

Increased mature and immature CCR3 messenger RNA⁺ eosinophils in bone marrow from patients with atopic asthma compared with atopic and nonatopic control subjects

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Background: Eosinophil infiltration of the bronchial mucosa is characteristic of asthma. Eosinophils differentiate from CD34⁺ progenitors. Animal models suggest cooperation between IL-5 and eotaxin to allow rapid mobilization of a pool of bone marrow eosinophils followed by recruitment to the airway mucosa. **Objective:** The purpose of this study was to enumerate CD34⁺ cell numbers in blood and bone marrow from atopic asthmatics and control subjects and to test the hypothesis that there is an increased bone marrow pool of CCR3⁺ eosinophils in patients with atopic asthma, as compared with control subjects. **Methods:** Bone marrow aspirates and peripheral blood were obtained from volunteers with asthma and control volunteers. CD34⁺ cell numbers were evaluated by flow cytometry, and eosinophil colony-forming activity was evaluated by methylcellulose cultures. Mature eosinophils, eosinophil myelocytes, metamyelocytes, and band forms (immature eosinophils) were enumerated by morphologic findings and immunocytochemistry for eosinophil cationic protein. CCR3 and eotaxin mRNA expression was examined by *in situ* hybridization, and protein expression was examined by immunocytochemistry. CCR3⁺ cells were further identified with Chromotrope 2R staining. **Results:** CD34⁺ cell numbers in bone marrow were increased in atopic subjects. Numbers of eosinophil colony-forming units in blood and bone marrow did not differ between groups. Percentages of both mature and immature eosinophils were increased in bone marrow from patients with atopic asthma, but not atopic patients with no asthma or normal control subjects. CCR3 was expressed by immature and mature bone marrow eosinophils. Eotaxin was expressed by bone marrow cells from all 3 groups, but there was no increase in subjects with asthma. **Conclusion:** These findings suggest that in humans there is an increased bone marrow pool of CCR3⁺ mature and immature

eosinophils available for rapid mobilization in subjects with asthma but not in atopic subjects with no asthma. (*J Allergy Clin Immunol* 1999;103:99-106.)

Key words: Asthma, atopy, eosinophil, progenitor cell, bone marrow.

Selective accumulation and activation of eosinophils in the bronchial mucosa are now considered central events in the pathogenesis of asthma.¹ The development of a sustained tissue eosinophilia, as occurs in asthma, requires an increase in the production and delivery of these cells.

In common with other leukocytes, eosinophils develop from pluripotent progenitors in the bone marrow (BM). These progenitors express the sialylated glycoprotein CD34² on their surface and have been shown to be capable of generating multiple cell lineages *in vitro*³ and to reconstitute hematopoiesis in humans.⁴ In normal adults, hematopoietic progenitors reside largely in the BM, and their proliferation and differentiation are regulated by a complex network of cytokines. Among those, IL-3 and GM-CSF cause CD34⁺ cells to differentiate to the eosinophil/basophil lineage, although IL-5 was initially described as acting at a later stage, to promote terminal differentiation of the committed eosinophil precursor; recent data also suggest that CD34⁺ cells can express IL-5 receptors.^{5,6} Previous reports have documented increased numbers of CD34⁺ cells in BM and peripheral blood (PB) of atopic subjects, irrespective of asthmatic status,^{6,7} and have shown increased expression of IL-5R α mRNA by CD34⁺ cells in BM after inhalation challenge of sensitized patients with asthma.⁶ Together with data from humans and experimental animals that show increased eosinophil colony-forming activity in BM and blood after allergen challenge,⁸⁻¹⁰ this work suggests a link between airway inflammation and BM turnover of eosinophil progenitors.

Eotaxin is a C-C chemokine that is chemotactic for eosinophils.^{11,12} Eotaxin acts through a specific receptor, CCR3, present in high numbers on eosinophils, and may therefore regulate the selective recruitment of eosinophils to sites of inflammation.^{13,14} Data from animal models suggest that eotaxin and IL-5 may act cooperatively to promote the recruitment of eosinophils into tissues.¹⁵ It is proposed that IL-5 provides the signal for the release of a pool of eosinophils from the BM, although eotaxin signals for eosinophil localization to the site of inflammation.^{15,16}

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TABLE I. Clinical characteristics of subjects studied

	AAs (n = 10)	ACs (n = 12)	NCs (n = 9)
Age (y)	24 (20-47)	23 (18-30)	24 (21-26)
Sex (M:F)	2:8	8:4	6:3
FEV ₁ (% predicted)	97.5 (65-120)	104 (94-130)	104 (95-121)
PEFR (% predicted)	95 (70-123)	95 (81-123)	90 (82-98)
Histamine (PC ₂₀ mg/mL)	3.6 (1-6)	>16	>16
IgE (IU/mL)	409* (235-1039)	112 (41-223)	38 (4-73)
PB eosinophil count ($\times 10^9$ /L)	0.3* (0.1-0.5)	0.1 (0.1-0.3)	0.1 (0.1-0.2)

All values are medians (range in parentheses).

PC₂₀, Provocative concentration producing a 20% fall in FEV₁.

*P < .05 compared with NCs and ACs.

Abbreviations used

AA:	Atopic asthmatic subject
AC:	Atopic control subject
APAAP:	Alkaline phosphatase antialkaline phosphatase
BM:	Bone marrow
Eo-CFU:	Eosinophil colony-forming units
IL-5R α :	α -Subunit of the IL-5 receptor
NC:	Nonatopic control subject
PB:	Peripheral blood
PEFR:	Peak expiratory flow rate

The purpose of this study was to confirm increased BM numbers of CD34⁺ cells, to enumerate eosinophils in the BM of subjects with asthma, and to determine the expression of CCR3, the eotaxin receptor.

METHODS

Subjects

Ten atopic asthmatic subjects (AAs), 12 atopic control subjects without asthma (ACs), and 9 nonatopic control subjects (NCs) were examined (Table I). Patients were recruited from the outpatient department of the Royal Brompton Hospital and from volunteers responding to advertisements. Asthma was defined on the basis of a history of variable breathlessness and/or wheeze, documented 20% reversibility in peak expiratory flow rate (PEFR) or FEV₁, and airway hyperresponsiveness defined by a histamine PC₂₀ of 6 mg/mL or less.¹⁷ Atopy was defined by a positive skin prick test response (defined as >3 mm weal diameter compared with control at 15 minutes) or a positive radioallergen sorbent test response of more than 0.7 IU/mL (Phadebas; Pharmacia, Uppsala, Sweden) to one or more of a panel of common aeroallergens. Six of the AAs and 3 of the ACs had seasonal symptoms; all were studied out of season. Three of the 10 AAs had mild symptoms (not requiring inhaled corticosteroids) at the time of BM aspiration. None of the ACs or NCs had current symptoms. Pregnant women and smokers were excluded. None of the subjects were currently taking oral or inhaled corticosteroids, and none had received allergen immunotherapy. The study was performed with the approval of the Ethics Committee of the Royal Brompton Hospital, and informed written consent was obtained from all participants.

BM aspiration

BM aspiration was performed by a consultant hematologist after the patient had received local anesthesia. BM was aspirated under aseptic conditions from the posterior iliac crest, with a standard aspiration needle, and transferred to a sterile universal container with 1000 units of heparin. Heparinized BM was then mixed with McCoy's 5A

medium (Gibco, Paisley, Scotland) in a ratio of 1:10. Total eosinophil counts from blood and BM were obtained by Kimura staining.

BM slides were prepared with a Shandon 2 cytopsin device (Shandon; Southern Instruments, Runcorn, UK) and stained with May-Grunwald Giemsa (Sigma, Poole, UK) to estimate the numbers of mature and immature eosinophils. Immature eosinophils in the BM were enumerated by standard hematologic criteria. The eosinophil myelocyte is the earliest distinct stage of eosinopoiesis that can be distinguished on the basis of characteristic eosinophilic cytoplasmic granules.¹⁸ In preparations stained with May-Grunwald Giemsa, we considered myelocytes cells of variable size with a large round or oval nucleus, but without any nucleoli, and with pink granules in the cytoplasm. The transition from myelocyte to metamyelocyte is characterized by an indentation of the nucleus and the increase of cytoplasmic granules. At the stage where the nucleus becomes kidney shaped and elongates further, the cell comes to be known as a "band" form. Eosinophil myelocytes, metamyelocytes, and band forms were enumerated together as immature eosinophils. The fully mature eosinophil is a smaller cell with nuclear material divided into 2 or more (rarely 3) segments that are joined by thin strands of chromatin. Numerous granules of uniform size appear to pack the cytoplasm and stain pink with May-Grunwald Giemsa. To confirm eosinophil lineage of both mature and immature cells, counts were also performed by combining morphologic criteria with immunocytochemistry for the eosinophil-specific eosinophil cationic protein (Fig 1).

Colony-forming assays

At the same time as the BM aspiration, 100 mL of PB was drawn into 2 heparin-containing syringes and mixed with McCoy's 5A medium in a ratio of 1:2. Colony-forming assays were performed on both blood and BM samples. Before being mixed with McCoy's medium, eosinophil counts were obtained with Kimura staining, with a hemocytometer. Samples were layered on Ficoll (Pharmacia) and centrifuged at 400g for 25 minutes. The mononuclear population was collected from the interface and washed twice with McCoy's 5A medium. Then samples were incubated in this medium (17 mL) with 15% (3 mL) FCS (Sigma), 100 U/mL penicillin (Sigma), 100 μ g/mL streptomycin (Sigma), and 50 μ mol/L 2-mercaptoethanol (Gibco) for 2 hours in plastic flasks (Nunc A/S, Roskilde, Denmark) at 37°C and 5% CO₂ to exclude the monocytes that interfere with the growth of progenitor cells in semisolid cultures.

Nonadherent mononuclear cells were harvested and washed twice with McCoy's 5A medium, then cultured in aliquots of 1×10^6 cells per 35 $\times$ 10 mm culture dish (Nunc), in Iscove's modified Dulbecco's medium (Gibco) containing 0.9% methylcellulose (Sigma), 100 U/mL penicillin, 100 μ g/mL streptomycin, 50 μ mol/L 2-mercaptoethanol, 20% FCS, and 1 ng/mL of IL-3 (R&D Systems, Abingdon, UK) alone, 1 ng/mL of IL-5 (R&D Systems) alone, or the combination of both. Dose-response experiments were first performed to

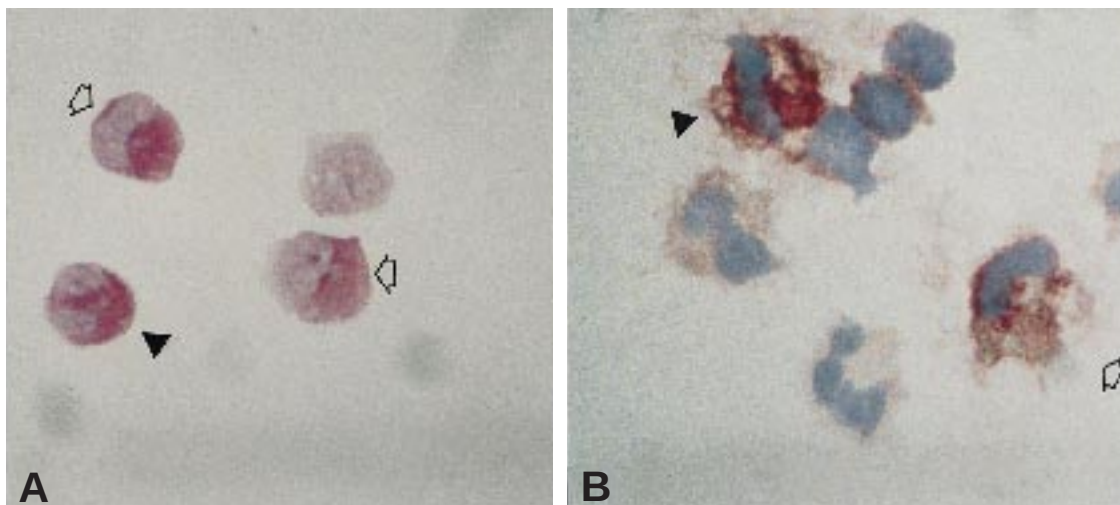


FIG 1. A, Photomicrograph of immature and mature eosinophil in BM cytocentrifuge preparations stained chromotrope 2R (original magnification, $\times 1000$). **B,** Photomicrograph of immature and mature eosinophil in BM cytocentrifuge preparations stained with EG2 mAb to EG2 (original magnification, $\times 1000$). Immature forms are large cells with a large, round nucleus (arrows), whereas mature eosinophils (arrowheads) show a more compact, bi-lobed nucleus.

assess the optimal concentrations of cytokines. Duplicate cell cultures were set up for each condition, and colonies (aggregates of 50 cells or more) were counted after 14 days of incubation at 37°C and 5% CO_2 . CFUs were identified by inverted microscopy, and only compact, tight, and round clusters were considered as eosinophil colony-forming units (Eo-CFUs).¹⁹ Eo-CFUs were picked at random, placed on slides, and confirmed as eosinophil colonies by chromotrope 2R staining. All counts were performed by one investigator, who was blinded to the clinical data of the patients.

Cytofluorometric analysis

Nonadherent mononuclear cell suspension (100 μL) containing up to 5×10^5 cells was mixed with 10 μL of FITC-conjugated anti-human CD34 mAb (HCPA-2, mouse IgG1; Becton-Dickinson, San Jose, Calif). Cells were incubated on ice for 15 minutes. After incubation cells were washed twice with phosphate buffer solution with 0.1% wt/vol azide and 0.5% wt/vol BSA and fixed with 0.5% formaldehyde-isoton solution (Coulter Corporation, Hialeah, Fla). FITC-conjugated mouse IgG1 isotype control mAb (Becton-Dickinson) was used to assess the degree of nonspecific binding to cell preparations. Stained cells were analyzed on a Coulter Epics Elite ESP flow cytometer (Coulter), with identical gain settings for each sample.

The cytometer flow cell was fully aligned and calibrated with Immuno-Check fluorospheres (Coulter) before each run. A total of at least 100,000 cells were analyzed to obtain a reliable number of events. Data were collected by gating on CD34+ FITC staining cells (Fig 2). This flow cytometry staining protocol was initially devised to allow triple color staining with CD33 and IL-5R α antibodies (data not shown). Nonspecific staining of platelets and granulocytes has been reported in whole blood samples, and it has been suggested that gating on CD34 be combined with exclusion of CD45+ cells²⁰ or CD14 and CD66e.²¹ However, when gating strategies that were based on the identification of cells based on forward and side scatter and CD34 staining were compared with additional staining of CD45+ cells, we obtained similar percentages for CD34+ popu-

lations, both from PB and BM, and saw no evidence of nonspecific staining (data not shown).

In situ hybridization

Cytospins were prepared from BM aspirates at 0.5×10^6 cells/slide coated with 0.1% poly-L-lysine (BDH, Leicester, UK), and in situ hybridization was performed as has been previously described.²² All reagents were from Sigma Chemicals, unless otherwise indicated. Based on sequencing, a 307-bp sequence of human eotaxin²³ (3' untranslated region, 894-1201 bp) and a 1056-bp sequence of human CCR3¹⁴ (encoding region) cDNA fragments were inserted into PCR Bluescript and pSP72 vectors, respectively. Riboprobes were prepared from cDNA for eotaxin and CCR3 as previously described.^{22,24} Briefly, riboprobes (antisense or sense) were synthesized in the presence of adenine triphosphate, guanosine triphosphate, cytosine triphosphate, and ³⁵S-uridine triphosphate, and appropriate RNA polymerases (T7, SP6, or T3), respectively. Slides were defrosted for the experimental procedure. Permeabilization, prehybridization, and hybridization protocols were as described previously.^{22,25} Incubation in N-ethyl maleimide, iodoacetamide, and triethanolamine reduced nonspecific binding of the ³⁵S-labeled probes. Negative controls used hybridization with the sense probe and pretreatment of slides with RNase A (Promega, Southampton, UK) before hybridization with the antisense probe. For autoradiography, slides were dipped into K-5 emulsion (Ilford, Basildon, UK) and exposed at 4°C for 2 weeks in absolute darkness in a desiccated environment. The slides were developed (D-19 developing solution; Eastman Kodak Co, Rochester, NY), rinsed, and counterstained with Harris' hematoxylin. Dense deposits of silver grains on autoradiographs were present over cells expressing cytokine mRNA. In some experiments slides were counterstained with chromotrope 2R to identify whether mature and immature eosinophils (according to the morphologic evidence) express CCR3 mRNA. Slides were counted in duplicate blind to the patients' clinical status with an eyepiece graticule, as previously described.²⁵ Results were expressed as the total number of positive cells per 200 total BM cells and percentages of CCR3 mRNA+/chromotrope 2R+ cells. The coefficient of variability of the duplicate counts obtained from all slides was less than 5%.

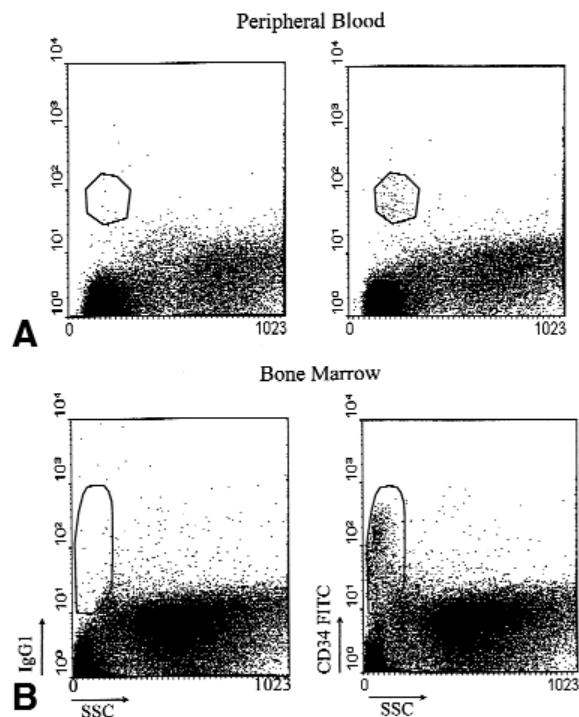


FIG 2. Flow cytometric analysis of CD34⁺ populations among non-adherent mononuclear cells in PB (**A**) and BM (**B**) from a subject with asthma. Cells were isolated by density gradient centrifugation and a 2-hour plastic adherence step at 37°C. Staining was with FITC-labeled anti-CD34 (HPCA2; Becton-Dickinson) or isotype control antibody (IgG1-FITC), and the cells were gated on the basis of forward and side scatter followed by CD34 and side scatter. Clear positive populations were identified, as shown, and expressed as percentage of 10,000 total mononuclear cells analyzed.

Immunocytochemistry

Immunocytochemistry was performed with alkaline phosphatase antialkaline phosphatase (APAAP) as described previously,²⁵ with some modifications. The monoclonal anti-CD34 (BIRMA-K3), rabbit anti-mouse Ig, APAAP, and control IgG1 were purchased from Dako (High Wycombe, UK). Optimal concentrations of all antibodies used were determined in pilot experiments. Briefly, the cytopsins were incubated with primary antibodies (1 in 50 dilution) in PBS for 60 minutes, incubated with rabbit anti-mouse Ig, and incubated with APAAP for 30 minutes each at room temperature. Positive cells stained red after development with Fast Red stain. Omission or substitution of the primary antibody with an irrelevant antibody of the same species was used as a negative control. The slides were counted in duplicate, without knowledge of the patients' clinical status. Results were expressed as the percentages of CD34 positive cells. The coefficient of variability of the duplicate counts obtained from all slides was less than 5%. No immunoreactivity was observed in cytopsins stained with omission of the primary rat antibody or substitution of this antibody with an irrelevant antibody of the same species.

To confirm eosinophil lineage of cells identified as immature and mature eosinophils on BM cytopsins, slides were stained with mAb against eosinophil cationic protein (EG2). Slides were incubated with EG2 antibody (Pharmacia, Bucks, UK) for 30 minutes, followed by rabbit anti-mouse antibody and then APAAP as described earlier. Staining was developed with Fast Red (Sigma).

Eotaxin immunocytochemistry was also performed with the

APAAP technique, with the use of the antieotaxin monoclonal antibody 2G6.²⁵ CCR3 immunoreactivity was detected by peroxidase-antiperoxidase staining. Slides were first treated with 0.3% hydrogen peroxide in PBS with 0.1% sodium azide for 20 minutes to block endogenous peroxidase, then incubated with monoclonal anti-CCR3, 7B11^{13,25} for 2 hours at room temperature. Staining was detected with rabbit anti-mouse Ig (Dako), followed by peroxidase-antiperoxidase staining (1:30) for 30 minutes, and was developed with diaminobenzidine tetrahydrochloride (Sigma). Positive cells stained brown, and this allowed counter-staining with chromotrope 2R. Negative controls, excluding the primary antibody, confirmed the blocking of nonspecific peroxidase activity.

Statistical analysis

Between patient groups, comparisons were performed with the nonparametric Mann-Whitney *U* test with the aid of a commercially available statistical package (Minitab for Windows, Minitab Release 9.2; Minitab Inc, State College, Pa). *P* values less than .05 were considered significant.

RESULTS

Increased percentage of both mature and immature eosinophils in BM from AAs compared with ACs and NCs

The percentages and total numbers of eosinophils in PB were significantly elevated in AAs compared with both ACs ($P < .01$) and NCs ($P < .01$; Table I and Fig 3, A).

There was also an increased percentage of total eosinophils in the BM of AAs when compared with that of ACs and NCs by Kimura staining (Fig 3, B). There was no significant difference in eosinophil percentage between ACs and NCs. Among all atopic subjects (both with asthma and with no asthma), there was a positive correlation between percentage of eosinophils in the BM and PB (Fig 3, C).

Immature eosinophils, including myelocytes, metamyelocytes, and band forms, were readily distinguished from mature, bi-lobed eosinophils in May-Grunwald Giemsa, and EG2-stained cytocentrifuge preparations of BM (Fig 1). Eosinophil lineage of these cells was confirmed by EG2 staining. The percentage of immature eosinophils in BM was significantly elevated in the AAs when compared with both ACs and NCs by both staining methods, but there was no difference in the percentage between ACs and NCs (Fig 4). Immature eosinophils were not detected in PB from any of the 3 patient groups (data not shown).

CCR3 expressed by both mature and immature eosinophils in BM

In situ hybridization was performed to detect mRNA encoding the eotaxin receptor CCR3. The percentage of BM cells expressing CCR3 mRNA was increased in the AA group, when compared with both ACs and NCs (Fig 5). Both mature and immature eosinophils expressed mRNA for CCR3 (Fig 5). Immunocytochemistry confirmed increased percentages of cells expressing CCR3 in BM from AAs (median, 16%; range, 12.5 to 23.5) when compared with AC (median, 4.7%; range, 1.5 to 10; $P < .01$ compared with AAs) and NC (median, 3.5%; range, 2 to 4; $P < .005$ compared with AAs) control groups. There

TABLE II. Numbers of Eo-CFUs in BM and PB

	AAs (n = 7)	ACs (n = 9)	NCs (n = 8)
BM Eo-CFUs			
IL-3	0 (0-48)	1 (0-76)	12 (0-42)
IL-5	18 (0-48)	6 (0-44)	16.5 (0-72)
IL-3+IL-5	18 (0-104)	38 (0-194)	40 (0-76)
PB Eo-CFUs			
IL-3	36 (0-60)	56 (2-234)	30 (0-114)
IL-5	40 (0-52)	16 (4-140)	27 (0-92)
IL-3+IL-5	38 (0-64)	70 (24-306)	51 (0-108)

Values are expressed as median (range in parentheses).

was no significant difference in percentage of CCR3⁺ cells in BM from ACs when compared with NCs.

The combination of in situ hybridization and chromotrope 2R⁺ staining enabled us to estimate the percentage of CCR3 mRNA⁺ cells in BM that were eosinophils. Although most (more than 90%) of CCR3 mRNA⁺ cells were eosinophils, there was a small percentage of CCR3 mRNA⁺ cells (less than 9%) that were chromotrope 2R negative. More than 90% of CCR3⁺ cells detected by immunocytochemistry were chromotrope 2R⁺, and both mature and immature eosinophils expressed CCR3.

Eotaxin expression not increased in the BM of AAs

Cells expressing eotaxin mRNA were detected in the BM of all the groups studied. The median percentage of eotaxin mRNA⁺ positive BM cells was 1.2% (range, 0.2% to 16.7%) in the AA group, and 2.2% (range, 0.2% to 8.6%) and 0.8% (range, 0.4% to 2.1%) in the AC and NC control groups, respectively. There were no significant differences in the percentages of eotaxin mRNA-positive cells detected between the 3 groups. These findings were confirmed by immunocytochemistry with the antieotaxin mAb 2G6. There were no significant differences between the 3 groups (median, 4%; range, 2 to 8 for AAs; median, 2%; range, 2 to 12 for ACs; and median, 1%; range, 0 to 8 for NCs). Cells expressing mRNA for eotaxin were predominately mononuclear cells, but we did not further phenotype these cells.

CD34⁺ progenitors in BM increased in ACs when compared with NCs

Increased percentages of CD34⁺ progenitor cells were detected by flow cytometry in the BM of both AAs and ACs (median percentage of nonadherent mononuclear cells, 1.5% [range, 1.1% to 3.6%] and 1.5% [range, 0.6% to 3.8%], respectively), when compared with NCs (median, 1.1%; range, 0.4% to 1.6%). Similar results were also obtained if numbers of CD34⁺ cells were expressed as the absolute number/ 1×10^6 nonadherent mononuclear cells. Elevated numbers of CD34 immunoreactive cells were also detected in the AA and AC groups when enumerated by immunocytochemistry (data not shown).

The percentage of CD34⁺ cells in PB, enumerated by

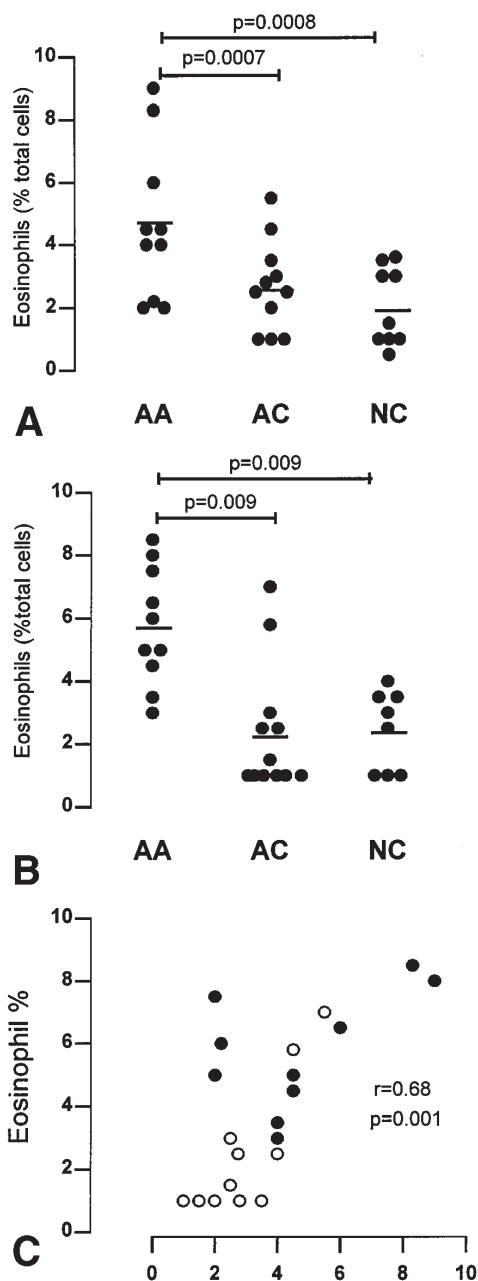


FIG 3. Percentage of eosinophils in PB (**A**) and BM (**B**) from AAs (n = 10), ACs (n = 12), and NCs (n = 9). Cells were stained with Kimura stain, and total eosinophils were counted by an observer blinded to subject details. **C**, The relationship between percentage of eosinophils in BM and blood for AAs (closed circles) and ACs (open circles). Cells were counted after Kimura staining. Correlation was Spearman's rank correlation.

flow cytometry, was 0.1% (range, 0.1% to 0.2%) in the AA group, 0.2% (range, 0.1% to 1.2%) in the AC group, and 0.1% (range, 0.1% to 0.2%) in the NC group. There were no significant differences among the groups.

Eo-CFUs

Eosinophil progenitors were identified by their ability

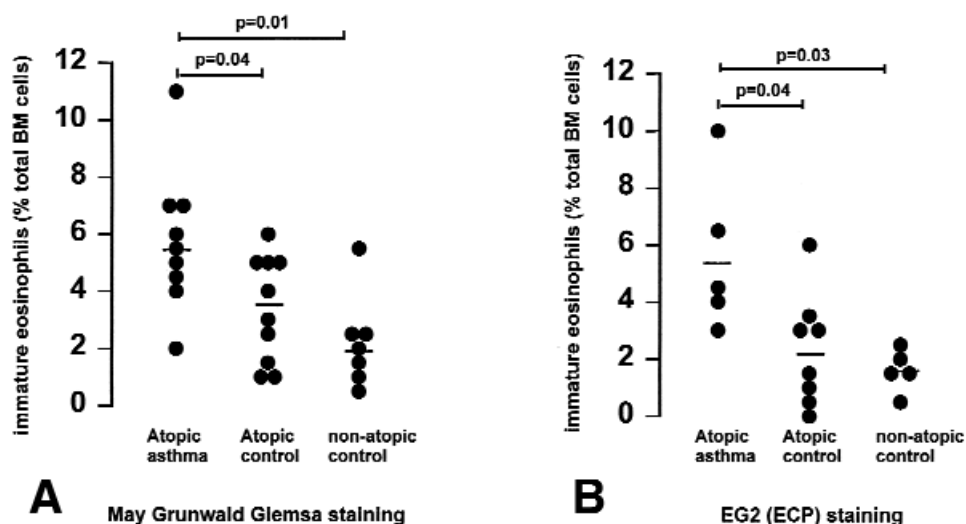


FIG 4. Percentages of immature eosinophils in BM from AAs (n = 10), ACs (n = 12), and NCs (n = 9). Cytocentrifuge preparations were stained with May-Grunwald Giemsa stain (**A**) or EG2 immunocytochemistry for eosinophil cationic protein (**B**); myelocyte, metamyelocyte, and band forms with clear eosinophil granules were counted. Comparison between groups was by Mann-Whitney *U* test.

to generate clonally derived colonies when cultured in semisolid media, under the influence of IL-3 and/or IL-5.

Numbers of Eo-CFUs identified at 14 days are shown in Table II. Eo-CFUs were seen in all groups, but no significant differences were found between the groups in both PB and BM. The Eo-CFU yield was higher than 80%, with approximately 5% basophil and 10% macrophage contamination. Only Eo-CFUs were counted. There were positive correlations between total CFUs with IL-3 and percentage CD34⁺ cells in BM ($r = 0.97$; $P < .001$) and between CFUs with IL-3 and IL-5 and percentage CD34⁺ in BM ($r = 0.82$; $P < .05$).

DISCUSSION

To investigate the potential contribution of the BM to the delivery of eosinophils in response to signals from the asthmatic airway, we examined CD34⁺ progenitor cells and Eo-CFUs in PB and BM. We also studied the presence of mature and immature eosinophils and the expression of eotaxin and its receptor CCR3 at the mRNA and protein level in the BM. Our results demonstrate, for the first time, a significant increment in the numbers of BM mature and immature eosinophils in AA groups, when compared with AC or NC control groups. We also show that BM eosinophils express CCR3, which suggests that there is a pool of eosinophils in the BM of subjects with asthma that is capable of responding to eotaxin. In contrast to the finding with eosinophils, the percentages of CD34⁺ in BM were elevated in atopic patients independently of their asthmatic status. We did not detect any increase in Eo-CFU in BM or PB in these asymptomatic atopic subjects.

Animal models of allergen challenge suggest that eotaxin and IL-5 may act cooperatively to promote the recruitment of eosinophils to the tissues. It has been proposed that during the inflammatory response, IL-5 may provide the

signal for the release of a pool of eosinophils from the BM, although eotaxin may elicit the signal for eosinophil localization to the site of inflammation.¹⁵ The proposed synergism between remotely acting IL-5 and locally acting eotaxin would induce a rapid recruitment of eosinophils in the allergen-stimulated tissue.¹⁶ In addition, it has recently been shown that eotaxin induces rapid release of eosinophils and their precursors from the BM in an animal model.²⁶ We show here that in asthma, but not atopy per se, there is evidence for an increased pool of both immature and mature CCR3⁺ eosinophils in the BM of human subjects, with the potential for such mobilization. Immature eosinophils were not detected in PB of subjects with asthma (not shown), thus it is likely that this BM eosinophil pool reflects local differentiation. Previous studies have documented the kinetics of eosinophil recruitment from blood to tissue in allergen challenged skin,²⁷ nasal mucosa,²⁸ and airway^{29,30} in atopic subjects, although there is no information on mobilization of BM eosinophils after allergen challenge in humans. The increased percentage of BM eosinophils in AAs, but not ACs, may reflect the relative concentrations of IL-5 circulating in these subjects.³¹ It seems likely that IL-5 acts in eosinophil differentiation and proliferation to provide the increased pool of available eosinophils in the BM and increased circulating cell numbers. Further, dynamic studies will be required to document changes in the BM eosinophil pool after allergen challenge.

To investigate the potential of BM eosinophils to respond to chemotactic stimuli, we examined the expression of eotaxin and CCR3 receptor. Eotaxin is a specific eosinophil chemoattractant¹¹ that regulates the selective recruitment of eosinophils through the CCR3 receptor, can act in the release of BM eosinophils in animal models,²⁶ and is constitutively expressed in a number of tissues.³² CCR3 expression is largely restricted to eosinophils and basophils.³³ Here

we demonstrate that CCR3 is expressed by the BM pool from both mature and immature eosinophils present in asthma. We cannot comment on the relative density of expression of CCR3 between immature and mature eosinophils or between eosinophils from AA and control groups using the technique of immunocytochemistry. However, it seems likely that, as for PB eosinophils,¹³ most of these cells will be responsive to eotaxin. Further studies are in progress to determine chemotactic responses of BM eosinophils. So far, it is not known at what stage of their differentiation eosinophils acquire the CCR3 receptor and become capable of responding to this chemotactic factor; this area is currently the subject of a separate study. Acquisition of CCR3 by the cell line HL-60 during differentiation towards an eosinophil-like cell has been reported.³⁴ A small percentage of BM cells expressing mRNA for CCR3 were not stained with chromotrope 2R. These may represent cells earlier in the eosinophil lineage or the small percentage of T cells reported to express CCR3.³⁵ We recently reported an increased expression of eotaxin in the bronchial mucosa of AAs compared with control subjects.²⁵ Our current data do not show any increase in eotaxin mRNA or protein expression in BM cells from AAs compared with ACs and NCs. This finding may provide evidence for a gradient of eotaxin favoring recruitment to the airway in asthma. However, it is of note that eotaxin mRNA was primarily detected in epithelial and endothelial cells in bronchial biopsies²⁵ and that BM aspirates may contain few such cells. An assessment of eotaxin in stromal cells or BM trephines would give further information.

In agreement with the findings of Sehmi et al⁷ and in contrast to our findings with eosinophils, we show increased CD34 numbers in BM in atopic subjects regardless of asthmatic status. The CD34⁺ gating strategy used here differed from that of Sehmi et al,⁷ and we did not use CD45 counter staining, which has been recommended to exclude nonspecific staining when whole blood is analyzed.²⁰ However, experiments comparing these 2 flow cytometry gating strategies on nonadherent mononuclear cells from both PB and BM showed that they gave similar percentages of CD34⁺ cells, without nonspecific staining, and our immunocytochemical data confirmed the differences observed between groups (data not shown). The agreement with the findings of Sehmi et al⁷ and the correlation between colony-forming counts and CD34 percentages suggest that our data do allow valid comparison among groups.

The stimulus that promotes the increment in CD34⁺ cell numbers in atopy is unknown but may represent a general stimulus from sites of inflammation. In contrast to previous reports, we found no increases in CD34⁺ cells in PB from atopic donors or increases in Eo-CFUs to IL-5 in PB or BM. It is of note that we found higher numbers of Eo-CFU to IL-5 in control subjects than were reported by Sehmi et al⁷ and that this may relate to methodologic considerations. These results may reflect the lack of current symptoms in the subjects in our study, because Eo-CFU have been shown to increase after allergen exposure, and CD34/IL-5R α ⁺ cells were only increased in BM of subjects with asthma after allergen challenge.⁶

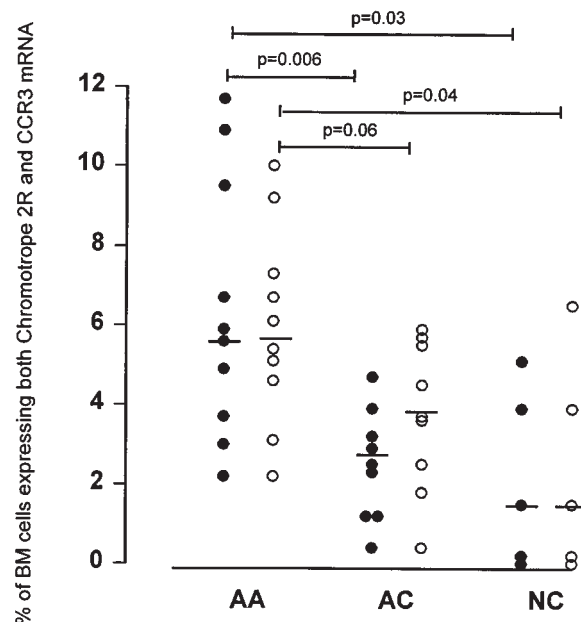


FIG 5. Percentages of BM cells staining with chromotrope 2R and expressing mRNA for CCR3 by in situ hybridization. Mature (closed circles) and immature (open circles) eosinophils were distinguished on the basis of cell size and nuclear morphologic findings (see Fig 1). Comparison between groups was by Mann-Whitney *U* test.

Recently, the allergen challenge was shown to result in elevated numbers of IL-5R α -bearing CD34 cells,⁶ although baseline numbers of CD34 cells expressing IL-5R α were very low before allergen challenge (less than 1% total) in this study. We also found very low levels of IL-5R α ⁺/CD34⁺ cells in the BM and PB of AAs, ACs, and NCs (data not shown). It is not known what factors drive CD34⁺ cells to IL-5R α expression and thus potential commitment to eosinophil lineage, although IL-3 and GM-CSF may contribute.^{5,36} We have recently studied factors regulating up-regulation of IL-5R α during eosinophil development from CD34⁺ cells and show that IL-5 itself switches to membrane expression of IL-5R α in these cells (unpublished data). Taken together with our current data, we propose that the increased CD34⁺ cell numbers in atopic subjects represent a response to inflammation and that a small percentage of these cells express IL-5 receptors in a stochastic manner. Release of IL-5 from the bronchial mucosa in response to allergen exposure would then drive the up-regulation of IL-5R α in such cells, allowing commitment to the eosinophil lineage and mobilizing an accumulated pool of eosinophils from the BM to the circulation. If this process is indeed dependent on a proinflammatory stimulus to IL-5 production, this may explain our data that show increased CD34⁺ cells and a pool of mature and immature eosinophils in BM, but no detectable increase in Eo-CFUs in baseline AAs without allergen challenge. The processes regulating eosinophil differentiation in the BM will need further study but represent a clear target for therapeutic intervention in asthma.

In conclusion, we have demonstrated that there is an increased number of eosinophils in the BM in asthma. This eosinophil pool consists of immature and mature cells and likely reflects local differentiation. Both immature and mature BM eosinophils express CCR3. The relative roles of eotaxin and IL-5 in differentiation and mobilization of these BM eosinophils and factors contributing to CD34⁺ cell differentiation towards the eosinophil lineage in asthma remain to be established.

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