Mechanisms of Acute Eosinophil Mobilization from the Bone Marrow Stimulated by Interleukin 5: The Role of Specific Adhesion Molecules and Phosphatidylinositol 3-Kinase

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Summary

Mobilization of bone marrow eosinophils is a critical early step in their trafficking to the lung during allergic inflammatory reactions. We have shown previously that the cytokine interleukin (IL)-5, generated during an allergic inflammatory reaction in the guinea pig, acts systemically to mobilize eosinophils from the bone marrow. Here, we have investigated the mechanisms underlying this release process. Examination by light and electron microscopy revealed the rapid migration of eosinophils from the hematopoietic compartment and across the bone marrow sinus endothelium in response to IL-5. Using an in situ perfusion system of the guinea pig hind limb, we showed that IL-5 stimulated a dose-dependent selective release of eosinophils from the bone marrow. Eosinophils released from the bone marrow in response to IL-5 expressed increased levels of β_2 integrin and a decrease in L-selectin, but no change in α_4 integrin levels. A β_2 integrin–blocking antibody markedly inhibited the mobilization of eosinophils from the bone marrow stimulated by IL-5. In contrast, an α_4 integrin blocking antibody increased the rate of eosinophil mobilization induced by IL-5. In vitro we demonstrated that IL-5 stimulates the selective chemokinesis of bone marrow eosinophils, a process markedly inhibited by two structurally distinct inhibitors of phosphatidylinositol 3-kinase, wortmannin and LY294002. Wortmannin was also shown to block eosinophil release induced by IL-5 in the perfused bone marrow system. The parallel observations on the bone marrow eosinophil release process and responses in isolated eosinophils in vitro suggest that eosinophil chemokinesis is the driving force for release in vivo and that this release process is regulated by α_4 and β_2 integrins acting in opposite directions.

Key words: eosinophil • bone marrow • integrin • phosphatidylinositol 3-kinase • interleukin 5

The cytokine IL-5 regulates the development and function of eosinophils. In the bone marrow, IL-5 stimulates the expansion of eosinophil precursors (1) and is a late differentiation factor for eosinophils (2, 3). As a consequence, IL-5 transgenic mice exhibit a marked blood and tissue eosinophilia (4). IL-5 also regulates certain functions of mature eosinophils. In particular, IL-5 has the ability to prime eosinophils, increasing their responsiveness to mediators that stimulate degranulation (5), the respiratory burst, and chemotaxis (6, 7). Finally, IL-5 is an important survival factor for eosinophils because it inhibits their apoptosis (8).

IL-5 mRNA is upregulated in tissues, including the airways (9, 10), skin (11), intestinal mucosa (12), bladder (13), and heart (14), during eosinophilic inflammatory reactions. IL-5 protein has been detected in the bronchoalveolar lavage fluid of allergen-challenged sensitized mice (15) and in the blood of asthmatics (16). In animal models of allergic inflammation, recruitment of eosinophils into the lungs and airway hyperreactivity is suppressed by neutralizing Abs to IL-5 (17, 18). Similarly, IL-5 gene disruption abolishes eosinophilia, airway hyperreactivity, and lung damage in a mouse model of asthma (19). These observations support the concept that IL-5 is an important endogenous eosinophil chemoattractant (20).

Aerosolized allergen challenge of sensitized guinea pigs results in a recruitment of eosinophils into the lung tissue. We showed that bronchoalveolar lavage fluid from these guinea pigs contained an eosinophil chemoattractant activity that did not correspond to IL-5, and sequencing revealed eotaxin, a novel CC chemokine (21–23). The kinetics of

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eotaxin generation correlated with the recruitment of eosinophils into the lung tissue (24). However, recruitment, but not eotaxin generation, was inhibited by pretreatment of guinea pigs with the anti–IL-5 mAb TRFK-5 (24). Thus, it appears that in this model IL-5 plays an important role in eosinophil recruitment, but not as a significant chemoattractant.

We demonstrated that intravenous administration of IL-5 into guinea pigs resulted in a rapid blood eosinophilia due to the mobilization of eosinophils from a storage pool in the bone marrow (25). Significantly, intradermally injected IL-5 did not induce eosinophil recruitment (25). This blood eosinophilia had a marked potentiating effect on the numbers of eosinophils recruited to the sites of intradermally injected eotaxin. Thus, IL-5 and eotaxin act cooperatively, with IL-5 mobilizing eosinophils from the bone marrow and eotaxin recruiting eosinophils locally (25). Similar cooperative effects of eotaxin and IL-5 have been shown in the mouse (26). Indeed, intradermal administration of eotaxin did not induce eosinophil accumulation in IL-5–deficient mice, and in one study eotaxin-stimulated eosinophil accumulation in the lungs was only consistently observed in IL-5 transgenic mice that have a pronounced basal blood eosinophilia (26). We subsequently showed that when sensitized guinea pigs were challenged with aerosolized allergen, eosinophils were mobilized from the bone marrow into the blood and subsequently accumulated in the lung (24). Bone marrow eosinophil release, blood eosinophilia, and lung eosinophilia were abrogated by pretreatment of guinea pigs with anti–IL-5 mAb (24). Our studies suggest that IL-5 plays a central role in the mobilization of eosinophils from the bone marrow, an important early step in eosinophil trafficking during the allergic inflammatory response.

The molecular mechanisms regulating the release of eosinophils from the bone marrow are poorly understood. Egress from the bone marrow may involve downregulation of specific adhesive interactions, increased motility, migration through the hematopoietic compartment, and transmigration across the sinus endothelium. This paper explores the mechanisms underlying mobilization of eosinophils from the bone marrow induced by IL-5.

Materials and Methods

Animals. Male Dunkin Hartley guinea pigs (250–350 g) were obtained from Harlan Olac, Ltd. (Bicester, Oxfordshire, UK).

Materials. Human recombinant IL-5 was a gift from Dr. T.N.C. Wells, (Serona Pharmaceutical Research Institute, Geneva, Switzerland). Anti-α_4 integrin chain (CD49d) mAb (HP1/2) and nonbinding isotype-matched control mAb (1e6) were gifts from Dr. R. Lobb (Biogen Inc., Boston, MA). Anti-β_2 integrin chain (CD18) mAb (6.5E) was a gift from Dr. M. Robinson (Celltech Therapeutics, Ltd., Slough, UK). 6.5E $F(ab')_2$ fragments were produced from the whole IgG_1 Ab by D. King (Celltech Therapeutics, Ltd.) using bromelain digestion. Isotype-matched control $F(ab')_2$ fragments [ENA2 $F(ab')_2$] were also a gift from Dr. M. Robinson. Anti-L-selectin (CD62L) mAb (MEL-14) was purchased from Serotec Ltd. (Kidlington, Oxford, UK). HBSS with and without Ca^{2+}/Mg^{2+} and Hepes was purchased from Life Technologies (Paisley, UK). Hypnorm (fentanyl citrate 0.315 mg/ml, fluanisone 10 mg/ml) was purchased from Janssen Pharmaceuticals, Ltd. (Oxford, UK). Hypnovel (Midazolam, 5 mg/ml) was purchased from Roche (Welwyn, UK). Expiral (sodium pentobarbitone 200 mg/ml) was purchased from May and Baker (Dagenham, UK). EasyLyse erythrocyte lysis kits were purchased from Universal Biologicals (London, UK). Methylene blue, eosin, May-Grunwald, and Giemsa stains were purchased from Merck (Dagenham, UK). Transwell inserts with 3-μm pores were purchased from Millipore (Watford, UK). Kimura's stain for positive identification of eosinophils was prepared as previously described (27). Wortmannin, LY294002, rapamycin, and all other reagents were purchased from Sigma Chemical Co. (Poole, UK).

Modified Krebs-Ringer bicarbonate buffer of the following composition was used in perfusion experiments: 10 mM d-Glucose, 2.50 mM $CaCl_2$, 0.49 mM $MgCl_2 \cdot 6H_2O$, 4.56 mM KCl, 120 mM NaCl, 0.7 mM Na_2HPO_4, 1.5 mM NaH_2PO_4, and 24 mM $NaHCO_3$, supplemented with Ficoll T-70 4% and BSA 0.1% and gassed with 95% O_2, 5% CO_2.

Measurement of Intrasinus Eosinophils by Light Microscopy. Guinea pigs were sedated with Hypnorm (0.2 ml i.m.) and injected intravenously with IL-5 (30 pmol/kg) or vehicle (PBS/0.1% very low endotoxin BSA). After 30 min, the guinea pigs were killed with Expiral (250 mg/kg by cardiac puncture) and the femurs were removed quickly. The ends of the femur were removed and femoral marrow was removed from the femoral shaft very gently using an applicator stick so as to not disrupt the cytoarchitecture of the marrow. The femoral marrow was fixed immediately in a 3.7% paraformaldehyde solution for 2 h. The tissue was then dehydrated in an ethanol series (30–100%) before being embedded in JB-4 resin as per the manufacturer's instructions (Polysciences, Warrington, UK). 3-μm sections were cut using a Reichart microtome and stained with May-Grunwald and Giemsa to visualize eosinophils. More than 500 intrasinus leukocytes were counted per section of femoral marrow and classified as eosinophils or other leukocytes based on positive or negative staining, respectively, with May-Grunwald ($n = 3$ sections/marrow, 7–10 animals).

Transwell Migration Assay. Guinea pigs were killed with Expiral and the femurs were removed quickly. The femoral shaft was flushed with 5 ml of cell buffer (HBSS without Ca^{2+}/Mg^{2+} containing 30 mM Hepes and 0.25% BSA, pH 7.4) containing 10 U/ml of heparin. Displaced cells were gently resuspended and centrifuged (200 *g* for 7 min at 20°C), and the cell pellet was resuspended in 1 ml of cell buffer. Erythrocytes were removed using hypotonic shock lysis (addition of 10 ml 0.2% NaCl followed by 10 ml of 1.6% NaCl to restore isotonicity). After centrifugation (200 *g* for 7 min at 20°C), the leukocyte pellet was resuspended in assay buffer (HBSS with Ca^{2+}/Mg^{2+} containing 30 mM Hepes and 0.25% BSA, pH 7.4). Bone marrow leukocytes (3×10^6 cells in 0.2 ml assay buffer) were placed in the upper chamber of Transwell filters (3-μm pore diameter) that were in turn placed in individual wells of a 24-well cell culture plate containing 0.3 ml of assay buffer. To demonstrate chemokinesis of guinea pig bone marrow eosinophils, IL-5 (0–3 nM) was placed in the upper and lower chambers in a checkerboard pattern. In some experiments bone marrow leukocytes were incubated with wortmannin, LY294002, or rapamycin for 30 min at 37°C before being placed in the upper Transwell chamber. Chambers were incubated for 60 min at 37°C. Cells that migrated into the bottom chamber after 60 min were counted using a flow cytometer

(FACScan®, Becton Dickinson, San Jose, CA), with relative cell counts obtained by acquiring events for a set time period of 60 s. This counting method was highly reproducible and enabled gating on the different leukocyte populations and the exclusion of debris. Counts obtained in this way closely matched those obtained by light microscopy.

In Situ Perfusion of the Guinea Pig Hind Limb. The guinea pig hind limb was perfused as previously described in detail (28). Guinea pigs were anesthetized and the external iliac artery and vein were exposed. The following arteries and their satellite veins were ligated with 5/0 braided silk suture: caudal abdominal artery, superficial iliac circumflex artery, and pudendoepigastric trunk. The animals were killed with Expiral (250 mg/kg by cardiac puncture). Cannulas polyethylene (0.8 mm outside diameter; Portex, London, UK) were immediately inserted into the external iliac artery and vein and pushed down under the inguinal ligament into the femoral artery and vein. Cannulas were tied in with 5/0 braided silk suture. Modified Krebs-Ringer bicarbonate buffer (37°C, gassed with 95% O_2, 5% CO_2; composition detailed above) was infused (3.4 ml/min) via the arterial cannula and removed from the venous cannula using a Minipuls peristaltic pump (Anachem, Luton, UK). Perfusate fractions were collected every 10 min and centrifuged (300 *g* for 10 min at 20°C), and the cell pellet was resuspended in Kimura's stain. Nucleated leukocytes and Kimura-positive eosinophils were counted in an Improved Neubauer Hemacytometer. In some experiments, cytocentrifuge preparations of leukocytes in each fraction were stained with methylene blue and eosin to perform a differential leukocyte count.

Measurement of Eosinophil Surface Adhesion Molecule Expression. The guinea pig hind limb was set up for in situ perfusion as described above, and IL-5 was infused (0.4 nM) for 120 min. The leukocytes released were collected onto ice and immediately centrifuged (300 *g* for 10 min at 4°C), and the cell pellet was resuspended at 10^6 leukocytes/ml in labeling buffer (ice-cold HBSS without Ca^{2+}/Mg^{2+} containing 30 mM Hepes and 0.25% BSA, pH 7.4). A control population of leukocytes from nonperfused femoral bone marrow was prepared as directed for the Transwell filter migration assay described in detail above. Bone marrow leukocytes were resuspended as a single cell population at 10^6 leukocytes/ml in labeling buffer and were incubated with mAb against L-selectin (MEL-14, 300 μg/ml), β_2 integrin (6.5E, 10 μg/ml), α_4 integrin (HP1/2, 10 μg/ml), or isotype-matched control Ab for 30 min on ice. MEL-14, 6.5E and HP1/2 have been shown to cross-react with their respective guinea pig antigens (29, 30). Leukocytes were washed twice in labeling buffer and incubated with FITC-conjugated secondary Ab [1:20 dilution of $F(ab')_2$ goat anti–murine IgG-FITC or goat anti–rat IgG-FITC; DAKO, High Wycombe, Buckinghamshire, UK] for 30 min on ice. Leukocytes were washed twice in labeling buffer and resuspended in FACS® flow for analysis using a FACScan® flow cytometer. Bone marrow eosinophils were gated by the characteristic forward/side scatter profile of these leukocytes, as previously described (28).

Transmission Electron Microscopy. The guinea pig hind limb was set up for in situ perfusion as described above. IL-5 was infused (0.8 nM) for 60 min, at which point the hind limb was perfused-fixed with 2.5% gluteraldehyde buffered with sodium cacodylate. The femur was removed and placed in fresh cacodylate-buffered 2.5% gluteraldehyde fixative for 12 h. The femur was then sawn in half and the fixed marrow was carefully pushed out with an applicator stick. The marrow was rinsed with 0.1 M cacodylate buffer, postfixed in 2% OsO_4 buffered with 0.1 M sodium cacodylate, and then dehydrated through an ethanol series (30–100%). During dehydration, the marrow was stained en bloc with a saturated solution of uranyl acetate in 50% ethanol. Marrow samples were embedded in Araldite (Ciba Chemical Co.), and ultrathin sections were prepared using a Reichart ultramicrotome. Ultrathin sections were placed on 200-mesh copper grids and further stained with uranyl acetate and lead citrate. Sections were examined and photographed in a Philips EM 301 transmission electron microscope. Correlative light microscopy was carried out using semithin sections stained with toluidine blue.

Eosinophil Accumulation in the Femoral Bone Marrow. Guinea pig peritoneal eosinophils were prepared to >95% purity and labeled with ^{111}In as previously described in detail (31). Before injection into recipient guinea pigs, ^{111}In-labeled eosinophils were pretreated with the anti-α_4 (HP1/2, 10 μg/ml) or isotype-matched control mAb (1e6, 10 μg/ml) for 20 min at 37°C. Recipient guinea pigs were sedated (Hypnorm, 0.2 ml i.m.) and injected with ^{111}In-labeled eosinophils (10^6 eosinophils/kg) via the marginal ear vein. After 1 h, the animals were killed and both femurs were removed, cleaned of connective tissue and muscle, and weighed. The number of ^{111}In-labeled eosinophils accumulated in each femur was measured using an automatic gamma counter (Canberra Packard, Pangbourne, UK) as previously described (31).

Statistical Analysis. For analysis of two groups, the unpaired two-way Student's *t* test was used. For analysis of three or more groups, one-way analysis of variance followed by either Bonferroni's multiple comparisons test or Dunnett's test for comparison with a control group was used. $P < 0.05$ was considered significant. All data are expressed as arithmetic mean ± SEM for *n* observations.

Results

IL-5 Stimulates a Rapid Migration of Eosinophils from the Hematopoietic Compartment into the Venous Sinusoids. Eosinophils mobilized from the bone marrow may originate from either the extravascular hematopoietic compartment or a marginating intravascular pool within the bone marrow sinuses. To distinguish between these two possibilities, femoral bone marrow was removed from guinea pigs 30 min after intravenous injection of IL-5 (30 pmol/kg) or PBS, and the location of the eosinophils within the bone marrow was determined by light microscopy. In the PBS-injected guinea pigs, very few intrasinus eosinophils were evident, suggesting that there was not a significant marginating intravascular pool of eosinophils (Fig. 1). The number of eosinophils present in the venous sinusoids, expressed as a percentage of the total number of leukocytes in the

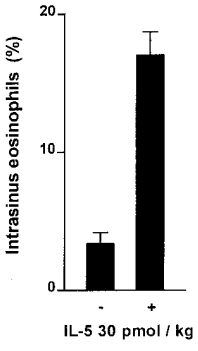

Figure 1. Rapid elevation of intrasinus eosinophil numbers after intravenous IL-5. Guinea pigs were injected with IL-5 (30 pmol/kg) or vehicle (PBS/0.1% BSA). The femoral bone marrow was removed after 30 min for histological analysis of intrasinus eosinophil levels. Data expressed as the percentage of eosinophilic intrasinus leukocytes, mean ± SEM (n = 7–10 animals).

venous sinusoids, increased fivefold 30 min after intravenous IL-5 injection (Fig. 1). There was no significant increase in peripheral blood eosinophil numbers at this time point (data not shown). These results demonstrate that mobilization involves the migration of eosinophils from the hematopoietic compartment into the bone marrow sinuses.

Transmission Electron Microscopy Demonstrates Possible Stages of Eosinophil Mobilization from the Bone Marrow. To examine this release process in more detail, we perfused the guinea pig hind limb with IL-5 (0.8 nM) for 60 min and then rapidly perfused-fixed it. Ultrathin sections of the bone marrow were stained with lead citrate and uranyl acetate, and examined by transmission electron microscopy. Eosinophils, readily identified by their characteristic secondary cytoplasmic granules, could be seen at different stages of emigration as shown in the electron micrographs. Fig. 2 *a* shows an eosinophil located within the hematopoietic compartment, abutting a thin fenestrated region of the

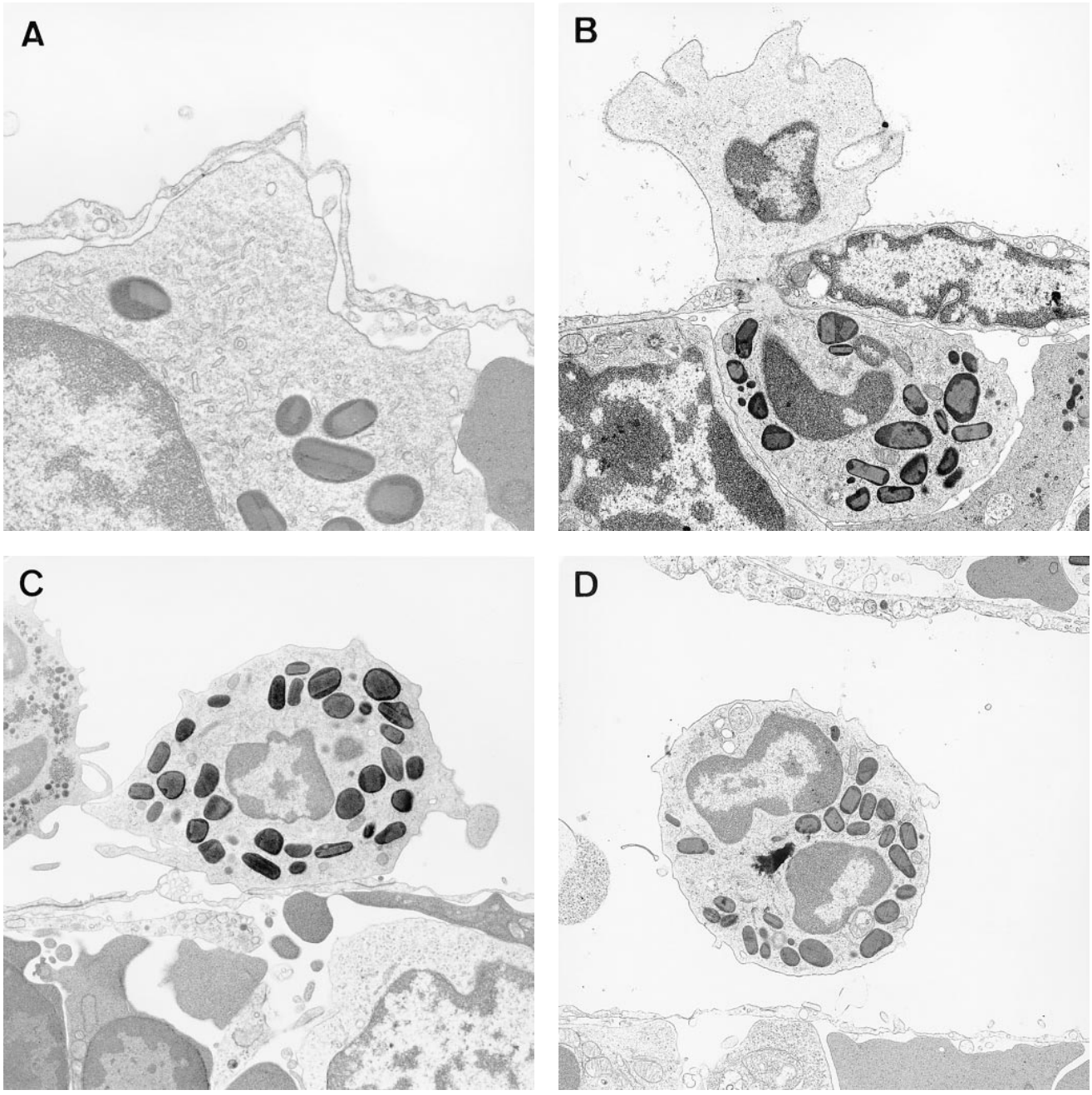

Figure 2. Transmission electron microscopy illustrating egress of eosinophils from the femoral bone marrow. Guinea pig hind limb was infused with IL-5 (0.8 nM) for 60 min and perfused fixed, and ultrathin sections were prepared for observation by transmission electron microscopy. Original magnification: *A*, ×18,600; *B*, ×9,400; *C*, ×7,300; *D*, ×6,900.

sinusoidal endothelium. Fig. 2 *b* shows an eosinophil in the process of transmigration through the sinusoidal endothelium, apparently not through the endothelial cell junctions. There is marked deformation of the eosinophil as it traverses the endothelium, consistent with passage through a tight fitting migration pore. Fig. 2 *c* shows an eosinophil within the sinus lumen, attached to the luminal surface of the sinusoidal endothelium. Fig. 2 *d* shows an eosinophil within the sinus lumen, apparently not attached to the sinus endothelium. Although these transmission electron micrographs are static images, when assembled in this sequence they permit reconstruction of the probable stages by which eosinophils emigrate from the bone marrow.

IL-5 Stimulates Mobilization of Eosinophils from the Guinea Pig Hind Limb. To investigate directly the kinetics and molecular mechanisms of eosinophil release from bone marrow, we used the in situ hind limb perfusion system in the guinea pig. During perfusion with PBS there was a steady release of leukocytes from the perfused hind limb ($1.04 \times 10^7 \pm 1.24 \times 10^6$ in 2 h). Differential leukocyte counts from cytospin preparations showed that these leukocytes were predominantly neutrophils (>90%) both of the mature, segmented band form (~80%) and the less mature, unsegmented band form (~20%). Very few eosinophils were released under basal conditions ($0.5 \times 10^6 \pm 0.1 \times 10^6$ in 2 h) (Fig. 3 *a*). In contrast, infusion with IL-5 (0.1–0.8 nM, indicated by the solid bar) stimulated a dose-dependent release of eosinophils (Fig. 3 *a*). IL-5–stimulated release of eosinophils was rapid, with a maximum rate of release attained after 1 h of IL-5 infusion (Fig. 3 *a*). The rate of eosinophil release did not change significantly between 1 and 2 h when IL-5 was infused at a concentration of 0.1 and 0.4 nM. When IL-5 was infused at 0.8 nM, the rate of eosinophil release attained at 1 h was higher than for the lower concentration of IL-5 but reduced at later time points. This may reflect depletion of a finite pool of mobilizable eosinophils. Cytospin preparations showed that the eosinophils released by IL-5 had a bilobed nucleus, characteristic of terminally differentiated eosinophils (data not shown). The total number of noneosinophilic leukocytes released was unaffected by infusion with IL-5 (Fig. 3 *b*).

Surface Adhesion Molecule Expression on Eosinophils Released from the Femoral Bone Marrow. The in situ hind limb perfusion system was used to determine whether there was a change in the expression of adhesion molecules on eosinophils mobilized in response to IL-5. IL-5 (0.4 nM) was infused into the hind limb for 120 min and the leukocytes released were collected on ice. Leukocytes from a nonperfused bone marrow were collected and prepared as described in Materials and Methods to provide the control population and kept on ice as above. The leukocytes were labeled with mAbs raised against L-selectin and β_2 and α_4 integrins, and binding was analyzed by flow cytometry.

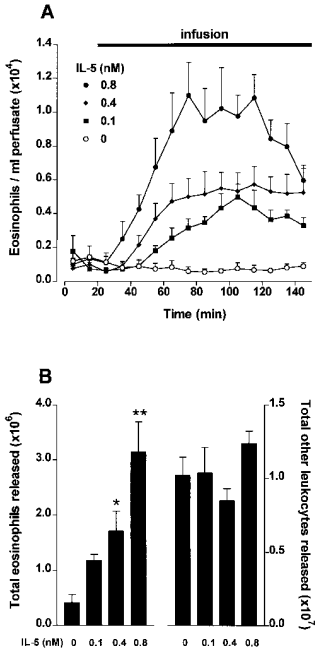

Figure 3. IL-5–stimulated mobilization of eosinophils from the femoral bone marrow measured using the in situ perfused hind limb preparation. (*A*) Kinetics of eosinophil release stimulated by a 120-min infusion (*solid bar*) of IL-5 (0.1–0.8 nM) or vehicle (PBS/0.1% BSA). Results expressed as the number of eosinophils per milliliter of perfusate in each 10-min fraction, mean ± SEM ($n = 5$ perfusions). (*B*) Total eosinophil and total other leukocyte release induced by a 120-min infusion of IL-5 (0.1–0.8 nM) or vehicle. Results are expressed as the total number of eosinophils or total number of other leukocytes released during the 120-min infusion period, mean ± SEM ($n = 5$ perfusions). $^*P < 0.05$, $^{**}P < 0.01$.

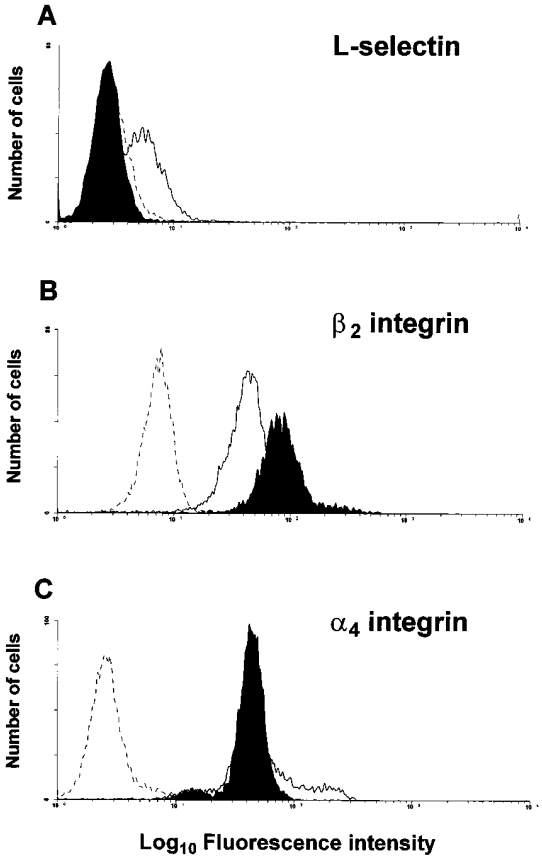

Figure 4. Changes in surface adhesion molecule expression on eosinophils mobilized from the bone marrow by IL-5. Binding of mAb recognizing L-selectin (*A*), β_2 integrin (*B*), or α_4 integrin (*C*), to eosinophils mobilized from perfused femoral marrow by IL-5 (0.4 nM, 120-min infusion) was analyzed by indirect immunofluorescence flow cytometry. Binding to eosinophils mobilized from the bone marrow is shown by the solid histograms, binding to control bone marrow eosinophils is shown by the open histograms. Binding of isotype matched control Ab is shown by the dotted line. Each histogram is representative of three separate experiments.

Fig. 4 shows binding of mAb against L-selectin (Fig. 4 *a*), β_2 integrin (Fig. 4 *b*), or α_4 integrin (Fig. 4 *c*) to eosinophils released from the perfused hind limb in response to IL-5 (0.4 nM, 120-min infusion, *filled histograms*) or eosinophils from control, nonperfused guinea pig bone marrow (*open histograms*). The binding of control Ab to eosinophils from control, nonperfused, guinea pig bone marrow is shown by the dotted line histogram. As shown in Fig. 4 *a* there is no detectable expression of L-selectin on eosinophils mobilized in response to IL-5 as compared with control bone marrow eosinophils (Fig. 4 *a*), suggesting that L-selectin shedding is associated with eosinophil mobilization. Conversely, eosinophils mobilized by IL-5 were shown to express increased levels of the β_2 integrin when compared with control bone marrow eosinophils (99% increase) (Fig. 4 *b*). There was no significant difference in the expression of the α_4 integrin between IL-5–mobilized eosinophils and control bone marrow eosinophils (Fig. 4 *c*).

The Effect of an Anti-β_2 mAb on IL-5–stimulated Eosinophil Mobilization from Femoral Bone Marrow. Perfusion of the femoral bone marrow in situ with IL-5 (0.4 nM) or PBS in the presence of either the β_2-blocking mAb 6.5E (10 μg/ml) or an isotype-matched control mAb (10 μg/ml) was performed to investigate the role of the β_2 integrin in the IL-5–stimulated mobilization of eosinophils. Infusion of 6.5E markedly reduced the rate of IL-5–stimulated eosinophil release from the perfused hind limb, reducing the total number of eosinophils released in response to IL-5 by 40% over the 2-h perfusion period (Fig. 5). As noted above, there is a basal release of leukocytes other than eosinophils (comprising >90% neutrophils) that is not affected by the infusion of IL-5. However, the release of these noneosinophilic leukocytes from the perfused hind limb was significantly reduced by the infusion of 6.5E (IL-5 plus control mAb released $9.0 \pm 0.55 \times 10^6$ noneosinophilic leukocytes; IL-5 plus 6.5E released $5.1 \pm 0.9 \times 10^6$ noneosinophilic leukocytes, $P < 0.01$ for $n = 5$ experiments). To exclude the possibility of Fc receptor cross-linking, we manufactured anti-β_2 F(ab′)$_2$ fragments and investigated whether these would have the same effect on leukocyte release as the whole (IgG_1) Ab. Indeed, anti-β_2 F(ab′)$_2$ (10 μg/ml) inhibited noneosinophil leukocyte release by 31% and IL-5–stimulated eosinophil release by 29% (data not shown). These results suggest that both basal leukocyte release and IL-5–stimulated mobilization of eosinophils from the bone marrow is dependent on the β_2 integrin.

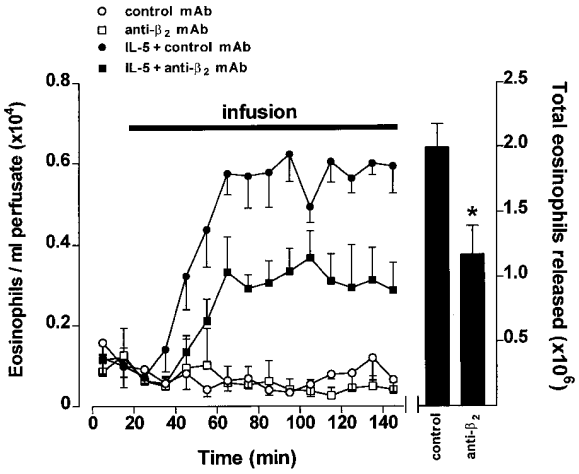

Figure 5. Effect of anti-β_2 integrin mAb on IL-5–stimulated eosinophil mobilization from the perfused hind limb. (*A*) Kinetics of eosinophil mobilization are shown on the left hand axis. The hind limb was infused with IL-5 (0.4 nM) together with anti-β_2 mAb (10 μg/ml, *filled squares*) or isotype-matched control mAb (10 μg/ml, *filled circles*). Infusion of PBS vehicle together with anti-β_2 integrin mAb and isotype-matched control mAb are shown by open squares and open circles, respectively. Data expressed as the number of eosinophils per milliliter of perfusate in each 10-min fraction, mean ± SEM ($n = 4$ separate perfusions). Total eosinophil mobilization induced by a 120-min infusion of IL-5 (0.4 nM) or vehicle in the presence of anti-β_2 integrin mAb (10 μg/ml) or isotype-matched control mAb (10 μg/ml) is shown on the right hand axis. Results are expressed as the total number of eosinophils mobilized during the 120-min infusion period, mean ± SEM ($n = 4$). *$P < 0.05$.

The Effect of an Anti-α_4 mAb on IL-5–stimulated Eosinophil Mobilization from the Bone Marrow. Perfusion of the femoral bone marrow in situ with IL-5 (0.4 nM) or PBS in the presence of either the α_4-blocking mAb, HP1/2 (10 μg/ml) or an isotype-matched control mAb (10 μg/ml) was performed to investigate the role of the α_4 integrin in the IL-5–stimulated mobilization of eosinophils. Basal release of eosinophils and noneosinophilic leukocytes in the PBS-infused group was not altered by infusion of anti-α_4 mAb (Fig. 6 *a* and data not shown). However, infusion of anti-α_4 mAb together with IL-5 (0.4 nM) resulted in a significantly increased initial rate of eosinophil release when compared with IL-5 infused together with the control mAb (Fig. 6 *a*). At later time points, the rate of eosinophil release in the presence of IL-5 and anti-α_4 mAb was reduced to control levels. This reduced rate of release is similar to that seen in Fig. 3 (0.8 nM IL-5) and may reflect depletion of the mobilizable pool of eosinophils. The total number of eosinophils mobilized by IL-5 was increased by 40% in the presence of the anti-α_4 mAb (Fig. 6 *a*).

The Effect of an Anti-α_4 mAb on IL-5–stimulated Blood Eosinophilia In Vivo. The effect of the anti-α_4 mAb HP 1/2 on IL-5–stimulated blood eosinophilia was examined in guinea pigs in vivo. Guinea pigs were coinjected intravenously with anti-α_4 mAb or an isotype-matched control mAb together with either IL-5 or PBS. Peripheral blood samples were collected before and 5, 10, 15, 30, and 60 min after the intravenous injection, and the numbers of circulating eosinophils were determined. Intravenous injection of PBS together with either the control mAb or anti-α_4 mAb had no effect on the basal number of circulating eosinophils at any time point. Intravenous injection of IL-5 (30 pmol/kg) stimulated an increase in the number of circulating eosinophils, reaching an 11-fold elevation by 60 min. The anti-α_4 mAb accelerated the blood eosinophilia response, such that a significant increase in circulating eosinophils was observed first at 30 min compared with 60 min in the absence of mAb (Fig. 6 *b*). Anti-α_4 mAb had no significant effect on blood eosinophil levels measured 60 min after IL-5 injection.

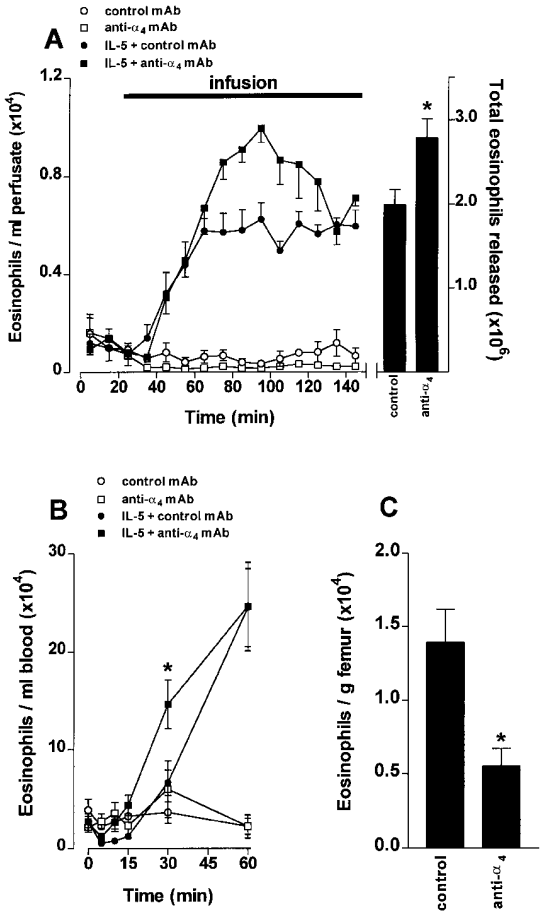

Figure 6. Effect of anti-α_4 integrin mAb on (*A*) IL-5–stimulated eosinophil mobilization from the perfused femoral bone marrow, (*B*) IL-5–stimulated blood eosinophilia in vivo, and (*C*) eosinophil accumulation in the femur. (*A*) Kinetics of eosinophil mobilization from the perfused hind limb stimulated by IL-5 (0.4 nM) in the presence of anti-α_4 integrin mAb (10 μg/ml, *filled squares*) or isotype-matched control mAb (10 μg/ml, *filled circles*) is shown on the left hand axis. Eosinophil mobilization stimulated by PBS vehicle in the presence of isotype-matched control mAb and anti-α_4 integrin mAb are shown by open circles and squares, respectively. Data expressed as the number of eosinophils per milliliter of perfusate in each 10-min fraction, mean ± SEM (n = 5–6 separate perfusions). Total eosinophil mobilization induced by a 120-min infusion of IL-5 (0.4 nM) or vehicle in the presence of anti-α_4 integrin mAb (10 μg/ml) or isotype-matched control mAb (10 μg/ml) is shown on the right hand axis. Results are expressed as the total number of eosinophils mobilized during the 120-min infusion period, mean ± SEM (n = 6). *$P < 0.05$. (*B*) Peripheral blood eosinophilia stimulated by IL-5 (30 pmol/kg i.v.) coinjected with either anti-α_4 integrin mAb (4 mg/kg i.v., *filled squares*) or isotype-matched control mAb (4 mg/kg i.v., *filled circles*). Blood eosinophil levels in PBS injected animals in the presence of anti-α_4 integrin mAb or isotype-matched control mAb is shown by open squares and circles, respectively. Data expressed as number of eosinophils per milliliter of blood, mean ± SEM (n = 4). *$P < 0.05$. (*C*) Accumulation of ^{111}In-labeled peritoneal eosinophils in the guinea pig femur. ^{111}In-labeled eosinophils were pretreated in vitro with either anti-α_4 integrin mAb (10 μg/ml) or isotype-matched control mAb (10 μg/ml) before intravenous injection into recipient guinea pigs. After 2 h, femoral ^{111}In-eosinophil accumulation was measured using a gamma counter. Data are expressed as the number of eosinophils per gram of femur, mean ± SEM (n = 5). *$P < 0.05$.

The Effect of an Anti-α_4 mAb on the Accumulation of Eosinophils in the Bone Marrow. Vascular cell adhesion molecule (VCAM)-1[1] is expressed constitutively on the sinus endothelium (32). One possible explanation for the above results is that there is a tendency for eosinophils that have migrated through the sinus endothelium to be retained on the luminal surface (as seen in Fig. 2 *c*), using α_4 integrins for attachment. To address this possibility, we used ^{111}In-labeled guinea pig peritoneal eosinophils as a surrogate for the newly migrated cells. Fig. 6 *d* shows that a significant proportion of these intravenously-injected cells localized in the bone marrow and that preincubation of these cells with anti-α_4 mAb significantly reduced this effect.

IL-5–stimulated Chemokinesis of Guinea Pig Femoral Bone Marrow Eosinophils. Using a Transwell filter assay, we investigated whether IL-5 could stimulate the selective migration of guinea pig bone marrow eosinophils in vitro. A mixed population of guinea pig bone marrow leukocytes was placed into the upper Transwell chamber. IL-5 (0.03–1 nM) was added to the upper and/or lower chambers of the Transwell system in a checkerboard analysis, and after 90 min the number of eosinophils migrated into the lower chamber was quantified by flow cytometry. The results in Table 1 show that IL-5 stimulated a dose-dependent migration of eosinophils from the upper chamber into the lower chamber. The migration was not dependent on a positive gradient of IL-5. These results demonstrate that IL-5 is chemokinetic and not chemotactic for guinea pig bone marrow eosinophils. IL-5 stimulated a significant increase in the migration of eosinophils at 30 pM with a maximal effect at 1 nM. IL-5 did not stimulate the migration of any other type of leukocyte, consistent with the selective mobilization of eosinophils by IL-5 in the in situ perfusion system and in vivo.

The Effect of Wortmannin, LY294002, and Rapamycin on IL-5–stimulated Chemokinesis of Bone Marrow Eosinophils. Using the Transwell filter assay, we examined the role of phosphatidylinositol (PI) 3-kinase in IL-5–stimulated bone marrow eosinophil chemokinesis. This was investigated using two specific inhibitors of PI 3-kinase, wortmannin and LY294002, which are structurally unrelated compounds that inhibit by different mechanisms (33–35). Femoral marrow leukocytes were incubated with wortmannin (1–50 nM) or LY294002 (1–20 μM) for 30 min at 37°C before being added to the upper Transwell chamber in the presence of IL-5 (3 nM). Eosinophils that accumulated in the lower chamber were quantified by flow cytometry after 1 h. Both wortmannin (25 and 50 nM) and LY294002 (1–20 μM) significantly inhibited IL-5–induced chemokinesis of guinea pig femoral marrow eosinophils (Fig. 7 *a*). These results indicate that IL-5–stimulated chemokinesis of guinea pig bone marrow eosinophils involves signaling through the PI 3-kinase pathway.

[1]*Abbreviations used in this paper:* PI 3-kinase, phosphatidylinositol 3-kinase; p70S6K, p70 S6-kinase; VCAM-1, vascular cell adhesion molecule 1; VLA_4, very late antigen 4.

Table 1. *IL-5–stimulated Chemokinesis of Guinea Pig Bone Marrow Eosinophils*

		Upper chamber IL-5				
		0	**0.03**	**0.1**	**0.3**	**1**
				nM		
Lower chamber IL-5 (nM)	**0**	1	3.74(0.41)	7.34(0.32)	9.28(0.77)	8.98(0.64)
	0.03	2.38(0.09)	5.28(0.09)	7.82(0.89)	8.45(0.97)	7.38(0.76)
	0.1	5.83(0.13)	6.90(0.69)	7.74(0.41)	8.17(0.73)	7.89(0.96)
	0.3	6.18(0.45)	6.89(0.05)	9.09(0.43)	8.34(0.89)	7.59(0.59)
	1	8.91(0.40)	8.37(0.41)	8.78(0.59)	9.11(0.75)	8.33(0.64)

Chemokinesis was demonstrated using checkerboard analysis of IL-5–stimulated eosinophil migration in the Transwell assay. A single cell suspension of 3×10^6 bone marrow leukocytes was placed in the upper chamber. IL-5 (0.03–1 nM) was placed in the upper and lower chambers in a checkerboard pattern. After 90 min, eosinophils accumulated in the lower chamber were identified by flow cytometry. Migration of eosinophils is expressed as the chemotactic index, mean ± SEM, representative experiment done in triplicate.

One of the downstream targets for PI 3-kinase is the serine/threonine kinase p70 S6-kinase (p70S6K). We examined whether this enzyme is involved in the IL-5–stimulated chemokinesis of bone marrow eosinophils using rapamycin, a selective inhibitor of p70S6K. Rapamycin (20 nM) did not significantly affect IL-5–stimulated chemokinesis of guinea pig bone marrow eosinophils when tested in the Transwell assay (Fig. 7 *a*).

The Effect of Wortmannin and Rapamycin on IL-5–stimulated Release of Eosinophils from the Perfused Femoral Bone Marrow. Using the in situ hind limb perfusion system, we examined if wortmannin or rapamycin could inhibit IL-5–stimulated mobilization of femoral marrow eosinophils. Wortmannin (100 nM), rapamycin (100 nM), or vehicle (PBS/0.1% BSA) were infused into the hind limb for 40 min and then IL-5 (3 nM) or vehicle (PBS/0.1% BSA) was infused into the hind limb for 10 min (10-min infusion indicated by the solid bar in Fig. 7 *b*). The perfusion was continued for an additional 50 min, with wortmannin, rapamycin, or vehicle being infused for the duration of the perfusion. The leukocytes released were collected in 10-min fractions, and stained with Kimura's stain for positive identification of eosinophils. Wortmannin (100 nM) had no effect on the basal release of noneosinophilic leukocytes (data not shown) or basal (PBS) release of eosinophils from the femoral marrow (Fig. 7 *b*). However, wortmannin (100 nM) significantly reduced the rate of IL-5–stimulated eosinophil release from the femoral marrow (Fig. 7 *b*). The total number of eosinophils mobilized in response to IL-5 was inhibited by 50% in the presence of wortmannin (100 nM) (IL-5 plus vehicle released $7.6 \pm 1.3 \times 10^5$ eosinophils; IL-5 plus wortmannin released $3.8 \pm 0.7 \times 10^5$ eosinophils; $P < 0.05$). Rapamycin (100 nM) did not affect the rate of IL-5–stimulated release of eosinophils from the femoral marrow when tested in the hind limb perfusion system (Fig. 7 *c*). These results suggest that IL-5–stimulated mobilization of bone marrow eosinophils involves signaling through PI 3-kinase.

Discussion

Mobilization of eosinophils is an important early step in their trafficking to the lungs during allergic inflammatory reactions. We have previously shown that IL-5, generated during allergic inflammatory reactions, acts systemically to release eosinophils selectively from the bone marrow (25). In this paper we have investigated the mechanisms underlying the acute mobilization of eosinophils from the bone marrow stimulated by IL-5. Examination of the process histologically by light and electron microscopy revealed that IL-5 stimulates a rapid movement of eosinophils from the bone marrow hematopoietic compartment into the sinuses. Our data suggest that transmigration across the bone marrow endothelium is a transcellular and not an intercellular event, as has been demonstrated for other leukocytes by serial thin sectioning (36–38) and has also recently been reported for the migration of eosinophils and neutrophils during their recruitment into inflammatory sites (39).

Using an in situ perfusion system of the guinea pig hind limb, we showed directly that infusion of IL-5 stimulates a dose-dependent selective release of eosinophils from the bone marrow. The release process is rapid and the kinetics of release in this model were comparable to the release in vivo after intravenous IL-5 injection (25). At the highest concentration of IL-5 tested, there appeared to be a depletion of the finite pool of mobilizable eosinophils from the bone marrow. In vivo this pool may be expanded, i.e., after sensitization with an allergen or due to infection with parasitic worms (40), thereby increasing the number of eosinophils available for rapid release.

Mature eosinophils are released in response to IL-5. Using this system, we have previously demonstrated that under these conditions IL-5 does not stimulate the release of colony-forming progenitor cells from the bone marrow (28). The mobilization of mature eosinophils in preference to immature eosinophils may reflect changes in eosinophils during maturation. These may include an increased motility and responsiveness to IL-5, an increased deformability

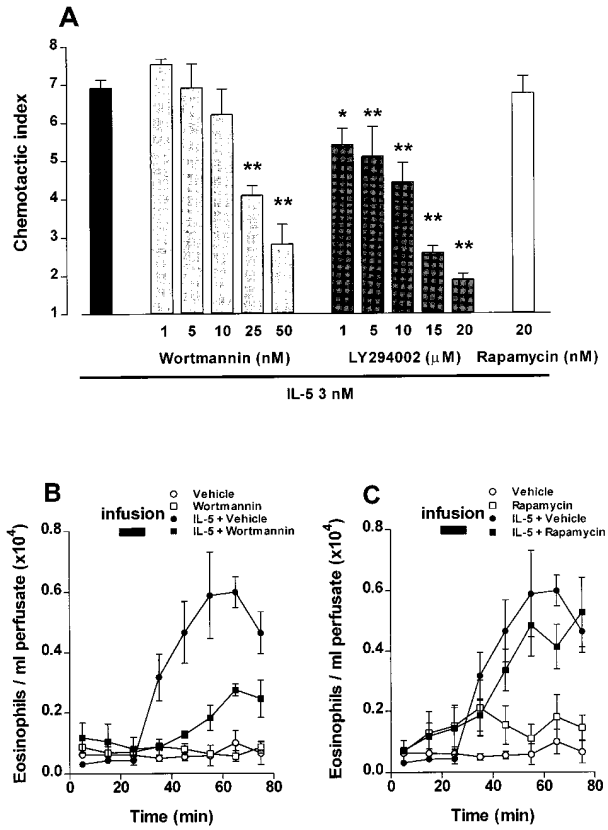

Figure 7. Effect of the PI 3-kinase inhibitors wortmannin and LY294002 on IL-5–stimulated eosinophil chemokinesis and mobilization from the femoral bone marrow. (*A*) Guinea pig femoral bone marrow leukocytes were incubated with wortmannin (1–50 nM), LY294002 (1–20 μM), or rapamycin (20 nM) for 30 min at 37°C before being placed in the upper Transwell chamber in the presence of IL-5 (3 nM). After incubation at 37°C for 1 h, eosinophils that accumulated in the lower Transwell chamber were quantified by flow cytometry. Eosinophil accumulation is expressed as the chemotactic index, with accumulation in response to vehicle having a chemotactic index of 1. Mean ± SEM (triplicate values of a representative experiment). $*P < 0.05$, $**P < 0.01$.(*B* and *C*) Kinetics of IL-5–stimulated eosinophil mobilization from the perfused hind limb in the presence of wortmannin (*B*) or rapamycin (*C*). The hind limb was infused with wortmannin (100 nM), rapamycin (100 nM), or vehicle (0.1% DMSO) for 40 min before infusion with IL-5 (3 nM, 10-min pulse, *black bar*) or vehicle. Data expressed as the number of eosinophils per milliliter of perfusate in each 10-min fraction, mean ± SEM ($n = 4$ separate perfusions).

aiding migration through tight fitting pores, and changes in the expression of adhesion molecules.

In this study, we show that there are changes in the expression of adhesion molecules on eosinophils as they are mobilized from the bone marrow in response to IL-5, by comparing eosinophils in bone marrow with those released. We found that levels of the α_4 integrin were the same in these two populations, but the eosinophils mobilized by IL-5 had no detectable expression of L-selectin and an increase in β_2 integrin expression. It has previously been shown that neutrophils shed L-selectin as they move from the hematopoietic compartment into the venous sinusoids of the bone marrow and it was postulated that this could control the release of neutrophils from the marrow (41). Our results are consistent with this theory; however, we have no direct evidence that L-selectin shedding is necessary for the egress of eosinophils from the bone marrow.

We report here that the expression of β_2 integrins was upregulated on eosinophils as they left the bone marrow in response to IL-5. Furthermore, a blocking Ab to the β_2 integrin significantly inhibited the IL-5–stimulated mobilization of eosinophils from the bone marrow. In vitro studies have previously demonstrated that IL-5 stimulates β_2 integrin–mediated adhesion of eosinophils to human umbilical vein endothelial cells (42) and in vivo the migration of eosinophils from the blood into tissues has been shown to be dependent on β_2 integrins (43). It is possible that β_2 integrins may be necessary for migration of the eosinophils within the hematopoietic compartment or their adhesion to and transmigration through the bone marrow endothelium.

In contrast to the effect of the blocking Ab to the β_2 integrin, the blocking Ab to the α_4 integrin significantly increased the rate of eosinophil mobilization in response to IL-5. This may be due to an inhibition of eosinophil adhesion to the bone marrow sinus endothelium, as electron micrographs show attachment of eosinophils to the luminal surface of the endothelium after exposure to IL-5 using the perfusion system. This is likely to be mediated by an attachment of eosinophil very late antigen $(VLA)_4$ to VCAM-1 expressed constitutively on the bone marrow endothelium (32), before eosinophils leave the bone marrow in response to IL-5. Consistent with this hypothesis was the finding that a proportion of intravenously injected ^{111}In-labeled guinea pig peritoneal eosinophils (used as a surrogate for newly migrated cells in vivo) localized in the bone marrow by a VLA_4-dependent mechanism. Furthermore, this hypothesis can explain the report that in vivo blocking of VLA_4 or VCAM-1, but not intercellular adhesion molecule (ICAM)-1, increases the blood eosinophil counts after allergen challenge of sensitized mice, although having no effect on blood eosinophil levels of unchallenged sensitized mice (44).

Our results show that the migration of eosinophils out of the bone marrow involves adhesive interactions with the α_4 and β_2 integrins. This may occur at several stages that remain to be identified, e.g., the adhesive interactions occurring between the abluminal surface of the endothelium and the leukocyte preparing to egress have not been defined and warrant further investigation.

The results of the light and electron microscopy provide evidence that eosinophil migration is a fundamental step in the mobilization process. Using an in vitro assay system we demonstrated that IL-5 is chemokinetic for guinea pig bone marrow eosinophils. This finding is consistent with previous studies that have shown that IL-5 is chemokinetic for human peripheral blood eosinophils (45). Chemokinesis is distinct from chemotaxis as it does not require a positive gradient of the chemoattractant and the resulting cell movement is random rather than directional. Our results suggest that stimulating the random migration of eosinophils within the hematopoietic tissue is sufficient to promote the egress of eosinophils. This may be due to the cy-

toarchitecture of the bone marrow where the hemopoietic islands are surrounded by branching venous sinusoids (46). We have previously demonstrated that eotaxin, a potent eosinophil CC-chemokine, is chemotactic for bone marrow eosinophils and can stimulate the mobilization of eosinophils from the bone marrow (28). Eotaxin has to establish a positive gradient across the sinus endothelium, by means of an elevated plasma concentration, to effect eosinophil release. In contrast, IL-5, because of its chemokinetic activity, will be effective when present in plasma or if generated extravascularly in the marrow. We found that IL-5 could act synergistically together with eotaxin in this mobilization process. Therefore, we hypothesize that a combination of both chemokinesis and chemotaxis may be the most effective means of mobilizing eosinophils from the bone marrow (28).

Despite the apparent similarity between mechanisms of eosinophil migration through the bone marrow sinus endothelium effecting release and migration through microvascular endothelial cells effecting recruitment to inflammatory sites, there is an interesting difference. In our studies both IL-5 and eotaxin can induce bone marrow eosinophil release, i.e., both chemokinesis and chemotaxis are effective. In contrast eotaxin, but not IL-5 is potent in stimulating recruitment at sites of inflammation (25), i.e., chemotaxis but not chemokinesis, is effective in this respect. This may be generally applicable to other leukocyte types.

IL-5 binds to and activates specific tyrosine kinase–linked IL-5 receptors expressed by eosinophils. A number of signal transduction molecules are activated in response to IL-5, including JAK1, JAK2, STAT1, Lyn, ERK2, and PI 3-kinase(47–49). In other cell types it has been demonstrated that PI 3-kinase plays a central role in regulating cytoskeletal changes and cell migration (50–52). In this study, we have demonstrated that the chemokinetic response of IL-5–stimulated bone marrow eosinophils was inhibited by wortmannin and LY294002, two selective inhibitors of PI 3-kinase. Furthermore, wortmannin (100 nM) markedly inhibited the IL-5–stimulated mobilization of eosinophils from the bone marrow. There are several potential molecular downstream targets of PI 3-kinase that have been identified in other cellular systems. These include protein kinase B, the rapamycin-sensitive p70S6K, and the focal adhesion–associated proteins p125 focal adhesion kinase and paxillin (53–56). It has been reported previously that rapamycin partially inhibits IL-5–mediated eosinophil survival (57). However, in our study, rapamycin had no effect on IL-5–stimulated eosinophil chemokinesis in vitro or in the in situ perfusion system. Thus p70S6K does not appear to be a downstream target of PI 3-kinase in this pathway.

The results of this study demonstrate that the emigration of eosinophils from the bone marrow is a multistep process. These steps may include release of mature eosinophils attached to bone marrow stromal cells and extracellular matrix, migration across the sinus endothelium, and release from the luminal surface of the endothelium. We have shown that adhesive interactions are important in regulating this process, α_4 and β_2 integrins acting in opposite directions. The identical effects of the reagents tested here on bone marrow eosinophil release and on eosinophil migration through an inert membrane in vitro reinforce the idea that it is chemokinesis of the eosinophil that is the primary response driving eosinophil mobilization in response to IL-5. The overriding conclusion from these studies is that eosinophil migration through the bone marrow sinus endothelium is the pivotal mechanism regulating release and as a consequence, this is an essential determinant of blood and tissue eosinophilia.

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References

1. Clutterbuck, E.J., E.M. Hirst, and C.J. Sanderson. 1989. Human interleukin-5 (IL-5) regulates the production of eosinophils in human bone marrow cultures: comparison and interaction with IL-1, IL-3, IL-6 and GM-CSF. *Blood*. 73: 1504–1512.
2. Sanderson, C.J., D.G. Warren, and M. Strath. 1985. Identification of a lymphokine that stimulates eosinophil differentiation in vitro. Its relationship to interleukin 3, and functional properties of eosinophils produced in cultures. *J. Exp. Med*. 162:60–74.
3. Yamaguchi, Y., T. Suda, J. Suda, M. Eguchi, Y. Miura, N. Harada, A. Tominaga, and K. Takatsu. 1988. Purified interleukin 5 supports the terminal differentiation and proliferation of murine eosinophilic precursors. *J. Exp. Med*. 167:43–56.
4. Dent, L.A., M. Strath, A.L. Mellor, and C.J. Sanderson.

1990. Eosinophilia in transgenic mice expressing interleukin 5. *J. Exp. Med.* 172:1425–1431.
5. Takafuji, S., K. Tadokoro, and K. Ito. 1996. Effects of interleukin (IL)-3 and IL-5 on human eosinophil degranulation induced by complement components C3a and C5a. *Allergy*. 51:563–568.
6. Warringa, R.A.J., H.J.J. Mengelers, P.H.M. Kuijper, J.A.M. Raaijmakers, P.L.B. Bruijnzeel, and L. Koenderman. 1992. *In vivo* priming of platelet-activating factor-induced eosinophil chemotaxis in allergic asthmatic individuals. *Blood*. 79: 1836–1841.
7. Warringa, R.A.J., R.C. Schweizer, T. Maikoe, P.H.M. Kuijper, P.L.B. Bruijnzeel, and L. Koenderman. 1992. Modulation of eosinophil chemotaxis by interleukin-5. *Am. J. Respir. Cell. Mol. Biol*. 7:631–636.
8. Yamaguchi, Y., T. Suda, S. Ohta, K. Tominaga, Y. Miura, and T. Kasahara. 1991. Analysis of the survival of mature human eosinophils: IL-5 prevents apoptosis in mature human eosinophils. *Blood*. 78:2542–2547.
9. Hamid, Q., S. Majumder, M. Shepphard, B. Corrin, C.M. Black, R.M. du Bois, and T.K. Jeffery. 1993. Expression of IL-4, IL-5, IFNγ and IL-2 mRNA in fibrosing alveolitis associated with systemic sclerosis. *Am. Rev. Respir. Dis*. 147: A479.(Abstr.)
10. Bachert, C., M. Wagenmann, U. Hauser, and C. Rudack. 1997. IL-5 synthesis is upregulated in human nasal polyp tissue. *J. Allergy Clin. Immunol*. 99:837–842.
11. Kay, A.B., S. Ying, V. Varney, M. Gaga, S.R. Durham, R. Moqbel, A.J. Wardlaw, and Q. Hamid. 1991. Messenger RNA expression of the cytokine gene cluster, interleukin 3 (IL-3), IL-4, IL-5, and granulocyte/macrophage colony-stimulating factor, in allergen-induced late-phase cutaneous reactions in atopic subjects. *J. Exp. Med*. 173:775–778.
12. Durham, S.R., S. Ying, V.A. Varney, M.R. Jacobson, R.M. Sudderick, I.S. Mackay, A.B. Kay, and Q.A. Hamid. 1992. Cytokine messenger RNA expression for IL-1, IL-4, IL-5, and granulocyte/macrophage-colony-stimulating factor in the nasal mucosa after local allergen provocation: relationship to tissue eosinophilia. *J. Immunol*. 148:2390–2394.
13. Dubucquoi, S., P. Desreumaux, A. Janin, O. Klein, M. Goldman, J. Tavernier, A. Capron, and M. Capron. 1994. Interleukin 5 synthesis by eosinophils: association with granules and immunoglobulin-dependent secretion. *J. Exp. Med*. 179:703–708.
14. Desreumaux, P., A. Janin, S. Dubucquoi, M. Copin, G. Torpier, A. Capron, M. Capron, and L. Prin. 1993. Synthesis of interleukin-5 by activated eosinophils in patients with eosinophilic heart diseases. *Blood*. 82:1553–1560.
15. Yamaguchi, S., H. Nagai, H. Tanaka, M. Tsujimoto, and N. Tsuruoka. 1994. Time course study for antigen-induced airway hyperreactivity and the effect of soluble IL-5 receptor. *Life Sci*. 54:471–475.
16. Corrigan, C.J., A. Haczku, V. Gemou-Engesaeth, S. Doi, Y. Kikuchi, K. Takatsu, S.R. Durham, and A.B. Kay. 1993. CD4 T-lymphocytes activation in asthma is accompanied by increased serum concentrations of interleukin-5. *Am. Rev. Respir. Dis*. 147:540–547.
17. Kung, T.T., D.M. Stelts, J.A. Zurcher, G.K. Adams III, R.W. Egan, W. Kreutner, A.S. Watnick, H. Jones, and R.W. Chapman. 1995. Involvement of IL-5 in a murine model of allergic pulmonary inflammation: prophylactic and therapeutic effect of an anti-IL-5 antibody. *Am. J. Respir. Cell. Mol. Biol*. 13:360–365.
18. Mauser, P.J., A. Pitman, A. Witt, X. Fernandez, J. Zurcher, T. Kung, H. Jones, A.S. Watnick, R.W. Egan, W. Kreutner, and K.G. Adams. 1993. Inhibitory effect of the TRFK-5 anti-IL-5 antibody in a guinea pig model of asthma. *Am. Rev. Respir. Dis*. 148:1623–1627.
19. Foster, P.S., S.P. Hogan, A.J. Ramsay, K.I. Matthaei, and I.G. Young. 1996. Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model. *J. Exp. Med*. 183:195–201.
20. Wang, J.M., A. Rambaldi, A. Biondi, Z.G. Chen, C.J. Sanderson, and A. Mantovani. 1989. Recombinant human interleukin-5 is a selective eosinophil chemoattractant. *Eur. J. Immunol*. 19:701–705.
21. Griffiths-Johnson, D.A., P.D. Collins, A.G. Rossi, P.J. Jose, and T.J. Williams. 1993. The chemokine, eotaxin, activates guinea-pig eosinophils *in vitro*, and causes their accumulation into the lung *in vivo*. *Biochem. Biophys. Res. Commun.* 197: 1167–1172.
22. Jose, P.J., D.A. Griffiths-Johnson, P.D. Collins, D.T. Walsh, R. Moqbel, N.F. Totty, O. Truong, J.J. Hsuan, and T.J. Williams. 1994. Eotaxin: a potent eosinophil chemoattractant cytokine detected in a guinea-pig model of allergic airways inflammation. *J. Exp. Med*. 179:881–887.
23. Jose, P.J., I.M. Adcock, D.A. Griffiths-Johnson, N. Berkman, T.N.C. Wells, T.J. Williams, and C.A. Power. 1994. Eotaxin: cloning of an eosinophil chemoattractant cytokine and increased mRNA expression in allergen-challenged guinea-pig lungs. *Biochem. Biophys. Res. Commun*. 205:788–794.
24. Humbles, A.A., D.M. Conroy, S. Marleau, S.M. Rankin, R.T. Palframan, A.E.I. Proudfoot, T.N.C. Wells, D. Li, P.K. Jeffery, D.A. Griffiths-Johnson, et al. 1997. Kinetics of eotaxin generation and its relationship to eosinophil accumulation in allergic airways disease: analysis in a guinea pig model in vivo. *J. Exp. Med*. 186:601–612.
25. Collins, P.D., S. Marleau, D.A. Griffiths-Johnson, P.J. Jose, and T.J. Williams. 1995. Co-operation between interleukin-5 and the chemokine eotaxin to induce eosinophil accumulation in vivo. *J. Exp. Med*. 182:1169–1174.
26. Mould, A.W., K.I. Matthaei, I.G. Young, and P.S. Foster. 1997. Relationship between interleukin-5 and eotaxin in regulating blood and tissue eosinophilia in mice. *J. Clin. Invest*. 99:1064–1071.
27. Kimura, I., Y. Moritani, and Y. Tanizaki. 1973. Basophils in bronchial asthma with reference to reagin-type allergy. *Clin. Allergy*. 3:195–202.
28. Palframan, R.T., P.D. Collins, T.J. Williams, and S.M. Rankin. 1998. Eotaxin induces a rapid release of eosinophils and their progenitors from the bone marrow. *Blood*. 91:2240–2248.
29. Teixeira, M.M., and P.G. Hellewell. 1997. The effect of the selectin binding polysaccharide fucoidin on eosinophil recruitment *in vivo*. *Br. J. Pharmacol*. 120:1059–1066.
30. Weg, V.B., T.J. Williams, R.R. Lobb, and S. Nourshargh. 1993. A monoclonal antibody recognizing very late activation antigen-4 (VLA-4) inhibits eosinophil accumulation in vivo. *J. Exp. Med*. 177:561–566.
31. Weg, V.B. and T.J. Williams. 1994. Chemical mediators and adhesion molecules involved in eosinophil accumulation *in vivo*. *Ann. NY Acad. Sci*. 725:146–155.
32. Schweitzer, K.M., A.M. Drager, P. van der Valk, S.F. Thijsen, A. Zevenbergen, A.P. Theijsmeijer, C.E. van der Schoot, and M.M. Langenhuijsen. 1996. Constitutive expression of E-selectin and vascular cell adhesion molecule-1 on

endothelial cells of hematopoietic tissues. *Am. J. Pathol*. 148: 165–175.
33. Kapeller, R., and L.C. Cantley. 1994. Phosphatidylinositol 3-kinase. *Bioessays*. 16:565–576.
34. Okada, T., L. Sakuma, Y. Fukui, O. Hazeki, and M. Ui. 1994. Blockage of chemotactic peptide-induced stimulation of neutrophils by wortmannin as a result of selective inhibition of phosphatidylinositol 3-kinase. *J. Biol. Chem*. 269: 3563–3567.
35. Vlahos, C.J., W.F. Matter, K.Y. Hui, and R.F. Brown. 1994. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J. Biol. Chem*. 269:5241–5248.
36. Becker, R.P., and P.P. De Bruyn. 1976. The transmural passage of blood cells into myeloid sinusoids and the entry of platelets into the sinusoidal circulation; a scanning electron microscopic investigation. *Am. J. Anat*. 145:183–205.
37. De Bruyn, P.P., S. Michelson, and T.B. Thomas. 1971. The migration of blood cells of the bone marrow through the sinusoidal wall. *J. Morphol*. 133:417–437.
38. De Bruyn, P.P. 1983. Transcellular cell movement and the formation of metastases. *Perspect. Biol. Med*. 26:441–450.
39. Feng, D., J.A. Nagy, K. Pyne, H.F. Dvorak, and A.M. Dvorak. 1998. Neutrophils emigrate from venules by a transendothelial cell pathway in response to fmlp. *J. Exp. Med*. 187:903–915.
40. Cook, R.M., H. Smith, and B.A. Spicer. 1993. Animal models of eosinophils. *In* Immunopharmacology of Eosinophils. H. Smith and R.M. Cook, editors. Academic Press, London. 193–216.
41. van Eeden, S.F., R. Miyagashima, L. Haley, and J.C. Hogg. 1997. A possible role for L-selectin in the release of polymorphonuclear leukocytes from bone marrow. *Am. J. Physiol*. 272:H1717–1724.
42. Walsh, G.M., A. Hartnell, A.J. Wardlaw, K. Kurihara, C.J. Sanderson, and A.B. Kay. 1990. IL-5 enhances the *in vitro* adhesion of human eosinophils, but not neutrophils, in a leucocyte integrin (CD11/18)-dependent manner. *Immunology*. 71: 258–265.
43. Das, A.M., T.J. Williams, R.R. Lobb, and S. Nourshargh. 1995. Lung eosinophilia is dependent on IL-5, and the adhesion molecules CD18 and VLA-4 in a guinea-pig model. *Immunology*. 84:41–46.
44. Nakajima, H., H. Sano, T. Nishimura, S. Yoshida, and I. Iwamoto. 1994. Role of vascular cell adhesion molecule 1/very late activation antigen 4 and intercellular adhesion molecule 1/lymphocyte function-associated antigen 1 interactions in antigen-induced eosinophil and T cell recruitment into the tissue. *J. Exp. Med*. 179:1145–1154.
45. Schweizer, R.C., B.A.C. van Kessel-Welmers, R.A.J. Warringa, T. Maikoe, J.A.M. Raaijmakers, J.J. Lammers, and L. Koenderman. 1996. Mechanisms involved in eosinophil migration. Platelet-activating factor-induced chemotaxis and interleukin-5-induced chemokinesis are mediated by different signals. *J. Leukocyte Biol*. 59:347–356.
46. De Bruyn, P.P., P.C. Breen, and T.B. Thomas. 1970. The microcirculation of the bone marrow. *Anat. Rec*. 168:55–68.
47. Pazdrak, K., S. Stafford, and R. Alam. 1995. The activation of the Jak-STAT 1 signaling pathway by IL-5 in eosinophils. *J. Immunol*. 155:397–402.
48. Pazdrak, K., D. Schreiber, P. Forsythe, L. Justement, and R. Alam. 1995. The intracellular signal transduction mechanism of interleukin 5 in eosinophils: the involvement of lyn tyrosine kinase and the Ras-Raf-1-MEK-microtubule-associated protein kinase pathway. *J. Exp. Med*. 181:1827–1834.
49. Coffer, P.J., R.C. Schweizer, G.R. Dubois, T. Maikoe, J.J. Lammers, and L. Koenderman. 1998. Analysis of signal transduction pathways in human eosinophils activated by chemoattractants and the T-helper 2-derived cytokines interleukin-4 and interleukin-5. *Blood*. 91:2547–2557.
50. Wennstrom, S., A. Siegbahn, K. Yokote, A.K. Arvidsson, C.H. Heldin, S. Mori, and L. Claesson Welsh. 1994. Membrane ruffling and chemotaxis transduced by the PDGF beta-receptor require the binding site for phosphatidylinositol 3′ kinase. *Oncogene*. 9:651–660.
51. Wennstrom, S., P. Hawkins, F. Cooke, K. Hara, K. Yonezawa, M. Kasuga, T. Jackson, L. Claesson Welsh, and L. Stephens. 1994. Activation of phosphoinositide 3-kinase is required for PDGF-stimulated membrane ruffling. *Curr. Biol*. 4:385–393.
52. Turner, L., S.G. Ward, and J. Westwick. 1995. RANTES-activated human T lymphocytes. A role for phosphoinositide 3-kinase. *J. Immunol*. 155:2437–2444.
53. Franke, T.F., S.I. Yang, T.O. Chan, K. Datta, A. Kazlauskas, D.K. Morrison, D.R. Kaplan, and P.N. Tsichlis. 1995. The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell*. 81:727–736.
54. Burgering, B.M., and P.J. Coffer. 1995. Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature*. 376:599–602.
55. Rankin, S., R. Hooshmand Rad, L. Claesson Welsh, and E. Rozengurt. 1996. Requirement for phosphatidylinositol 3′-kinase activity in platelet-derived growth factor-stimulated tyrosine phosphorylation of p125 focal adhesion kinase and paxillin. *J. Biol. Chem*. 271:7829–7834.
56. Chung, J., T.C. Grammer, K.P. Lemon, A. Kazlauskas, and J. Blenis. 1994. PDGF- and insulin-dependent pp70S6k activation mediated by phosphatidylinositol-3-OH kinase. *Nature*. 370:71–75.
57. Hom, J.T., and T. Estridge. 1993. FK506 and rapamycin modulate the functional activities of human peripheral blood eosinophils. *Clin. Immunol. Immunopathol*. 68:293–300.