

# A Comparison of C3a and C5a-Mediated Stable Adhesion of Rolling Eosinophils in Postcapillary Venules and Transendothelial Migration In Vitro and In Vivo<sup>1</sup>

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The comparative ability of the complement anaphylatoxins C3a and C5a to mediate leukocyte adhesion and transendothelial migration in vivo and in vitro was investigated. Superfusion of IL-1 $\beta$ -stimulated rabbit mesentery with C3a resulted in a rapid and stable adhesion of rolling eosinophils, but not neutrophils, to postcapillary venules. However, C3a failed to evoke subsequent transmigration of the adherent eosinophils. In contrast, C5a induced both the rapid activation-dependent firm adhesion and transmigration of eosinophils and neutrophils through venular endothelium. C3a induced selective shedding of L-selectin and an increase in  $\alpha_M\beta_2$  integrin expression on eosinophils but not neutrophils, while C5a induced shedding of L-selectin and up-regulation of  $\alpha_M\beta_2$  integrin on both eosinophils and neutrophils. Both C3a- and C5a-dependent adhesion to venular endothelium was blocked by ex vivo treatment of eosinophils with anti- $\alpha_4$  and anti- $\beta_2$  integrin mAbs. In vitro, both C3a (but not C3a<sub>desArg</sub>) and C5a (including C5a<sub>desArg</sub>)-dependent transmigration of eosinophils across IL-1 $\beta$ -stimulated endothelial monolayer was mediated by  $\alpha_4\beta_1$  and  $\alpha_M\beta_2$  integrins. Overall these studies suggest that C3a is eosinophil-specific chemotactic mediator that influences selectively eosinophil adhesion but not transmigration in vivo. C5a in contrast is a complete activator of integrin-dependent adhesion as well as transmigration of eosinophils and neutrophils. *The Journal of Immunology*, 1999, 162: 1127–1136.

The accumulation of eosinophils into extravascular regions of allergic inflammation is dependent on sequential interaction of circulating leukocytes with inflamed endothelial cells lining the vasculature. This complex cascade of transient and activation-dependent adhesion events, under conditions of physiologic flow, is mediated by the selective engagement of adhesion molecules expressed by the eosinophil membrane with counterreceptors on the endothelial surface (1, 2). Among the several mediators of eosinophil activation and recruitment are the complement anaphylatoxins, C3a and C5a. These are small cationic activation peptides ( $M_r$  9,000–11,000) released from components C3 and C5 by the action of C3/5 convertases. The anaphylatoxins of human complement are important as general effectors of inflammation, evoking such varied responses as spasmogenic activity on smooth muscle, tissue edema, granulocyte activation, and chemotaxis (see Refs. 3 and 4 for reviews). However, whereas C5a is a potent chemotaxin for neutrophils and eosinophils, C3a is chemotactic only for eosinophils but not neutrophils (5, 6).

Recently, receptors for C3a and C5a have been cloned and sequenced. These receptors are members of the G-coupled receptor family and are found in a variety of cell types (7–11). The C3a and C5a receptors are membrane constituents of both neutrophils and

eosinophils; however, differing outcomes result from engagement of these receptors with their respective ligands.

Whereas C3a can evoke neutrophil calcium ion mobilization and a respiratory burst but is inactive chemotactically for these cells, C5a can mediate a greater range of neutrophil responses such as a respiratory burst, degranulation, and chemotaxis (6, 12, 13). However, the situation differs for eosinophils as both C3a and C5a are reported to be chemotaxins as well as mediators for degranulation and oxidant secretion (5, 14–16).

Most published findings demonstrating the chemotactic potential of C3a and C5a for eosinophils were based on static in vitro assays (5, 16). Because a greater range of complicating effects occurs in vivo including the physical influence of shear forces in blood vessels, it remains important to demonstrate the ability of the anaphylatoxins to mediate eosinophil adhesion and recruitment under in vivo conditions.

Although our current understanding of the recruitment of eosinophils to allergic inflammatory sites is far from complete, a general multistep paradigm has been described for leukocyte mobilization that provides insight into this phenomenon. According to this paradigm, the three sequential steps involve leukocyte rolling along the vascular wall, followed by activation-dependent firm adhesion of the leukocytes to the endothelium, and finally chemoattractant-directed transmigration of the leukocytes into the extravascular space (1, 17–19). For eosinophils, rolling is the first and rate-limiting step of the multistep adhesion cascade in vivo and is mediated by eosinophil L-selectin (CD62L) interaction (2, 44) with endothelial sialylated and fucosylated glycoconjugates. In addition  $\alpha_4\beta_1$  and  $\alpha_4\beta_7$  integrins mediate eosinophil rolling on VCAM-1 (CD106) (20–24, 44). Moreover, eosinophil rolling in blood vessels is also mediated by endothelial P-selectin (CD62P) (25) but not E-selectin (CD62E) (21, 26). Leukocyte rolling is followed by a stimulus dependent firm adhesion, a process that is accompanied by rapid of activation of  $\beta_2$  integrins (27). In case of eosinophils,  $\alpha_4$  integrins in addition could contribute

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to the adhesion process by interacting with inducible VCAM-1 on vascular endothelium (23, 24).

Transmigration of adherent leukocytes including eosinophils across cultured endothelial cells is dependent on the interaction of  $\alpha_L\beta_2$  and  $\alpha_M\beta_2$  integrins with their endothelial counterreceptors, ICAM-1 (CD54) and ICAM-2 (CD102) (17, 28–31). In addition, homophilic interactions between molecules of platelet endothelial cell adhesion molecule-1 (CD31) on leukocytes with those on endothelial cells have also been reported to be essential for leukocyte motility (32).

Although these studies define a plausible scheme for eosinophil-endothelial cell interactions in general, the underlining mechanisms by which rolling eosinophils respond to a specific chemotactic stimulus and sequester to sites of inflammation are not well understood. Although studies demonstrating that C3a and C5a can function as eosinophil active chemotaxins *in vitro* have also been published (5, 16), the comparative abilities of the anaphylatoxins to direct eosinophil adhesion and subsequent transmigration across the vascular endothelium have not been investigated. In the present report, we have examined the ability of  $\alpha_4$  and  $\beta_2$  integrins to mediate C3a and C5a dependent stable adhesion of rolling eosinophils and their subsequent transmigration across inflamed post-capillary venules of the rabbit mesentery *in vivo* and across cultured endothelial cells *in vitro*.

## Materials and Methods

### Reagents

Dextran 70 was supplied by Baxter Diagnostics Inc. (McGaw Park, IL). Ficoll-Paque PLUS (endotoxin-free) was purchased from Pharmacia Biotech (Piscataway, NJ). Earle's balanced salt solution was supplied by Life Technologies (Grand Island, NY). HEPES and Eosin Y were obtained from Sigma (St. Louis, MO). Goat anti-mouse IgG-coated magnetic beads and a magnetic separation unit were purchased from PerSeptive Biosystems (Framingham, MA). Unconjugated mouse anti-human mAbs against CD16 were purchased from Biosource International (Camarillo, CA). RPMI 1640 and FCS were from Bio-Whittaker (Walkersville, MD). IL-1 $\beta$  was purchased from R&D systems (Minneapolis, MN). Endothelial cell basal media (EBM),<sup>3</sup> endothelial cell growth media (EGM), and primary cultures of HUVECs were purchased from Clonetics (Walkersville, MD). Biocoat inserts (containing 3.0- $\mu$ m pores) were obtained from Becton Dickinson Labware (Bedford, MA). The fluorescent compound, 5- and 6-carboxy-fluorescein diacetate (CFDA), was from Molecular Probes (Eugene, OR). Loperamide hydrochloride (Immodium) was from McNeil-PPC (Fort Washington, PA), while xylazine hydrochloride and lidocaine were from Butler (Columbus, OH).

### Isolation of human C3a, C5a, and carboxypeptidase N (CPN)

Human C3a and C5a were generated from complement components C3 and C5 using fluid phase C3 or C5 convertases. C3a and C5a were isolated subsequently by ion exchange chromatography (33). CPN was purified from the same batch of human plasma used to isolate >20 other plasma proteins (33). Briefly, the steps included 4.5–12% polyethylene glycol precipitation of barium citrate-treated plasma, DEAE-Sephadex column chromatography, gel filtration through Sephacryl S300, and affinity chromatography using *p*-aminobenzoyl-L-arginine-Sepharose.

### Monoclonal Abs

Blocking mAbs with specificities to CD11a ( $\alpha_L$  integrin) (clone TS1/22.1.1.13) and CD11b ( $\alpha_M$  integrin) (clone 44aacb) were isolated from hybridoma cells obtained from American Type Culture Collection (Manassas, VA) (34, 35). Anti-CD11c ( $\alpha_X$  integrin) (clone CBR-p150/4G1) was from Biosource International. mAbs to CD29 ( $\beta_1$  integrin chain) (clone P4C10) (36) were from Life Technologies (Gaithersburg, MD). mAbs with specificity to the integrin chain CD49d ( $\alpha_4$  integrin) (clones P4G9 and P4C2) and those directed against CD51/CD61 ( $\alpha_V\beta_3$  integrin) (clone LM609) and CD51/ $\beta_5$  ( $\alpha_V\beta_5$  integrin) (clone P1F6) were from

Chemicon (Temecula, CA) (37, 38). mAbs against CD18 ( $\beta_2$  integrin) (clone IB4) (39) and human tenascin 81C6 (40) were also used. A mAb preparation against mouse  $\beta_7$  integrin (clone FIB504) with cross-reactivity to human  $\beta_7$  integrin (41) and anti-CD62L (L-selectin; clone DREG200) (42) were obtained from Dr. Eugene Butcher (Stanford University, Stanford, CA). Preparations of mAbs with endotoxin levels in range of 1–10 ng/mg as determined by amebocyte *Limulus* test were used. Phycoerythrin-conjugated secondary Abs to mouse and rat IgG were purchased from Jackson Research Laboratories (West Grove, PA). FITC-conjugated Ab to CD9 was obtained from PharMingen (San Diego, CA).

### Eosinophil preparation

Eosinophils were prepared from the peripheral blood of normal donors, having at least 3% peripheral blood eosinophilia, using essentially a two-step procedure. First, an eosinophil-enriched fraction was obtained by centrifugation through Ficoll-Paque PLUS. The second step involved removal of residual neutrophils using negative selection of CD16-positive cells by a magnetic bead separation technique (43). Eosinophils were >99% pure as assessed by eosin staining.

For both *in vitro* and *in vivo* experiments, eosinophils were labeled fluorescently using CFDA similarly to a procedure previously described (44). Eosinophils ( $1.5 \times 10^7$  cells) were suspended in 5 ml of buffer A: 10 mM HEPES, pH 7.35, 0.145 M NaCl, 5 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub> and reacted with a solution (100  $\mu$ l) of CFDA (2 mg/ml) for 30 min in the dark. The cells were washed once with buffer containing 1 mg/ml human serum albumin and suspended in the same buffer or in EBM at a concentration of  $10^6$  cells/ml.

### Animal preparation and superfusion of the mesenteric vascular bed with C3a and C5a

The circulation of CFDA-labeled human eosinophils in postcapillary venules of IL-1 $\beta$ -stimulated mesenteric blood vessels of New Zealand White rabbits was examined by intravital microscopy as described earlier (26, 44). The ability of C3a or C5a to mediate adhesion and transmigration of rolling eosinophils across IL-1 $\beta$ -stimulated mesenteric venules was determined. Between 6 and 10 h after IL-1 $\beta$  stimulation (*i.p.*), portions of the exposed mesentery were superfused with  $10^{-9}$ – $10^{-6}$  M C3a or C5a using a constant flow infusion syringe pump (Harvard Apparatus, South Natick, MA). The flow of the syringe pump was adjusted to match the flow of the superfusion buffer. CFDA-labeled eosinophils ( $0.2$ – $0.5 \times 10^7$  cells) were administered into the mesenteric circulation 5 min after the superfusion had ensued. These cells were injected successively through the side branch of the terminal superior mesenteric artery cannulated with a PE10 polyethylene catheter as previously described (27).

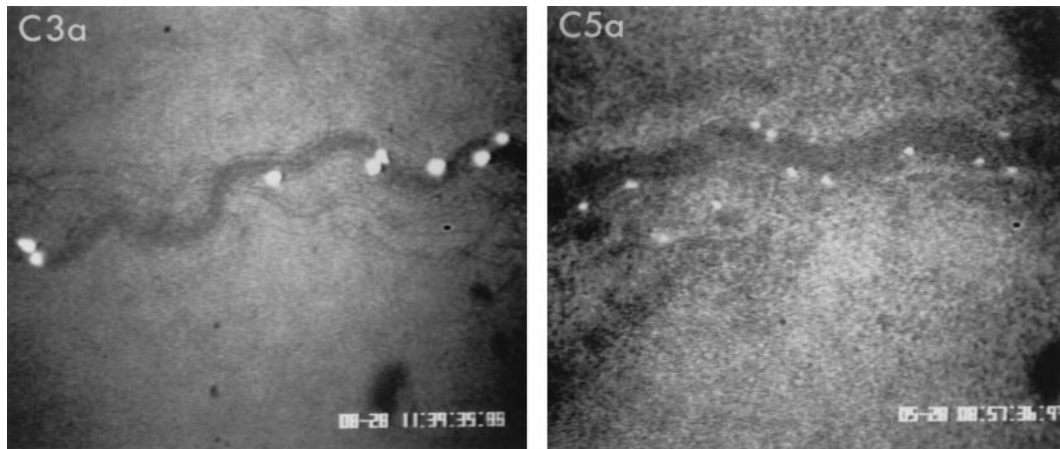
### Intravital microscopy and image analysis

The passage of CFDA-labeled eosinophils in the inflamed venules was made visible by stroboscopic epillumination as previously described, and the images were recorded with an SVHS video recorder (27). The video images were analyzed offline by manually counting the total number of CFDA-labeled eosinophils passing through a reference point in a venular segment (total flux). The tapes were rewound, and only those cells found to be visibly rolling along the venular wall were counted (rolling flux). Rolling fraction (RF) was calculated as the percentage of rolling cells in the total flux of eosinophils passing through a venular segment during a given injection. Adherent eosinophils were defined as cells that remained stationary in the venule for at least 30 s, and data are expressed as adherent cells/250- $\mu$ m length of the vessel. Eosinophil transmigration was quantified by enumerating the number of adherent cells that had emigrated away from the vessel wall in parallel with a 250- $\mu$ m length of a vessel segment.

### Inhibition of *in vivo* adhesion and transendothelial migration by anti-integrin mAbs

The abilities of anti- $\alpha_4$ , anti- $\beta_1$ , anti- $\beta_2$ , or anti- $\beta_7$  integrin mAbs to block eosinophil adhesion and transmigration across IL-1 $\beta$ -stimulated venular endothelium were determined as follows. Eosinophils were incubated *in vivo* with functional blocking anti- $\alpha_4$  integrin (mAb P4G9), anti- $\beta_1$  integrin (mAb P4C10), anti- $\beta_2$  integrin (mAb IB4), anti- $\beta_7$  integrin (mAb FIB504) Abs, or combinations of these at a concentration of 50  $\mu$ g/ml for 20 min at room temperature before injection into the rabbit mesentery, which was superfused with either C3a or C5a ( $10^{-9}$ – $10^{-6}$  M). As a control a mAb with a specificity vs human tenascin (clone 81C6) was used. Statistical analyses were made using analysis of variance and multiple comparisons tests. For all tests, *p* values <5% were considered significant. Data are presented as mean  $\pm$  SD. Statistical calculations were computed with a statistical software package (SigmaStat, Jandel Scientific, San Rafael, CA).

<sup>3</sup> Abbreviations used in this paper: EBM, endothelial cell basal medium; CFDA, carboxyfluorescein diacetate; CPN, carboxypeptidase N; EGM, endothelial cell growth medium; RF, rolling fraction.



**FIGURE 1.** Stable arrest in vivo of rolling eosinophils in response to activation with C3a and C5a. Superfusion of the IL-1 $\beta$ -stimulated rabbit mesentery with C3a ( $10^{-6}$  M) (left) and C5a ( $10^{-7}$  M) (right) resulted in firm adhesion of rolling CFDA-labeled eosinophils (bright spheres) to the endothelium of postcapillary venules. At all the concentrations tested, C5a induced greater adhesion of rolling eosinophils than C3a. CFDA-labeled eosinophils were made visible by stroboscopic epiillumination.

#### Flow cytometry

Granulocyte preparations, which were enriched for eosinophils (>10% eosinophils), were obtained according to the eosinophil isolation procedure using centrifugation through Ficoll-Paque PLUS (45). Hypotonic lysis to remove erythrocytes was not performed. Granulocytes were stimulated for 15 min at 37°C with either C3a (100 nM) or C5a (10 nM). The cells were immediately placed on ice and were incubated first with primary mAbs (5  $\mu$ g/ml) in HBSS containing 10% FBS for 45 min. The cells were washed and incubated with appropriate phycoerythrin-conjugated secondary Abs to either mouse or rat IgG (FIB27) followed by incubation with FITC-conjugated Ab to CD9, an eosinophil-specific granulocyte marker. The cells were washed again and analyzed for receptor expression using Becton Dickinson FACScan or FACSsort instruments with CellQuest software (Becton Dickinson, San Jose, CA). A minimum of 5000 eosinophils was collected and analyzed for each determination. Data from three experiments were normalized to the staining with nonspecific mouse IgG as a negative control and are expressed as arbitrary fluorescence units.

#### Eosinophil chemotaxis assays

Chemotaxis of eosinophils was performed using a two-chamber system. The upper chamber consisted of an insert that was separated from the lower chamber by a membrane of polyethylene terephthalate containing 3- $\mu$ m pores. For each determination, a total of  $10^5$  labeled eosinophils was placed in the upper insert. The lower well contained 700  $\mu$ l of buffer A containing 1 mg/ml human serum albumin with various concentrations of C3a ( $10^{-9}$ – $10^{-5}$  M) or C5a ( $10^{-10}$ – $10^{-6}$  M). The plates were incubated at 37°C for 2 h. Then EDTA was added to a final concentration of 10 mM to the bottom well, and the plates were allowed to stand for 10 min at room temperature before the inserts were removed from the wells. The cells, which had passed through the membrane and which were contained within a 1.1-mm<sup>2</sup> central area on the bottom well, were counted using an inverted fluorescence microscope (Leitz Fluovert FS, Wetzlar, Germany).

#### Preparation of HUVECs

Individual growth factor supplements were added to EBM to formulate the growth media EGM. These supplements include bovine brain extract, human epidermal growth factor, hydrocortisone, gentamicin, and FBS (final concentration, 2%). EGM (4 ml) containing  $1$ – $2 \times 10^5$  cells/ml of HUVEC was transferred to tissue culture flasks and incubated in a humidified atmosphere at 37°C and 5% CO<sub>2</sub>. Medium was changed every 2 days until reaching confluence between 6 and 10 days after plating. Confluent cells were trypsinized, washed, and resuspended in EGM. Aliquots of 250  $\mu$ l were added to the upper fibronectin-coated inserts of the transwell assemblies, and 700- $\mu$ l portions of EGM were added to the lower wells. The medium was changed every 2 days. FITC-labeled albumin was added to the upper well of both HUVEC-containing and empty Transwell (Corning Costar, Cambridge, MA) chambers to guide assessment of confluency. When confluent, EBM containing  $10^3$  U/ml IL-1 $\beta$  was added to both upper and lower chambers, and cells were cultured for an additional 18 h before transmigration assays.

#### Eosinophil transendothelial migration assays

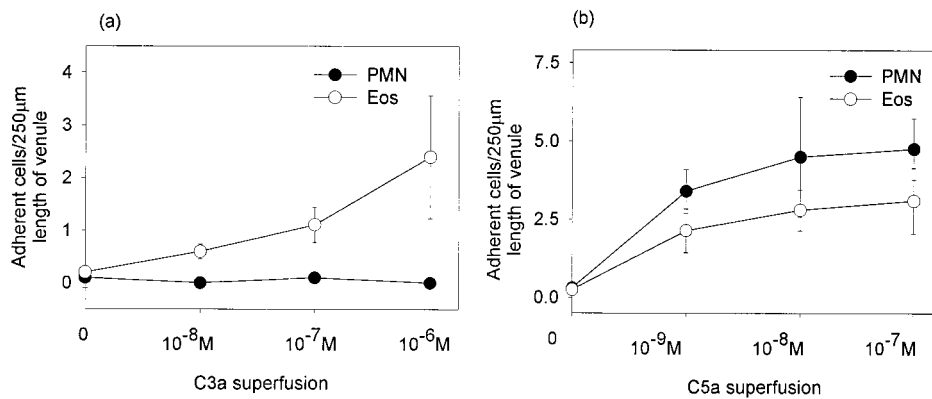
Transmigration of eosinophils through endothelial cells, which were grown as confluent monolayers on fibronectin-coated inserts, was performed similarly to the assays for chemotaxis through pores in polyethylene terephthalate membranes, except that EBM was used instead of buffer A. The inhibition of C3a- or C5a-mediated transmigration of eosinophils by various anti-integrin mAbs was performed by mixing the eosinophils with mAbs (5  $\mu$ g/ml) for 10 min at 23°C before these cells were used in transmigration assays. To examine the potential role of CPN on eosinophil transmigration evoked by C3a and C5a, CPN (10  $\mu$ g/ml) was placed into the bottom wells just before the addition of eosinophils, and transmigration assays were conducted as before.

## Results

#### Superfusion of IL-1 $\beta$ -stimulated mesenteric blood vessels with C3a and C5a results in stable adhesion of rolling leukocytes in postcapillary venules

To examine the possible effects of C3a and C5a on the behavior of circulating eosinophils and neutrophils, CFDA-labeled cells were administered into the rabbit mesentery microcirculation, and their ability to interact with IL-1 $\beta$ -stimulated postcapillary venules was visualized by intravital microscopy. CFDA-labeled eosinophils were observed to roll avidly (RF; range, 20–80%) along the walls of cytokine-stimulated postcapillary venules. In the absence of further activation, spontaneous adhesion of rolling eosinophils was not frequently observed in the majority of the venules examined. Although firm adhesion of a few eosinophils was occasionally noticed, this represented only 1–5% of the total number of rolling eosinophils. We next determined whether rolling eosinophils would firmly adhere in the postcapillary venules in response to extravascular stimulation by the complement anaphylatoxins. To examine this question, the microcirculation within the exteriorized mesentery, which had been pretreated for 6–10 h with IL-1 $\beta$ , was constantly superfused with C3a or C5a ( $10^{-6}$ – $10^{-9}$  M), and the interaction of the infused labeled eosinophils in the inflamed blood vessels was examined microscopically (Fig. 1). In contrast to a lack of spontaneous adhesion of eosinophils observed in majority of the blood vessels, administration of CFDA-labeled eosinophils into the mesentery superfused with C3a ( $10^{-6}$ – $10^{-8}$  M) resulted in the rapid and stable adhesion of rolling eosinophils (range, 2–4 adherent cells/250- $\mu$ m length at  $10^{-6}$  M) (Fig. 2). Adhesion was observed in venules but not arterioles (Fig. 1). In comparison with





**FIGURE 2.** Quantitative comparison of the effect of C3a and C5a on stable arrest of rolling of eosinophils and neutrophils in vivo. Rabbit mesentery was stimulated with IL-1 $\beta$  (i.p.) for 6–10 h. Baseline rolling of cells in the mesenteric circulation was first determined by administration of CFDA-labeled eosinophils or neutrophils through the cannulated side branch of superior mesenteric artery. Subsequently, the exteriorized mesentery was continuously superfused with C3a ( $10^{-6}$ – $10^{-8}$  M) (left) or C5a (right) ( $10^{-7}$ – $10^{-9}$  M), and the effects on injected neutrophils (●) or eosinophils (○) were determined. Cells were considered to be adherent if they remained stationary along the vessel wall for at least 30 s. Data represent mean  $\pm$  SD adherent cells/250- $\mu$ m length of venule derived from 2–5 venules/animal ( $n = 3$ –5 rabbits).

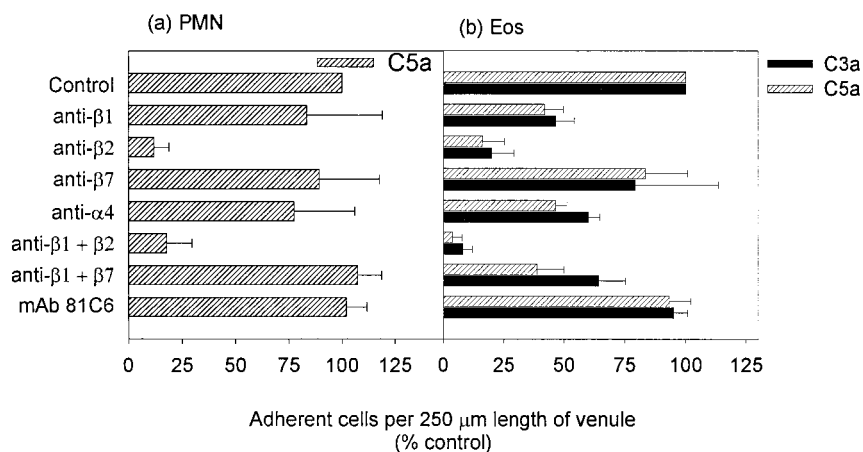
eosinophils, neutrophils failed to adhere in response to C3a, suggesting that this chemoattractant selectively activates rolling eosinophils, but not neutrophils (Fig. 2).

In contrast, administration of either eosinophils or neutrophils into the mesenteric blood stream superfused with C5a ( $10^{-7}$  M) resulted in a rapid and dose-dependent increase in firm adhesion of both rolling eosinophils (range, 2–4 adherent cells/250- $\mu$ m length of venule) and neutrophils (range, 3–6 adherent cells/250- $\mu$ m length of venule) (Figs. 1 and 2). Maximum adhesion was observed when rolling eosinophils were superfused at a concentration of  $10^{-6}$  M C3a, while C5a-induced adhesion was maximally observed between  $10^{-7}$  and  $10^{-8}$  M. In all experiments that we performed, C5a was more effective than C3a in inducing firm adhesion of rolling eosinophils on cytokine-stimulated venular endothelium.

#### Effect of anti-integrin mAbs on C3a- and C5a-mediated firm adhesion in vivo

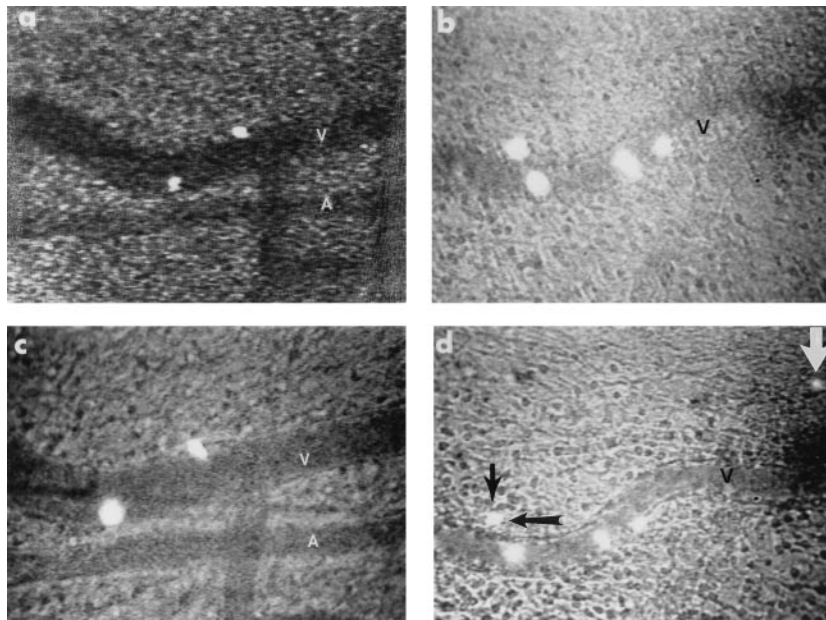
$\alpha_4$  ( $\alpha_4\beta_1/\alpha_4\beta_7$ ) and  $\beta_2$  integrins are known to mediate adhesion of leukocytes in vitro. Therefore, we hypothesized that the observed

activation-dependent firm adhesion of eosinophils or neutrophils to rabbit mesentery venules induced by C3a and C5a was dependent on the engagement of one or several of these adhesion molecules. To examine this, eosinophils and neutrophils were treated ex vivo with functional blocking mAbs directed against  $\alpha_4$ ,  $\beta_1$ ,  $\beta_2$ , and  $\beta_7$  integrins before administration into C3a- or C5a-stimulated rabbit mesentery, and the ability of rolling eosinophils to adhere to the venular endothelium was determined (Fig. 3). Pretreatment of eosinophils with anti- $\alpha_4$ , anti- $\beta_1$ , or anti- $\beta_2$  integrin mAbs resulted in ~40–80% inhibition of C3a-induced adhesion, and similarly these Abs inhibited C5a-mediated adhesion ~50–80%. A smaller effect was also observed with anti- $\beta_7$  integrin, which inhibited C3a- and C5a-induced adhesion ~20%. No further inhibition of adhesion was observed when eosinophils were pretreated with anti- $\beta_1$  integrin and anti- $\beta_7$  integrin mAbs in combination. The nearly complete inhibition of adhesion induced by preincubation of eosinophils with anti- $\beta_1$  and anti- $\beta_2$  integrin mAbs in combination is suggestive of the significant involvement of both  $\beta_1$  ( $\alpha_4\beta_1$ ) and  $\beta_2$  integrins in C3a- or C5a-mediated adhesion. An irrelevant control mAb 81C6 had little inhibitory effect on C3a- and C5a-induced



**FIGURE 3.** Inhibition of C3a-mediated adhesion of eosinophils and C5a-mediated adhesion eosinophils and neutrophils by mAbs to various integrins. CFDA-labeled neutrophils (PMNs) (a) or CFDA-labeled eosinophils (b) were preincubated with function blocking anti-CD49d ( $\alpha_4$  integrin), anti-CD29 ( $\beta_1$  integrin), anti-CD18 ( $\beta_2$  integrin), anti- $\beta_7$  integrin, anti- $\beta_1 + \beta_2$ , anti- $\beta_1 + \beta_7$ , or control mAb 81C6 (50  $\mu$ g/ml ex vivo) for 20 min at room temperature, before administration into the mesenteric circulation that was superfused continuously with C3a ( $10^{-6}$  M) or C5a ( $10^{-7}$  M). For neutrophils (PMNs), data are shown only for C5a because C3a did not affect these cells. Data represents adhesion (% control cells)/250- $\mu$ m length of venules (mean  $\pm$  SD).

**FIGURE 4.** C5a but not C3a mediates transmigration of adherent eosinophils across IL-1 $\beta$ -stimulated endothelium in the rabbit mesentery. *Left*, CFDA-labeled eosinophils adherent to postcapillary venules after C3a stimulation ( $10^{-6}$  M) at 5 min (*a*) and 30 min (*c*). *Right*, Demonstration of the ability of adherent CFDA-labeled eosinophils to transmigrate in response to C5a ( $10^{-7}$  M) was determined by observing emigration of adherent eosinophils between 5 min (*b*) and 30 min (*d*). Arrows point to individual eosinophils that migrated into the extravascular space in response to C5a. A refers to arterioles and V to venules. Magnification,  $\times 400$ .



adhesion of eosinophils. In contrast to eosinophils, neutrophil adhesion to the venular endothelium induced by C5a was inhibited only by mAbs against  $\beta_2$  integrins and not by those against  $\beta_1$ ,  $\alpha_4$ , or  $\beta_7$  integrins (Fig. 3).

#### *Demonstration of eosinophil and neutrophil transendothelial migration in vivo in response to C5a but not C3a*

Although C3a evokes chemotaxis of eosinophils in vitro (5), C3a was not observed to cause extravascular migration of the adherent eosinophils in vivo after the mesentery was superfused with this anaphylatoxin ( $10^{-7}$ – $10^{-6}$  M) (Fig. 4). It was not possible to evaluate the effect of C3a at a superfusion concentration of  $>10^{-6}$  M because at these levels of anaphylatoxin application to the rabbit mesentery resulted in intravascular coagulation, platelet aggregation, thrombus formation, and even cessation of blood flow. A clear evidence of extravascular migration was not discernible after superfusion of the mesentery with C3a (1 emigrated cell in  $n = 5$  rabbits, 3–5 venules/experiment) (Fig. 5). However, a careful inspection of the venular endothelium under higher resolution ( $\times 40$

objective) revealed that this anaphylatoxin did cause eosinophil penetration between adjacent venular endothelial cells. Nevertheless, these adherent eosinophils failed to emigrate from the vessel wall into the extravascular space even after 30 min of C3a exposure (Fig. 4).

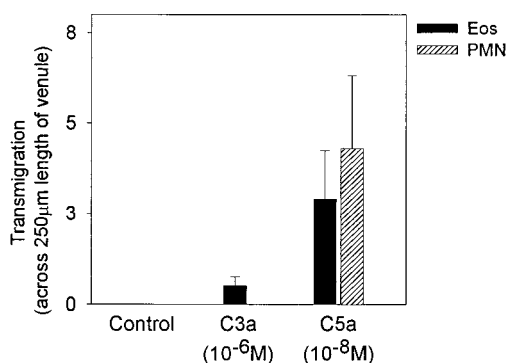
In contrast, C5a,  $10^{-6}$ – $10^{-9}$  M, did induce transmigration of adherent eosinophils across mesenteric venules into the extravascular space (Fig. 4). The extent of C5a-induced eosinophil migration was variable and ranged from 3 to 5 cells along a 250- $\mu$ m length of the venular wall (Fig. 5). Maximal transmigration of eosinophils was observed when C5a was superfused at a concentration of  $10^{-7}$ – $10^{-8}$  M. Likewise, the extent of C5a-induced neutrophil emigration varied from donor to donor (range, 3–7 cells along a 250- $\mu$ m length of the venular wall) (Fig. 5). Most adherent cells were observed to emigrate into the extravascular space after 10–15 min of exposure to C5a.

#### *Venular hemodynamics*

In addition to adhesion, we examined the effects of C3a and C5a superfusion on venular hemodynamics in the rabbit mesentery (Table I). Superfusion of C3a and C5a ( $10^{-6}$  and  $10^{-7}$  M, respectively) had no significant effect on either the velocity of rolling eosinophils and neutrophils or shear stress within the postcapillary venules.

#### *Modulation of the surface expression of eosinophil and neutrophil adhesion molecules in response to activation with C3a and C5a*

Since chemoattractants modulate activity and expression of cell surface receptors, we used flow cytometry to examine whether or not C3a and C5a affected the levels of several eosinophil and neutrophil adhesion molecules (Table II). C3a altered eosinophil but not neutrophil surface expression of several adhesion molecules that we examined. Exposure of eosinophils to C3a resulted in an  $\sim 20\%$  reduction of L-selectin, an appreciable augmentation ( $>100\%$ ) of  $\alpha_M$  integrin, and a smaller rise (20–40%) in  $\beta_2$ - and  $\alpha_X$  integrins. In contrast to the selective effect of C3a on the expression of eosinophil adhesion molecule expression, C5a had similar effects on both cell types. For both eosinophils and neutrophils C5a evoked a 20–50% reduction in L-selectin and a significant



**FIGURE 5.** Determination of C5a-mediated transmigration of emigrated eosinophils and neutrophils in the rabbit mesentery. The number of labeled cells invading the extravascular space in response to C3a and C5a was measured for each 250- $\mu$ m venule length. Only C5a generated measurable results for both eosinophils (black bars) and neutrophils (gray bars). Data represent numbers of transmigrating cells across a 250- $\mu$ m length of postcapillary venule (mean  $\pm$  SD).

Table I. *Effect of C3a and C5a on venular microhemodynamics in the rabbit mesentery<sup>a</sup>*

	PMN	PMN + C3a	PMN + C5a	Eos	Eos + C3a	Eos + C5a
Diameter ( $\mu\text{m}$ )	48.2 $\pm$ 13.9	31.2 $\pm$ 8.5	32.7 $\pm$ 13.6	36.6 $\pm$ 13.2	33.1 $\pm$ 8.7	48.6 $\pm$ 17.7
Rolling velocity (mm/s)	0.23 $\pm$ 0.19	0.26 $\pm$ 0.17	0.27 $\pm$ 0.18	0.30 $\pm$ 0.20	0.29 $\pm$ 0.18	0.25 $\pm$ 0.16
Shear rate ( $\text{s}^{-1}$ )	264 $\pm$ 115	303 $\pm$ 101	186 $\pm$ 51	221 $\pm$ 166	183 $\pm$ 61	217 $\pm$ 35
Shear stress ( $\text{dyn/cm}^2$ )	6.6 $\pm$ 2.9	7.6 $\pm$ 2.6	4.7 $\pm$ 1.3	5.5 $\pm$ 4.2	4.6 $\pm$ 1.5	5.4 $\pm$ 0.9

<sup>a</sup> Values are mean  $\pm$  SD obtained from 3–5 rabbits/group; 2–5 vessels per animal.

up-regulation (170–220%) of  $\alpha_M$  integrin. The up-regulation of  $\beta_2$  integrin by C5a was the only adhesion molecule that showed any appreciable cell type dependence, where the augmentation for neutrophils ( $\sim$ 160%) exceeded that of eosinophils ( $\sim$ 30%). For both cell types, C5a evoked moderately large increases (40–100%) in  $\alpha_X$  integrin expression. No significant changes in the expression of  $\alpha_L$ ,  $\alpha_4$ , or  $\beta_1$  integrins were observed after stimulation of either cell type with either anaphylatoxin.

#### *Comparative effects of C3a and C5a on eosinophil chemotaxis and transendothelial migration in vitro*

Since C3a and C5a were observed to evoke eosinophil adhesion and C5a induced transmigration in vivo, we proceeded further to elucidate mechanisms and regulation of these two complement mediators for eosinophil chemotaxis and transendothelial migration in vitro. Eosinophils were observed to be more responsive chemotactically to C5a than C3a when assayed for migration through 3- $\mu\text{m}$  pores in polyethylene terephthalate filters (Fig. 6). The effective range of concentration for chemotaxis induced by C5a was  $10^{-8}$ – $10^{-6}$  M, whereas that for C3a was  $10^{-7}$ – $10^{-5}$  M.

Furthermore, at peak activities, about 1.5-fold more eosinophils responded to C5a than C3a (Fig. 6).

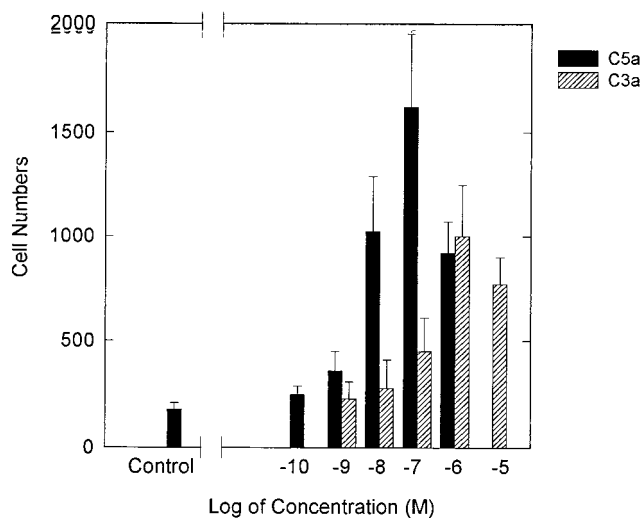
The superior effectiveness of C5a to C3a for induction of migration of eosinophils was demonstrated further for transmigration of eosinophils through HUVEC monolayers (Fig. 7). In these cases, at the optimal concentrations of the anaphylatoxins, more than twice as many eosinophils transmigrated in response to C5a than C3a. This effect occurred whether or not the endothelial cells were primed with IL-1 $\beta$ . Furthermore, the optimal concentration for transendothelial migration was an order of magnitude lower for C5a than C3a ( $10^{-7}$  M vs  $10^{-6}$  M).

Moreover, regardless of the mediator (i.e., C3a or C5a), more transmigration occurred when the endothelial cells were primed with IL-1 $\beta$  than when these cells were unstimulated or when chemotaxis occurred through uncoated plastic membranes (Figs. 6 and 7). Since the in vivo observations suggested the engagement of eosinophil  $\alpha_4/\beta_1$  and  $\beta_2$  integrins (with counterreceptors presented by activated endothelial cells), we examined potential inhibition by anti-integrin mAbs on transmigration of eosinophils through both unstimulated and IL-1 $\beta$ -primed HUVECs.

Table II. *Comparison of the effect of C3a and C5a on the surface expression of adhesion molecules on neutrophils and eosinophils*

Ag	Stimulus	Neutrophils		Eosinophils	
		MFI <sup>a</sup>	% change	MFI	% change
$\alpha_L$	None	590 $\pm$ 93		578 $\pm$ 89	
	C3a	609 $\pm$ 84	4 $\pm$ 2	602 $\pm$ 79	6 $\pm$ 3
	C5a	691 $\pm$ 16	17 $\pm$ 8	591 $\pm$ 60	5 $\pm$ 6
$\alpha_M$	None	1066 $\pm$ 217		464 $\pm$ 144	
	C3a	1238 $\pm$ 264	13 $\pm$ 9	964 $\pm$ 280	111 $\pm$ 15
	C5a	3179 $\pm$ 550	225 $\pm$ 69	1147 $\pm$ 261	172 $\pm$ 34
$\alpha_X$	None	105 $\pm$ 37		73 $\pm$ 25	
	C3a	90 $\pm$ 33	-12 $\pm$ 3	95 $\pm$ 35	22 $\pm$ 14
	C5a	217 $\pm$ 64	99 $\pm$ 19	102 $\pm$ 29	39 $\pm$ 8
$\alpha_4$	None	7 $\pm$ 3		217 $\pm$ 60	
	C3a	6 $\pm$ 1		224 $\pm$ 59	5 $\pm$ 3
	C5a	11 $\pm$ 3		223 $\pm$ 63	3 $\pm$ 5
$\beta_1$	None	119 $\pm$ 28		220 $\pm$ 33	
	C3a	128 $\pm$ 15	7 $\pm$ 2	225 $\pm$ 26	4 $\pm$ 6
	C5a	151 $\pm$ 19	24 $\pm$ 4	227 $\pm$ 31	5 $\pm$ 7
$\beta_2$	None	143 $\pm$ 31		261 $\pm$ 111	
	C3a	173 $\pm$ 34	21 $\pm$ 8	354 $\pm$ 134	39 $\pm$ 7
	C5a	377 $\pm$ 75	162 $\pm$ 54	320 $\pm$ 145	28 $\pm$ 14
$\beta_7$	None	11 $\pm$ 9		25 $\pm$ 12	
	C3a	10 $\pm$ 11		25 $\pm$ 11	0 $\pm$ 5
	C5a	11 $\pm$ 8		27 $\pm$ 14	11 $\pm$ 13
L-selectin	None	776 $\pm$ 47		435 $\pm$ 63	
	C3a	887 $\pm$ 108	9 $\pm$ 6	334 $\pm$ 56	-23 $\pm$ 4
	C5a	432 $\pm$ 75	-49 $\pm$ 7	330 $\pm$ 55	-24 $\pm$ 8

<sup>a</sup> MFI, mean fluorescence intensity.



**FIGURE 6.** Comparison of chemotaxis of eosinophils mediated by C3a and C5a. Anaphylatoxin-mediated chemotaxis of eosinophils through uncoated polyethylene terephthalate membranes containing 3- $\mu$ m pores was measured in response to various concentrations of C3a (hatched bars) or C5a (black bars).

#### Effect of anti-integrin mAb on C3a- and C5a-mediated transmigration in vitro

The profiles for the inhibition by anti-integrin mAbs of C3a and C5a induced transmigration of eosinophils through unstimulated endothelial cells were similar (Fig. 8). Both C3a- and C5a-induced eosinophil migration were inhibited strongly by mAbs to  $\alpha_L$  and  $\beta_2$  integrins, but only weak inhibition, if any, was caused by the other anti-integrin mAbs tested (Fig. 8).

The profiles for the inhibition of anaphylatoxin-induced transmigration of eosinophils through IL-1 $\beta$ -primed HUVECs differed from those of unstimulated endothelial cells (Figs. 8 and 9). When cytokine-stimulated endothelial cells were used, inhibition was caused not only by mAbs to  $\alpha_L$  and  $\beta_2$  integrins but also by those to  $\alpha_M$ ,  $\alpha_4$ , and  $\beta_1$  integrins, and to a lesser extent  $\beta_7$  integrin (Fig. 9). In contrast, other mAbs failed to block appreciably eosinophil transmigration. This spectrum of inhibition by mAbs was the same whether or not C3a or C5a was the mediator for eosinophil migration (Fig. 9).

#### Effect of CPN on C3a- and C5a-mediated eosinophil transendothelial migration in vitro

To explore possible differences in eosinophil migration evoked by C3a and C5a in vivo and in vitro, the influence of CPN, a plasma inhibitor of the anaphylatoxins, was examined. When this carboxypeptidase was present at a concentration of 10  $\mu$ g/ml along

with the anaphylatoxins, all migration of eosinophils to C3a (C3a<sub>desArg</sub>) was lost, whereas residual migration to C5a (C5a<sub>desArg</sub>) was retained (Fig. 10). The optimal concentration of C5a<sub>desArg</sub> for transmigration activity was about 1–2 orders of magnitude higher than that for intact C5a, and the number of cells that migrated at the optimal concentration of C5a<sub>desArg</sub> was almost one-half that at the optimal concentration of C5a.

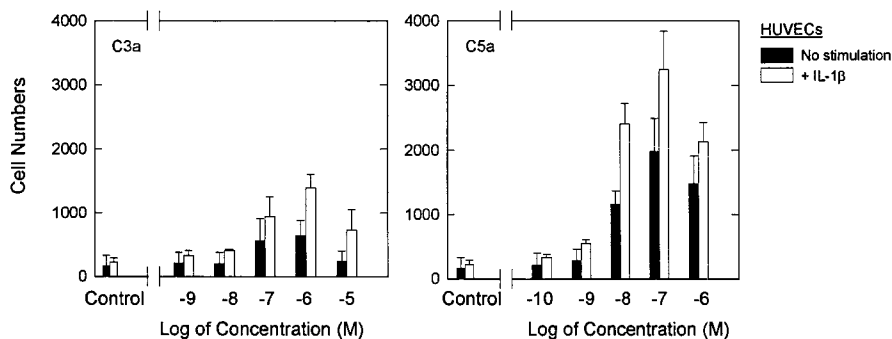
## Discussion

There are both similarities and differences in the mechanisms by which the two complement anaphylatoxins, C3a and C5a, recruit granulocytes. Because C3a and C5a were shown to be chemoattractants for eosinophils in vitro (5, 16), we extended these findings to examine the effects of these anaphylatoxins in vivo. Our observations have resulted in the identification of several important aspects of complement-mediated eosinophil recruitment. C3a can induce a rapid activation-dependent adhesion of rolling eosinophils but not neutrophils on IL-1 $\beta$ -stimulated mesenteric venules. Although C3a-stimulated eosinophils could penetrate the gap between adjacent endothelial cells, this anaphylatoxin did not evoke eosinophil transmigration into the extravascular space (Figs. 1–4).

In contrast, C5a induces rapid activation-dependent adhesion of both rolling neutrophils and eosinophils. Moreover, C5a-induced adhesion is associated with subsequent chemotaxis and transmigration of both eosinophils and neutrophils into tissue. These studies suggest that in vivo C3a selectively influences rolling eosinophils to adhere at sites of inflammation but may not be a potent chemoattractant in that it fails to induce transmigration of the adherent cells under the conditions reported. In contrast, C5a is a complete activator of integrin-dependent adhesion as well as an effective chemoattractant for eosinophils and neutrophils in vivo. The effects of C3a and C5a on eosinophils and neutrophils are biochemical and are not a result of alterations in hemodynamic properties (Table I). Parameters such as rolling velocity and shear rate of eosinophils and neutrophils did not vary appreciably to account for the effects of the anaphylatoxins.

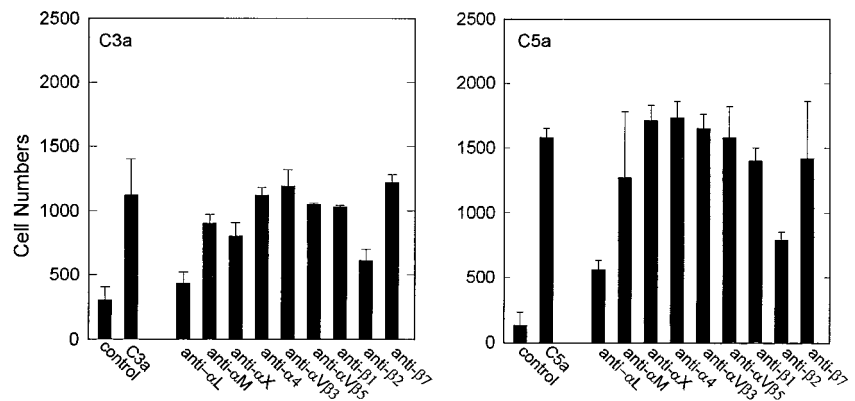
Variations in the spectrum of adhesion molecules on the surface of the eosinophil in response to C3a and C5a are expected to be important for the transitions that this cell type undergoes during emigration as described by the multistep paradigm. Alterations of the levels of several eosinophil and neutrophil membrane adhesion molecules were determined by flow cytometry after these cells were exposed to C3a or C5a. For eosinophils both anaphylatoxins acted similarly by evoking a shedding of L-selectin with a concomitant rise in  $\alpha_M$ ,  $\alpha_X$ , and  $\beta_2$  integrins, while  $\alpha_L$ ,  $\alpha_4$ , and  $\beta_1$  integrin profiles remained essentially unchanged (Table II). L-selectin is utilized for eosinophil rolling along the vascular endothelium (44), and its shedding corresponding with an up-regulation of eosinophil  $\alpha_M\beta_2$  integrin was found to occur during transendothelial migration (30). This suggests that alterations of these adhesion

**FIGURE 7.** Comparison of C3a- and C5a-induced eosinophil transmigration through unstimulated and IL-1 $\beta$ -treated endothelial cells. Endothelial confluent monolayers were grown on fibronectin-coated polyethylene terephthalate filters. Eosinophil transmigration evoked by C3a (left) or C5a (right) through untreated (black bars) and IL-1 $\beta$ -treated (white bars) endothelial monolayers was measured.





**FIGURE 8.** Inhibition of C3a- and C5a mediated transmigration through unstimulated endothelial cell monolayers by anti-integrin mAbs. Eosinophils were mixed with mAbs (5  $\mu\text{g/ml}$ ), which had specificities for various integrin chains, for 10 min at 23°C before usage in transmigration assays. Eosinophil transmigration evoked by  $10^{-6}$  M C3a (left) or  $5 \times 10^{-8}$  M C5a (right) through endothelial cell barriers was measured subsequently.



molecules may be important for the accumulation of eosinophils at sites of allergic inflammation. Accordingly, the transition from rolling to adherent eosinophils that occurs *in vivo* after application of either anaphylatoxin (Figs. 1 and 2) could be brought about in part by altered levels of L-selectin and  $\alpha_M\beta_2$  integrin.

The application of functional blocking mAbs to eosinophil adhesion molecules *in vivo* indicated that for both anaphylatoxins the observed transition from eosinophil rolling to firm adhesion is dependent not only on  $\beta_2$  integrins but also on  $\alpha_4$  integrins as well. Although, the levels of  $\alpha_4$  and  $\beta_1$  integrins are not up-regulated by the anaphylatoxins (Table II), we suggest that the most likely interpretation is an augmented functional adhesive state of  $\alpha_4\beta_1$  integrin. This integrin is known to be able to modulate its functional state and has been reported transition from a low avidity to high avidity state after GM-CSF stimulation of eosinophils (46, 47). Our data suggest that  $\alpha_4\beta_1$  integrin is more efficient than  $\alpha_4\beta_7$  integrin as an adhesion receptor during episodes of inflammation mediated by the anaphylatoxins C3a or C5a, although both  $\alpha_4\beta_1$  and  $\alpha_4\beta_7$  integrins can support eosinophil rolling on VCAM-1 *in vitro* and *in vivo*.<sup>4</sup>

When eosinophil chemotaxis and transendothelial migration were examined *in vitro*, we observed that the optimal concentration range of C3a ( $10^{-7}$ – $10^{-5}$  M) was an order of magnitude higher than that of C5a ( $10^{-8}$ – $10^{-6}$  M), and the number of eosinophils migrating in response to C3a was less than that to C5a (Figs. 6 and 7). Although the potential plasma concentration of C3a is 20 times that of C5a, our results indicated that whether chemotaxis through plastic membranes or transmigration through

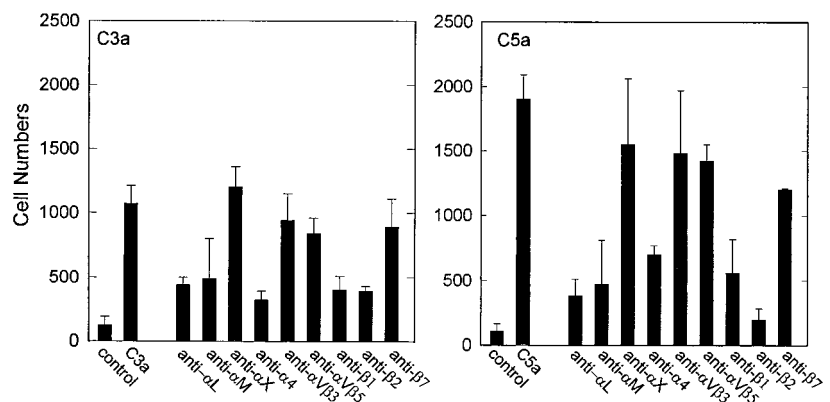
endothelial cells was measured, C5a evoked the migration of 1.5–3-fold more cells than did C3a (Figs. 6 and 7). An explanation for this could be rooted in eosinophil heterogeneity (48), and it is conceivable that only a subpopulation of eosinophils may respond chemotactically to C3a.

Both anaphylatoxins evoked greater motility for eosinophils through IL-1 $\beta$ -stimulated endothelial cells than through unstimulated endothelial cells or through pores in naked plastic (Figs. 6 and 7). The increased transmigration across IL-1 $\beta$  endothelial monolayers, in comparison with unstimulated cells, is likely to be a consequence of an augmented adhesion of the eosinophils to cytokine-stimulated endothelial cells. This effect is probably caused by an up-regulation of endothelial ICAM-1 (CD54), and induction of VCAM-1 (CD106) (48, 49), which interact with greater avidity with their eosinophil counterreceptors,  $\beta_2$  and  $\alpha_4$  integrins, respectively (20, 24, 50–53). The augmented interaction should result in greater traction, facilitating mobility of the eosinophil.

Inhibition studies using mAbs directed against several eosinophil adhesion molecules indicated that in the absence of cytokine stimulation, C3a- or C5a-mediated eosinophil transmigration across HUVEC monolayers was primarily dependent on  $\alpha_L\beta_2$  integrin (CD11a/CD18), which interacts with ICAM-1 and ICAM-2 (CD102), that are constitutively expressed at low levels on unprimed endothelial cells (28, 54, 55). However, when IL-1 $\beta$ -treated endothelial cells were used, significant inhibition was observed by mAbs with specificities against  $\alpha_L$  and  $\beta_2$  integrins as well as those against  $\alpha_M$ ,  $\beta_1$ , and  $\alpha_4$  integrins, and to a lesser degree against  $\beta_7$  integrin (Figs. 8 and 9). It is thus concluded that the engagement of several of the eosinophil integrins including  $\alpha_L\beta_2$ ,  $\alpha_M\beta_2$ , and  $\alpha_4\beta_1$  integrins is required for optimal eosinophil

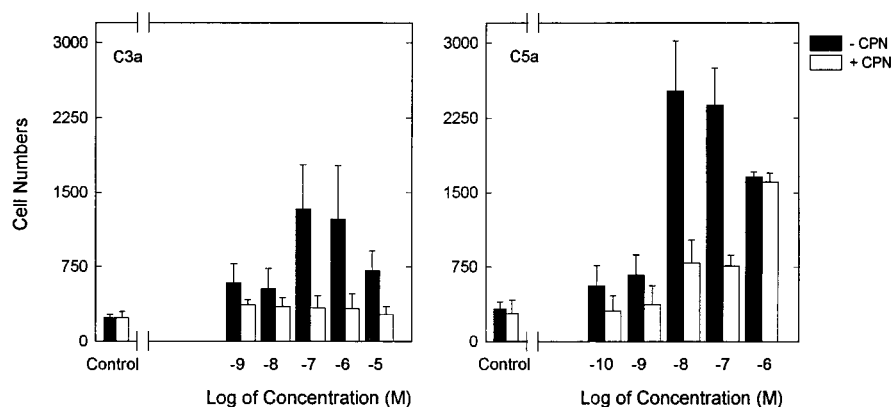
<sup>4</sup> P. Sriramarao, R. G. DiScipio, G. H. Hughes, M. Elices, K. P. Sung, C. Cybulsky, and D. H. Brodie (1998).  $\alpha_4\beta_7$  integrins support eosinophil rolling and adhesion on VCAM-1 *in vitro* and *in vivo*: influence of  $\beta_1$ -integrin activation on  $\alpha_4\beta_1$  stable arrest of rolling eosinophil under conditions of flow. Submitted for publication.

**FIGURE 9.** Inhibition of C3a- and C5a-mediated eosinophil transmigration through IL-1 $\beta$ -primed endothelial cell monolayers by anti-integrin mAbs. Eosinophils were mixed with mAbs (5  $\mu\text{g/ml}$ ) which had specificities for various integrin chains for 10 min at 23°C before usage in transmigration assays. Then eosinophil transmigration evoked by  $10^{-6}$  M C3a (left) or  $5 \times 10^{-8}$  M C5a (right) through endothelial cell monolayers was determined.





**FIGURE 10.** Influence of CPN on C3a and C5a transendothelial migration of eosinophils by C3a and C5a. Eosinophil transmigration mediated by C3a (*left*) or C5a (*right*) through IL-1 $\beta$ -treated endothelial cell monolayers was measured in the presence (white bars) or absence (black bars) of CPN (10  $\mu$ g/ml).



migration across cytokine-stimulated endothelium induced by either C3a or C5a. Similar results have been reported for the chemokine RANTES and for platelet-activating factor. These agents were found to induce greater eosinophil transendothelial migration through IL-1 $\beta$ -primed endothelial cells than through unprimed cells. Moreover, of anti-integrin mAbs to eosinophils resulted in a pattern of inhibition that highlighted the importance of  $\alpha_4$  and  $\beta_2$  integrins, for RANTES- and platelet-activating factor-mediated transmigration of this cell type (29, 56).

As noted, there is a correspondence in the inhibitory effects of several mAbs to eosinophil integrins for adhesion and transmigration in vitro and in vivo for both C3a and C5a stimulation (Figs. 3, 8, and 9). The ability of several mAbs to block eosinophil transmigration in vivo may be a consequence of prior blockade of the adhesion step or it may reflect a function of  $\alpha_4$  and  $\beta_2$  integrins to participate directly in the actual emigration process. Hence, the ability of eosinophils to accumulate in an inflammatory site will depend on the nature of the chemotaxin and the priming of the endothelium. However, the actual machinery of mobility, which involves the engagement of adhesion molecules, appears to be the same in all cases examined, which include our results with C3a and C5a as well as previously published findings for platelet-activating factor and RANTES (29, 56).

Although in vitro experiments demonstrate clearly that C3a and C5a can evoke chemotaxis and transmigration of eosinophils in buffer or serum-free medium (5, 16) (Figs. 6 and 7), it is realized that in vivo these anaphylatoxins could be regulated by blood components. Since plasma contains the anaphylatoxin inhibitor CPN (57), we examined the effect of this carboxypeptidase on C3a- and C5a-mediated eosinophil transmigration. The outcome of in vitro experiments, in which CPN was included with the anaphylatoxins in the chemotactic wells, was that transmigration to C3a (C3a<sub>desArg</sub>) was lost completely; however, residual activity to C5a (C5a<sub>desArg</sub>) was retained (Fig. 10). The concentration of CPN (10  $\mu$ g/ml), which was used for these experiments, is comparable to the estimated plasma concentration (15–30  $\mu$ g/ml) (57). The differences in the retained functional activity of C5a<sub>desArg</sub> but not C3a<sub>desArg</sub> in inducing eosinophil migration are similar to previously published results for a wide range of other functional effects on eosinophils and neutrophils. For example, C3a<sub>desArg</sub>, in contrast to intact C3a, was completely inactive in inducing guinea pig ileum spasmogenic activity, neutrophil respiratory burst, eosinophil enzyme release activity, and eosinophil respiratory burst (5, 12, 15, 58). In contrast to the inertness of C3a<sub>desArg</sub>, C5a<sub>desArg</sub>, although weaker than C5a, is nevertheless an effective chemotaxin for neutrophils and eosinophils (6, 58) (Fig. 10).

The lack of eosinophil transmigration induced by C3a across rabbit mesenteric venules could possibly be attributable of the ac-

tion of regulatory molecules such as CPN. This carboxypeptidase could reduce the effective concentration or the effective duration of C3a because in vitro studies indicate that C3a is completely inactivated when in the presence of CPN (Fig. 10). However, it is possible that C3a<sub>desArg</sub> could support eosinophil adhesion but not transmigration in vivo.

CPN is a large molecule and is expected to have limited access to interstitial fluid (59). Hence it is conceivable that C3a could operate in an extravascular microenvironment of an allergic inflammatory site free of regulation by CPN. Since C5a<sub>desArg</sub> retains some chemotactic activity but C3a<sub>desArg</sub> does not (Fig. 10), it is concluded that C3a is designed and regulated to act as a more short range or short term mediator than is C5a.

What is important is that C3a selectively causes stable adhesion of rolling eosinophils on cytokine-stimulated venular endothelium. In contrast to C5a, C3a had no effect on the stable adhesion of neutrophils in postcapillary venules (Fig. 2). Thus C3a can join a short list of mediators, which includes MCP-3, MCP-4, RANTES, and eotaxin (60–63), which are chemotactic for eosinophils and basophils but not neutrophils.

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