercellular adhesion molecule 1. Inhibition by pertussis toxin confirmed signaling via G protein-coupled receptors. Chemoattractants induced transient, while phorbol 12-myristate 13-acetate induced sustained actin polymerization. Disruption of actin filaments by cytochalasins inhibited increases in avidity of VLA-4 but not of Mac-1. Chemoattractants did not upregulate a $Mn^{2+}$-inducible β1 neoepitope defined by the mAb 9EG7, but induced prolonged expression of a Mac-1 activation epitope recognized by the mAb CBRM1/5. This mAb inhibited chemoattractant-stimulated adhesion of eosinophils to intercellular adhesion molecule 1. Thus, regulation of VLA-4 was dependent on the actin cytoskeleton, whereas conformational changes appeared to be crucial for activation of Mac-1. To our knowledge, this is the first demonstration that physiological agonists, such as chemoattractants, can differentially regulate the avidity of a β1 and a β2 integrin expressed on the same leukocyte.**

The emigration of circulating blood leukocytes into subendothelial tissues includes multiple steps. Tethering and rolling on the vessel wall, firm attachment, and transendothelial migration are mediated by sequential interactions of selectins with carbohydrates and integrins with immunoglobulin superfamily members and matrix components (1). Chemoattractants have been suggested to induce both integrin adhesiveness and directional cell movement across the endothelium. Eosinophils are major effector granulocytes in inflammatory reactions and are predominantly recruited into sites of chronic allergic inflammation (2–4). The classical peptide chemoattractants fMLP and C5a act on multiple leukocyte subsets, whereas the recently described families of α or CXC chemokines and β or CC chemokines activate specific leukocyte subpopulations by binding to G protein-coupled seven-transmembrane receptors (5, 6). Eosinophils are strongly attracted by C5a and by the CC chemokines *r*egulated on *a*ctivation *n*ormal *T e*xpressed and *s*ecreted (RANTES) and monocyte chemotactic protein 3 (MCP-3) (7–12). All granulocytes share the β2 integrins LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18), and p150,95 (CD11c/CD18). Binding of LFA-1 (αLβ2) and Mac-1 (αMβ2) to the immunoglobulin superfamily member intercellular adhesion molecule 1 (ICAM-1) requires surface density-independent activation of adhesiveness (13–16). Unlike neutrophils, eosinophils express the β1 integrin very late antigen 4 (VLA-4, CD29/CD49d) (17, 18), which mediates binding to vascular cell adhesion molecule 1 (VCAM-1), an immunoglobulin superfamily member induced by cytokines on endothelium (19), and to an alternatively spliced domain in fibronectin. VLA-4 (α4β1) binds to a sequence motif in fibronectin distinct from that recognized by VLA-5 (α5β1) (20). Both VCAM-1 and ICAM-1 contribute to adhesion of eosinophils to cytokine-activated endothelium (17, 18). β2 integrins and ICAM-1 are important in eosinophil migration to RANTES across resting and cytokine-stimulated endothelium, and VLA-4 on eosinophils is also important in migration across stimulated endothelium (21). ICAM-1 expression is upregulated on inflamed bronchial endothelium and airway epithelium, and a mAb to ICAM-1 attenuates airway eosinophilia and hyperresponsiveness *in vivo* (22).

The exact sequence of events controlling chemotaxis of leukocytes, and particularly the regulation of integrin avidity, is poorly understood. Since different integrins may function in different steps of eosinophil attachment to and migration through endothelium and the basement membrane, we hypothesized that integrins might differ in the kinetics of activation and subsequent deactivation of adhesiveness. Indeed, we find that chemoattractants can differentially regulate the avidity of β1 and β2 integrins in eosinophils, inducing transient activation and rapid deactivation of VLA-4 but prolonged activation of Mac-1. This differential regulation may involve distinct mechanisms.

## MATERIALS AND METHODS

**Reagents and mAb.** Human recombinant RANTES and C5a were from Genzyme, and synthetic MCP-3 was a kind gift of I. Clark-Lewis (23). 2′,7′-Bis-2-carboxyethyl-5-(6)-carboxyfluorescein-acetoxymethylester (BCECF/AM) was from Molecular Probes. Pertussis toxin was from GIBCO/BRL. The murine IgG myeloma X63 and mAb TS1/22 (CD11a) (24), CBRM1/29 and CBRM1/5 (CD11b) (16), and R6.5 (ICAM-1, CD54) (25) were previously reported and purified with protein A. Purified HP2/1 (CD49d) (26) was from AMAC (Westbrook, ME) and purified HAE-2a (VCAM-1, CD106) (27) was a kind gift from T. Tedder (Duke University, Durham, NC). A5-PUJ-1 (CD49e, ascites) and purified 9EG7 (28) were kind gifts from M. E. Hemler (Dana–Farber Cancer Institute, Boston). Goat anti-mouse IgG fluorescein isothiocyanate (FITC) was from Zymed. All other reagents were from Sigma.

**Blood Cell Isolation.** Blood was collected from healthy donors and citrate-anticoagulated. Leukocyte-rich plasma was

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Abbreviations: F-actin, filamentous actin; FITC, fluorescein isothiocyanate; ICAM-1, intercellular adhesion molecule 1; sICAM-1, soluble ICAM-1; MCP-3, monocyte chemotactic protein 3; PMA, phorbol 12-myristate 13-acetate; RANTES, *r*egulated on *a*ctivation *n*ormal *T e*xpressed and *s*ecreted; VCAM-1, vascular cell adhesion molecule 1.

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prepared by 0.6% dextran T500 sedimentation of erythrocytes for 45 min at 20°C. Granulocytes were prepared from leukocyte-rich plasma by Ficoll-Hypaque density gradient centrifugation. Following hypotonic lysis of erythrocytes, eosinophils were isolated to a purity of >95% using negative selection with CD16 mAb-conjugated immunomagnetic beads (Miltenyi Biotec, Sunnyvale, CA) (29).

**Purification of Soluble ICAM-1 (sICAM-1).** sICAM-1 expressed in mutant Chinese hamster ovary Lec 3.2.8.1 cells that express high mannose carbohydrates (30) was purified from supernatants by immunoaffinity chromatography with ICAM-1 mAb R6.5 coupled to Sepharose (5 mg/ml) (31). Fractions containing sICAM-1 were identified by SDS/PAGE and silver staining and concentrated to 1 mg/ml in microconcentrators (Amicon), as determined by $A_{280}$. Recombinant soluble 7-domain VCAM-1 was a kind gift of R. Lobb (32).

**Cell Adhesion Assay.** Cell attachment in stasis to VCAM-1 and fibronectin adsorbed at 2.5 $\mu$g/ml, sICAM-1 adsorbed at 10 $\mu$g/ml, or BSA adsorbed at 2.5 or 10 $\mu$g/ml was performed as described (33). Briefly, proteins were coated onto 96-well microtiter plates (Linbro/Titertek). Plates were blocked by the addition of 1% human serum albumin, which was pretreated at 56°C for 2 h. Cells were labeled with the fluorescent dye BCECF-AM (1 $\mu$g/ml) and for 30 min, washed and resuspended in HHMC (Hanks' balanced salt solution/10 mM Hepes, pH 7.4/1 mM $Mg^{2+}$/1 mM $Ca^{2+}$) with 0.5% human serum albumin. For mAb inhibition experiments, cells were preincubated with 5% heat-inactivated human serum to block Fc receptors. This improved inhibition of binding with blocking mAb but did not affect binding itself (data not shown). Some wells were preincubated with mAb to ICAM-1 (R6.5) or VCAM-1 (HAE-2a) for 20 min on ice and washed. Some cell aliquots were preincubated with mAb for 20 min, pertussis toxin (50 ng/ml) for 2 h, cytochalasin B or D (in DMSO) or DMSO (0.1%) for 30 min and washed. Labeled cells ($5 \times 10^4$ in 50 $\mu$l) were added to each well of a ligand-coated microtiter plate with or without chemoattractants or phorbol 12-myristate 13-acetate (PMA) on ice and allowed to settle for 20 min. Plates were rapidly warmed and incubated for indicated periods at 37°C. Fluorescence of input cells was quantified by a fluorescence concentration analyzer (Idexx Laboratories, Westbrook, ME). Nonadherent cells were removed by a standardized washing procedure (4 cycles, washing volume 200 $\mu$l, volume remaining after aspiration 50 $\mu$l, pressure 21,000 Pa) in an automated plate washer (Microplate Autowasher EL-404; Bio-Tek, Burlington, VT) using HHMC. The washing program was adjusted to minimize nonspecific background binding, assessed with BSA-coated control wells. Fluorescence of adherent cells was analyzed using the fluorescence concentration analyzer. After subtraction of background binding (typically less than 5%), specific binding was calculated as percentage of input. Data are reported as mean ± SD of three independent experiments performed in duplicate.

For restimulation experiments, cells were stimulated with RANTES on VCAM-1 for 15 min, nonadherent cells were removed by the standardized washing procedure, and attached cells were recovered by treatment with 5 mM EDTA on ice. Cells were washed and resuspended in HHMC with 0.5% human serum albumin and subjected to adhesion assays on ICAM-1 and stimulation with C5a for indicated periods, as described above. Conversely, cells stimulated with RANTES and attached to ICAM-1 at 30 min were recovered and subjected to adhesion assays on VCAM-1 and stimulation with C5a for indicated periods.

**Actin Polymerization Assay.** Polymerized actin [filamentous (F)-actin] was determined by staining with FITC-conjugated phalloidin. Briefly, $10^5$ cells were resuspended in L-15 medium and pretreated with or without cytochalasin B (10 $\mu$g/ml) for 30 min. Cells were stimulated with chemokines or PMA (100 nM) at 37°C and the content of F-actin was measured 0, 30, 60, 180, 600, or 1800 s later. At these time points, cells were fixed, permeabilized, and stained in a single step by adding a solution containing 100 ng FITC-phalloidin, 0.1 mg/ml L-$\alpha$-lysophosphatidyl-choline and 37% formaldehyde. Cells were incubated at 25°C for 10 min, washed with PBS and subjected to flow cytometry. Data were expressed as fluorescence intensity relative as percentage of the unstimulated control.

**Flow Cytometry.** Cells were stimulated with or without chemokines, $Mn^{2+}$ or the soluble ligands ICAM-1, fibrinogen, VCAM-1, or the 40-kDa fragment of fibronectin for 15, 30, or 60 min at 37°C, washed and reacted for 30 min with saturating amounts of mAb TS1/22, CBRM1/29, CBRM1/5, HP2/1, A5-PUJ1, 9EG7, or X63 (isotype control) in HHMC with 0.5% BSA on ice. Cells were stained with FITC goat anti-mouse IgG mAb and analyzed by FACS (Becton Dickinson). Cells were also preincubated with ICAM-1 mAb R6.5 or RR1/1 Fab, stimulated with chemoattractants and stained with isotype control mAb or CBRM1/5 mAb that were directly conjugated with FITC, as described (34). Concentrations of mAb used for blocking experiments in the adhesion assay were found to be saturating (data not shown).

## RESULTS

**Transient Regulation of VLA-4 Avidity by Chemoattractants in Eosinophils.** The CC chemokines RANTES and MCP-3 and the myeloid chemoattractant C5a induce chemotaxis, transendothelial migration, and activation of eosinophils (7–9). We studied regulation by these agonists of $\beta$1 integrin avidity for purified VCAM-1 and the extracellular matrix protein fibronectin. RANTES, MCP-3, and C5a rapidly stimulated adhesion of eosinophils to VCAM-1 2 to 3-fold at the earliest time points examined (Fig. 1*A*). The optimal concentrations for stimulation were 100 ng/ml for RANTES and MCP-3, and 10 ng/ml for C5a (Fig. 1*B*). With prolonged incubation of unstimulated eosinophils on VCAM-1 substrates, adhesion increased for up to 60 min, whereas continued stimulation with chemoattractants resulted in a 50% decline in adhesion by 30 min (Fig. 1*A*). Since cells were exposed to both chemoattractants and the substrate throughout the incubation period, it appeared that about half of the chemoattractant-stimulated cells that had attached at 15 min, detached at 30 min. Adhesion of both resting and stimulated cells was inhibited by mAb to VCAM-1 or VLA-4 at 15 min (Fig. 1*C*) or later time points (data not shown), showing dependence on interaction of VLA-4 with VCAM-1. By contrast to stimulation with chemoattractants, stimulation with PMA resulted in prolonged VLA-4 dependent adhesion to VCAM-1 (Fig. 1*D*).

Similar results were obtained on fibronectin substrates. RANTES, MCP-3, and C5a transiently increased but subsequently reduced adhesion of eosinophils to fibronectin (Fig. 2*A*). Comparable chemoattractant dose-responses were found for stimulation of binding to VCAM-1 or fibronectin at 15 min (data not shown), and for the decrease in adhesion to fibronectin at 45 min (Fig. 2*B*) or later time points (data not shown). Inhibition with mAb revealed that both resting and stimulated adhesion at early time points was mainly mediated by VLA-4, with a contribution of less than 20% by VLA-5 at 15 min (Fig. 2*C*). By 45 min, there was little VLA-4-dependent adhesion (Fig. 2*D*), suggesting that VLA-4 had been deactivated. Our data suggest that adhesiveness of VLA-4 for VCAM-1 and fibronectin is transiently activated and rapidly reduced by chemoattractants in eosinophils.

**Sustained Activation of Mac-1 Avidity by Chemoattractants in Eosinophils.** $\beta$2 integrin-mediated adhesion of chemoattractant-stimulated eosinophils to purified ICAM-1 was modulated differentially from VLA-4-dependent binding. RANTES, MCP-3, and C5a induced prolonged increases in binding of eosinophils to ICAM-1 (Fig. 3*A*). Enhanced binding was evident at 15 min, plateaued at 30 min, and persisted at 60 min.

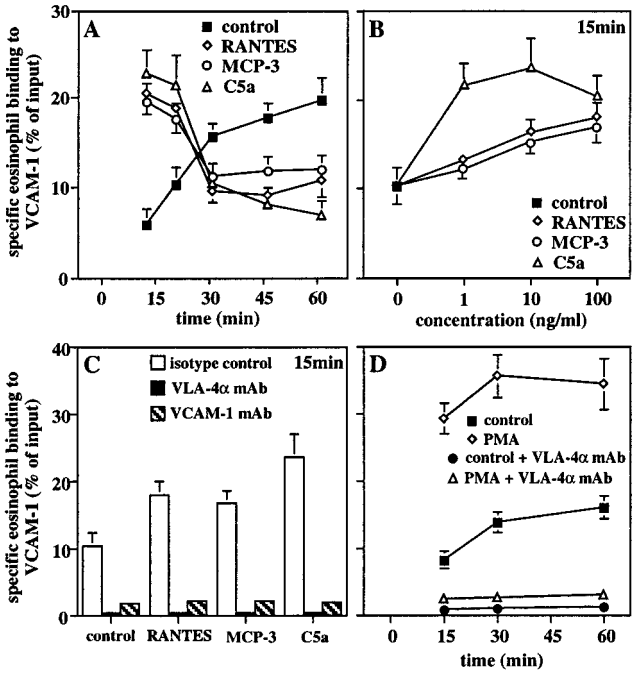


FIG. 1. Chemoattractants but not PMA transiently increase and subsequently decrease VLA-4-dependent binding of eosinophils to VCAM-1. Shown are kinetics (*A*), dose-response of the decrease in adhesion at 15 min (*B*), and inhibition with mAb (*C*) of effects induced by chemoattractants and kinetics of stimulation with PMA (*D*). Isolated eosinophils were subjected to adhesion assays in 96-well plates coated with VCAM-1 or BSA (all 2.5 $\mu$g/ml) at 37°C in presence of assay medium (control), RANTES, MCP-3 (both 100 ng/ml), C5a (10 ng/ml), or PMA (100 nM) for indicated periods (*A*, *C*, and *D*) or at indicated concentrations for 15 min (*B*). Cells were preincubated with VLA-4 mAb (HP2/1) or isotype control (X63), or wells were preincubated with VCAM-1 mAb (HAE-2a, all 10 $\mu$g/ml) for 20 min (*C* and *D*). Data are mean ± SD of three independent experiments performed in duplicate.

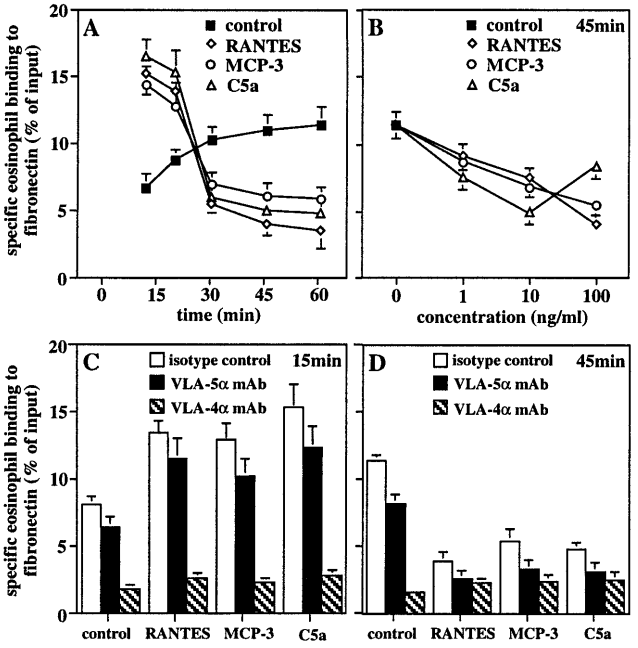


FIG. 2. Chemoattractants transiently increase and subsequently decrease VLA-4-dependent binding of eosinophils to fibronectin. Shown are kinetics (*A*), dose-response of the decrease in adhesion at 45 min (*B*), and inhibition with mAb (*C* and *D*). Isolated eosinophils were subjected to adhesion assays in 96-well plates coated with fibronectin or BSA (all 2.5 $\mu$g/ml) at 37°C in presence of assay medium (control), RANTES, MCP-3 (both 100 ng/ml), or C5a (10 ng/ml) for indicated periods (*A*, *C*, and *D*) or at indicated concentrations for 45 min (*B*). Cells were preincubated with mAb to VLA-4 (HP2/1, 10 $\mu$g/ml), VLA-5 (A5-PUJ1, 1:100 ascites) or isotype control (X63, 10 $\mu$g/ml) for 20 min (*C* and *D*). Data are mean ± SD of three independent experiments performed in duplicate.

Again, RANTES and MCP-3 were most active at 100 ng/ml, and C5a was most active at 10 ng/ml (Fig. 3*B*). Both basal and stimulated adhesion to ICAM-1 was inhibited by mAb to Mac-1 or ICAM-1 (Fig. 3*C*). In contrast, mAb to LFA-1 only slightly reduced both basal and stimulated binding (data not shown). This indicates that increased adhesion was mediated by activation of Mac-1. However, PMA- and $Mn^{2+}$-stimulated adhesion to ICAM-1 was dependent on both Mac-1 and LFA-1, showing that LFA-1 function can be activated (data not shown). Similar patterns of regulation with transient activation and rapid deactivation of VLA-4 avidity but prolonged activation of Mac-1 avidity were also found following stimulation with fMLP or leukotriene B4 (data not shown).

To confirm that binding to VCAM-1 and ICAM-1 is differentially regulated in a cell population binding to both integrin ligands, adherent cells were recovered and restimulated with a chemoattractant to which the cells had not been desensitized. Cells bound to VCAM-1 at 15 min or to ICAM-1 at 30 min after stimulation with RANTES were eluted with EDTA and stimulated with C5a on ICAM-1 and VCAM-1, respectively (Fig. 3 *E* and *F*). C5a induced sustained adhesion to ICAM-1 of cells that had previously adhered to VCAM-1 in the presence of RANTES (Fig. 3*E*). Conversely, C5a induced an early and transient increase and a subsequent decrease in adhesion to VCAM-1 of cells that had previously adhered to ICAM-1 in the presence of RANTES (Fig. 3*F*). These findings show that a single cell population can undergo differential regulation of VLA-4 and Mac-1.

**Distinct Mechanisms of Integrin Activation by Chemoattractants.** Chemottractants signal via G protein-coupled receptors (6). Pretreatment of eosinophils with pertussis toxin prevented increases in adhesion to ICAM-1 (Fig. 3*D*), early increases (Fig. 4*A*) and later decreases (Fig. 4*B*) in adhesion to VCAM-1 induced by chemoattractants. This indicated that regulation of VLA-4 and activation of Mac-1 was mediated by G protein-coupled receptors. By contrast, stimulation of binding by PMA was not altered (Figs. 3*D* and 4*A*).

Polymerization of cytoskeletal actin is important for LFA-1- or $\beta$1 integrin-mediated adhesion stimulated by PMA (35, 36). Using FITC-phalloidin we measured the content of F-actin in eosinophils. While PMA induced increases in the content of F-actin persisting over 30 min, C5a, and RANTES very rapidly and transiently increased the F-actin content with a peak at 30 s (Fig. 4*C*). Cytochalasin B, which disrupts actin filaments, inhibited increases in F-actin induced by PMA or chemoattractants (Fig. 4*D*). Hence, we used cytochalasins to address similarities in the transient mode of actin polymerization and VLA-4 activation stimulated by chemoattractants. Both cytochalasin B and D at concentrations as low as 3 $\mu$g/ml inhibited activation of VLA-4 adhesiveness to VCAM-1 by chemoattractants (Fig. 4*E* and data not shown), but did not affect stimulation of Mac-1 avidity for ICAM-1 at concentrations up to 25 $\mu$g/ml (Fig. 4*F* and data not shown). Adhesion of unstimulated cells to VCAM-1 or ICAM-1 was not altered by cytochalasins at 15 min or later (Fig. 4 *E* and *F* and data not shown). This implicates cytoskeletal events associated with actin polymerization in VLA-4 activation but not in sustained activation of Mac-1 avidity by chemoattractants.

Increased avidity of Mac-1 occurs independently of upregulated surface expression (13–15) and may be due to conformational changes, resulting in the induction of an activation epitope, as shown in neutrophils stimulated by $Mn^{2+}$, PMA, or fMLP (16). In eosinophils, stimulation with RANTES, MCP-3,

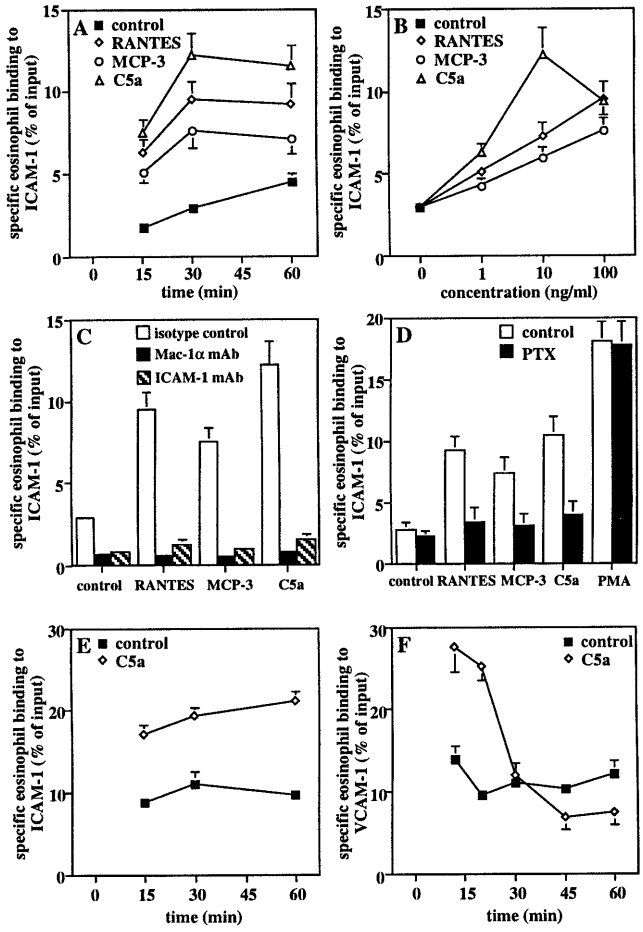


FIG. 3. Chemoattractants induce a prolonged increase in Mac-1-mediated binding of eosinophils to ICAM-1. Shown are kinetics (*A*), dose-response at 30 min (*B*), and inhibition with mAb (*C*), or pertussis toxin (*D*). Isolated eosinophils were subjected to adhesion assays in 96-well plates coated with ICAM-1 or BSA (all 10 $\mu$g/ml) at 37°C in presence of assay medium (control), RANTES, MCP-3 (both 100 ng/ml), C5a (10 ng/ml), or PMA (100 nM) for indicated periods (*A*), for 30 min (*B*, *C*, and *D*) or at indicated concentrations (*B*). Cells were preincubated with Mac-1 mAb (CBRM1/29) or isotype control (X63), or wells were preincubated with ICAM-1 mAb (R6.5, all 20 $\mu$g/ml) for 20 min (*C*) or with pertussis toxin (PTX, 50 ng/ml) for 2 h (*D*). (*E* and *F*) Restimulation of previously adherent cells. Cells stimulated with RANTES (100 ng/ml) that had adhered to VCAM-1 at 15 min (*E*) or that had adhered to ICAM-1 at 30 min (*F*) were recovered with EDTA and subjected to adhesion assays on ICAM-1 (*E*) or VCAM-1 (*F*) or BSA in the presence of C5a (10 ng/ml) for the indicated periods. Data are mean ± SD of three independent experiments performed in duplicate.

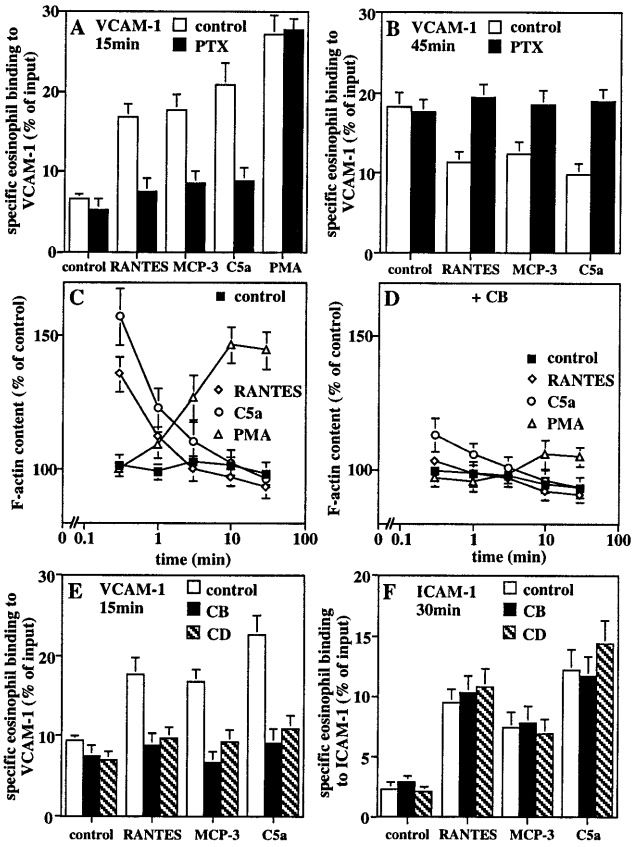


FIG. 4. Mechanisms of integrin activation. (*A* and *B*) Effect of pertussis toxin on eosinophil adhesion to VCAM-1 at 15 min (*A*) or 45 min (*B*). (*C* and *D*) Kinetics of actin polymerization induced by chemoattractants or PMA and inhibition with cytochalasin B (CB). Isolated eosinophils stimulated without (control) or with RANTES (100 ng/ml), C5a (10 ng/ml), or PMA (100 nM) for indicated periods were subjected to actin polymerization assays after preincubation without (*C*) or with CB at 10 $\mu$g/ml (*D*) for 30 min. Data are mean ± SD of three independent experiments. (*E* and *F*) Effect of cytochalasins on eosinophil adhesion to VCAM-1 (*E*) or ICAM-1 (*F*). (*A*, *B*, *E*, and *F*) Isolated eosinophils were subjected to adhesion assays in 96-well plates coated with VCAM-1 (2.5 $\mu$g/ml; *A*, *B*, and *E*), ICAM-1 (10 $\mu$g/ml; *F*) or BSA (2.5 or 10 $\mu$g/ml) at 37°C in presence of assay medium (control), RANTES, MCP-3 (both 100 ng/ml), C5a (10 ng/ml), or PMA (100 nM) for 15 min (*A* and *E*), 30 min (*F*), or 45 min (*B*). Cells were preincubated with or without pertussis toxin (PTX, 50 ng/ml) for 2 h (*A* and *B*), cytochalasin B (CB) or D (CD) at 10 $\mu$g/ml in 0.1% DMSO or 0.1% DMSO (control) for 30 min (*E* and *F*). Data are mean ± SD of three independent experiments performed in duplicate.

or C5a for 15, 30, or 60 min increased surface expression of Mac-1 but did not affect expression of LFA-1, VLA-4 or VLA-5 (Fig. 5*A* and data not shown). Surface expression of VLA-5 was significantly lower than that of VLA-4. In parallel to sustained activation of Mac-1 avidity, chemoattractants induced the prolonged expression of an activation-specific Mac-1 epitope recognized by the mAb CBRM1/5 in a subpopulation of Mac-1 molecules (Fig. 5 *A* and *B*). This epitope was inducible by $Mn^{2+}$ or PMA but not by soluble ICAM-1 or fibrinogen, and preincubation of eosinophils with ICAM-1 mAb did not affect induction of the CBRM1/5 epitope (data not shown). This shows that unlike the activation epitope recognized by the LFA-1 mAb 24 (34), induction of the CBRM1/5 epitope is not dependent on the presence of ligands. CBRM1/5 inhibited chemoattractant-stimulated adhesion of eosinophils to ICAM-1 (Fig. 5*C*), indicating that it was mediated by the activated subset of Mac-1, rather than by increased surface expression. A $\beta$1 integrin neoepitope that can be induced by $Mn^{2+}$ or soluble ligands has recently been defined by the mAb 9EG7 (28, 37). In contrast to the CBRM1/5 epitope, chemoattractants failed to upregulate this neoepitope in eosinophils, even in the presence of soluble ligands, whereas $Mn^{2+}$, soluble VCAM-1 or the 40-kDa fragment of fibronectin-induced 9EG7 expression on eosinophils (Fig. 5*A* and data not shown). Thus, chemoattractants appear to regulate VLA-4 and Mac-1 by distinct mechanisms.

## DISCUSSION

We have found that chemoattractants differentially regulate the $\alpha 4\beta 1$ and $\alpha M\beta 2$ integrin-mediated adhesiveness of eosinophils. The CC chemokines RANTES and MCP-3, and the anaphylatoxin C5a transiently increase but subsequently reduce VLA-4-dependent eosinophil adhesiveness to VCAM-1, without changing surface expression of VLA-4. In contrast to

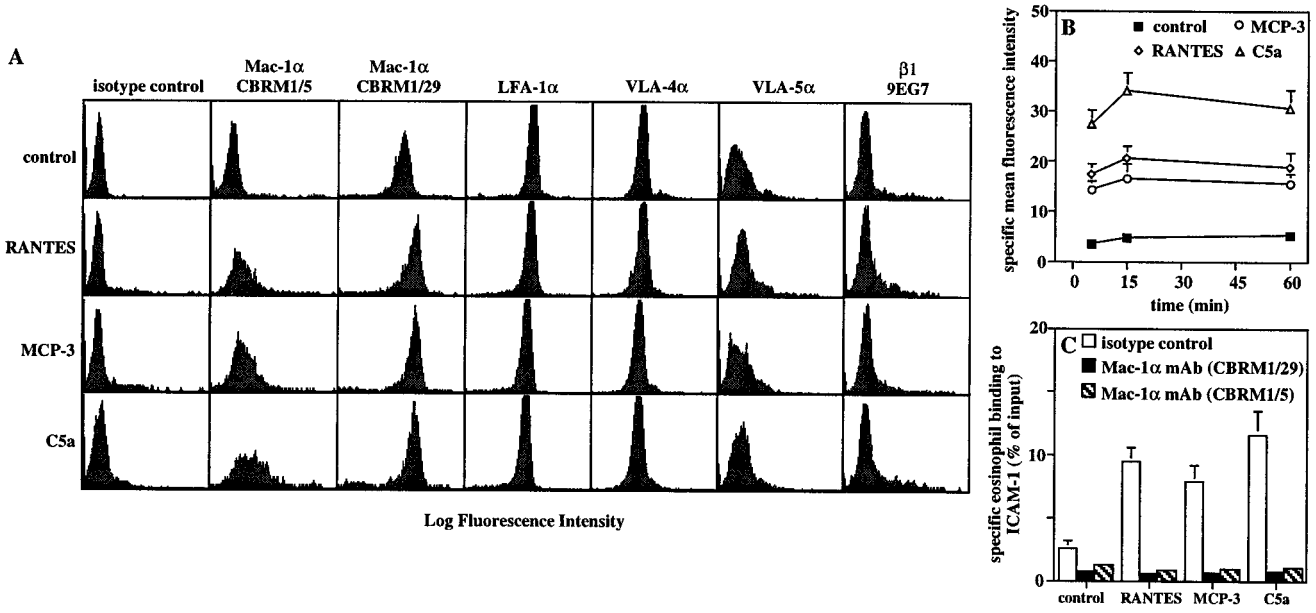


FIG. 5. Chemoattractants induce a Mac-1 activation epitope that mediates adhesion to ICAM-1 in eosinophils. (*A*) Effect of chemoattractants on integrin surface expression. (*B*) Kinetics of Mac-1 activation epitope expression. (*C*) Inhibition with mAb of eosinophil adhesion to ICAM-1. (*A* and *B*) Isolated eosinophils were stimulated with RANTES, MCP-3 (both 100 ng/ml), or C5a (10 ng/ml) or were held unstimulated for 15 min (*A*) or indicated periods (*B*) at 37°C and reacted with saturating amounts of CBRM1/29 (Mac-1$\alpha$), CBRM1/5 (Mac-1$\alpha$ activation epitope), TS1/22 (LFA-1$\alpha$), HP2/1 (VLA-4$\alpha$), A5-PUJ1 (VLA-5$\alpha$), 9EG7 ($\beta$1 neoepitope), or X63 (isotype control) in HHMC with 0.5% BSA. Cells were stained with goat anti-mouse IgG-FITC and analyzed by FACS. Histograms are representative of three independent experiments (*A*). Specific mean fluorescence intensities are mean ± SD of three independent experiments (*B*). (*C*) Isolated eosinophils were subjected to adhesion assays in 96-well-plates coated with ICAM-1 or BSA (10 $\mu$g/ml each) at 37°C in presence of assay medium (control), RANTES, MCP-3 (both 100 ng/ml), or C5a (10 ng/ml) for 30 min after preincubation with Mac-1 mAb (CBRM1/29), Mac-1 activation epitope mAb (CBRM1/5) or isotype control (X63, all 20 $\mu$g/ml) for 20 min. Data are mean ± SD of three independent experiments performed in duplicate.

previous studies that demonstrated transient upregulation of adhesiveness of VLA-4 in fMLP receptor transfectants (38) or after stimulation of lymphocytes with mAb to CD31 (39), our study utilized a blood leukocyte with physiologic agonists, and demonstrates that such regulation may be directly relevant to binding of leukocytes to the vessel wall and emigration from the bloodstream. In contrast to the transient modulation of VLA-4 avidity in eosinophils, chemoattractants stimulated prolonged increases in avidity of the $\beta$2 integrin Mac-1 for ICAM-1. To our knowledge, this is the first report to provide evidence that two integrins expressed on the same cell can be differentially regulated by the same agonist. This demonstrates further versatility in integrin regulation than previously appreciated, and could be of great physiologic importance in modulating adhesion to the many different types of cell surface and matrix integrin ligands encountered by leukocytes as they migrate through tissues. The differences in behavior of $\alpha$4$\beta$1 and $\alpha$M$\beta$2 integrins on chemoattractant-stimulated eosinophils were consistently found with different concentrations and classes of chemoattractants. RANTES and MCP-3 have been suggested to bind to the same receptor on eosinophils (8), whereas C5a binds to a distinct receptor. The C5a and RANTES/MCP-3 receptors function independently—i.e., they do not cross-desensitize one another (7). Thus, the mode of avidity regulation was specific for the integrin not the chemoattractant.

The regulation of VLA-4 and Mac-1 avidity by chemoattractants appears to involve distinct mechanisms. Clearly the pathways for triggering adhesiveness of these integrins differ, because chemoattractants and PMA stimulated transient and prolonged activation of VLA-4, respectively, whereas these agents both stimulated prolonged activation of Mac-1. The kinetics of regulation of VLA-4 adhesiveness for VCAM-1 and fibronectin were remarkably similar, suggesting a similar regulatory mechanism. The regulation of VLA-4 avidity was associated with the actin cytoskeleton, wheras that of Mac-1 was not. Cytochalasins B and D abrogated the increase in VLA-4 but not Mac-1 adhesiveness. Furthermore, chemoattractants and PMA stimulated transient and prolonged F-actin polymerization in eosinophils, respectively, correlating with effects on VLA-4 and not Mac-1. The kinetics of F-actin polymerization and upregulation of adhesiveness differed, but this may be related to assay differences and adhesiveness may be linked to but not directly regulated by F-actin polymerization.

In another contrast between the two integrins, alterations in activation epitopes were detected in Mac-1 and not VLA-4. Chemoattractants stimulated expression of an activation-specific Mac-1 epitope defined with the CBRM1/5 mAb. This epitope, present on a subpopulation of Mac-1 molecules on the surface of activated eosinophils, was closely associated with functional activity, as shown by inhibition of binding to ICAM-1 with CBRM1/5 mAb. Previous studies with neutrophils stimulated with fMLP, PMA or $Mn^{2+}$ suggested that the CBRM1/5 epitope reflected a conformational change in the Mac-1 I domain inducing high avidity binding (16). To test for conformational alterations in VLA-4, we used the 9EG7 mAb to the $\beta$1 subunit (28). The 9EG7 neoepitope, which can be induced by $Mn^{2+}$ or soluble ligand, was not upregulated by chemoattractants or PMA. In previous studies, expression of this epitope was not correlated to integrin activity, when adhesion was stimulated by PMA without increasing ligand binding (37). VLA-4 has been found to exist in multiple activation states with distinct affinities (33, 40). Lateral interaction sites have been defined on $\alpha$4 that are crucial for clustering of VLA-4 and affect adhesion strengthening but not monovalent ligand binding (C. Pujades, S.-K. Kraeff, R. Alon, A. Masumoto, L. Burke, T. A. Springer, L. B. Chen, R. R. Lobb & M. E. Hemler, unpublished data). Likewise, PMA stimulates $\beta$1 integrin-mediated cell adhesion to fibronectin without altering affinity for soluble fibronectin and inhibition by cytochalasin D implicated cytoskeletal events post-ligand binding (e.g., spreading) (36). Thus, adhesiveness of VLA-4

may be regulated by lateral multimerization to strengthen attachment in areas of ligand apposition, and this may be attributed to cytoskeletal reorganization or interaction of the cytoplasmic domain of $\alpha 4$ or $\beta 1$ with cytoskeletal proteins (41). Relative to other $\beta 1$ integrin $\alpha$ subunits, the $\alpha 4$ cytoplasmic tail appears specialized to engage in weaker cytoskeletal interactions (42). This may allow transient activation by chemoattractants. Distinct mechanisms for activation of Mac-1 and VLA-4 may be due to differences in the cytoplasmic domains of their $\alpha$ or $\beta$ subunits that are essential for association with specific regulatory proteins or coupling to distinct signaling pathways.

Migration of eosinophils across resting endothelium induced by RANTES is mediated by $\beta 2$ integrins and ICAM-1; however, interaction of VLA-4 and VCAM-1 also contributes to migration across stimulated endothelium (21). Freezing VLA-4 and VLA-5 in a high affinity state with an activating $\beta 1$ mAb has been shown to block eosinophil migration across fibronectin or endothelium (43). Thus, after L-selectin and VLA-4 promote rolling on and attachment of eosinophils to endothelium (44), appropriate regulation of integrin avidity may be required to allow firm adhesion to the vessel wall to be followed by diapedesis (i.e., migration across endothelium and the basement membrane into tissue). The differential regulation of VLA-4 and Mac-1 by chemoattractants shown here may be important in the choreography of diapedesis. For example, transient adhesion through VLA-4 of eosinophils arrested on endothelium might support locomotion to intercellular junctions by reversible adhesion and detachment events, whereas prolonged activation of Mac-1 might be crucial to complete the process of extravasation at intercellular junctions. By comparison to sustained activation of VLA-4 with the activating $\beta 1$ mAbs 8A2 (43) or TS2/16 (45), which induced eosinophil binding to VCAM-1 of up to 60% of input cells in our assay (data not shown), the sustained adhesion of Mac-1 to ICAM-1 stimulated by chemoattractants appears to be a relatively weak interaction and may therefore not impair diapedesis. Alternatively, deactivation of Mac-1 may be triggered by a signal from the endothelium. VCAM-1 and fibronectin would be encountered sequentially during eosinophil diapedesis and it is unclear why adhesiveness of VLA-4 for these ligands is regulated identically. However, the stimulated adhesiveness of VLA-5 for fibronectin is much more prolonged than of VLA-4, as better illustrated with monocytes, which show more VLA-5-dependent adhesion than eosinophils (45). Furthermore, the concentration of chemoattractants to which eosinophils would be exposed *in vivo* would be expected to change dramatically during diapedesis, and could also contribute to regulation of this complex and currently only rudimentarily understood process.

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