Eosinophil recruitment into sites of delayed-type hypersensitivity reactions in mice

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Abstract: The selective accumulation of eosinophils in tissue is a characteristic feature of allergic diseases where there is a predominance of lymphocytes expressing a Th2 phenotype. In an attempt to define factors determining specific eosinophil accumulation *in vivo*, we have used a radiolabeled technique to assess the occurrence and the mechanisms underlying ^{111}In-eosinophil recruitment into Th1- and Th2-predominant, delayed-type hypersensitivity (DTH) reactions. Eosinophils were purified from the blood of IL-5 transgenic mice, labeled with ^{111}In and injected into nontransgenic CBA/Ca mice. Th1- and Th2-predominant, DTH reactions were induced in mice by immunization with methylated bovine serum albumin (MBSA) in Freund's complete adjuvant or with *Schistosoma mansoni* eggs, respectively. In these animals, ^{111}In-eosinophils were recruited in skin sites in an antigen-, time-, and concentration-dependent manner. Depletion of CD4$^+$ lymphocytes abrogated ^{111}In-eosinophil recruitment in both reactions. Pretreatment of animals with anti-IFN-γ mAb abrogated ^{111}In-eosinophil recruitment in MBSA-immunized and -challenged animals, whereas anti-IL-4 inhibited ^{111}In-eosinophil recruitment in both models. Local pretreatment with an anti-eotaxin polyclonal antibody inhibited the MBSA and SEA reactions by 51% and 39%, respectively. These results demonstrate that, although eosinophilia is not a feature of Th1-predominant, DTH reactions, these reactions produce the necessary chemoattractants and express the necessary cell adhesion molecules for eosinophil migration. The control of the circulating levels of eosinophils appears to be a most important strategy in determining tissue eosinophilia. *J. Leukoc. Biol.* 69: 353–360; 2001.

Key Words: *chemokines · bone marrow · interleukin-4 · lymphocytes*

INTRODUCTION

Tissue eosinophilia is a marked characteristic of allergic diseases and is often associated with organ dysfunction. For example, in asthma, the presence of activated eosinophils in the respiratory mucosa is associated with significant lung dysfunction, and effective anti-inflammatory therapies are usually accompanied by resolution of pulmonary eosinophilia [1, 2]. Similarly, in atopic dermatitis, deposits of eosinophil-derived granules have been demonstrated extensively and associated with diseased skin, although eosinophils are not seen in histological sections routinely [3]. These studies support the idea that eosinophils play a central role in the pathophysiology of allergic diseases and suggest that the development of strategies aimed at inhibiting eosinophil recruitment to sites of inflammation *in vivo* may be a relevant therapeutic approach in the treatment of allergic diseases [4–6].

In response to an appropriate inflammatory stimulus, circulating blood eosinophils interact with endothelial cells initially and then enter the tissue. The process of eosinophil migration is regulated tightly by the expression of cell adhesion molecules (CAMs) on endothelial cells and on leukocytes. In addition, eosinophils must be activated by chemoattractant molecules [e.g., chemokines, leukotriene B_4 (LTB_4)] acting on seven-transmembrane receptors on the leukocyte surface prior to their entry into the tissue [4, 7]. However, there are other factors that control the accumulation of eosinophils *in vivo*, and these include their release from the bone marrow and circulation in blood, and survival in the tissue [4]. For example, the pretreatment of guinea pigs with interleukin (IL)-5 increases blood levels of primed eosinophils and facilitates the recruitment of these cells in the skin [8]. Similarly, the recruitment of eosinophils in the lungs of IL-5 transgenic mice after challenge with the chemokine eotaxin is increased markedly compared with nontransgenic animals [9, 10]. Finally, the importance of survival for the accumulation of leukocytes in tissues is illustrated by studies demonstrating the efficacy of apoptosis-inducing strategies at reducing eosinophilic inflammation in sensitized and challenged animals [11, 12].

A marked characteristic of allergic diseases, such as asthma and atopic dermatitis, is the predominance of cytokines with a

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Th2 phenotype secreted mainly by activated T lymphocytes [13]. It is thought that the production of these Th2 cytokines, and especially IL-4 and IL-5, drives the local expression of cell adhesion molecules and chemokines necessary for eosinophil accumulation usually seen in these diseases [4, 14]. In contrast, tissue eosinophilia is not usually observed in diseases or models associated with a predominant Th1 cytokine response, although Th1 cytokines may be expressed in allergic tissues [15, 16]. The explanations for the lack of tissue eosinophilia in inflammatory responses associated with a Th1 response are not entirely known and include the lack of the local expression of the necessary chemoattractant molecules [17] and CAMs [18, 19]. However, the possibility that the IL-5-driven blood eosinophilia plays a more central role in determining tissue eosinophilia has not been examined in detail. The present study was undertaken to examine the ability of eosinophils to migrate into sites of Th1- or Th2-predominant, delayed-type hypersensitivity (DTH) reactions. To evaluate the central role of primed blood eosinophils for eosinophil recruitment *in vivo*, radiolabeled eosinophils purified from the blood of IL-5-transgenic mice [20, 21] were injected into nontransgenic, immunized mice, and their recruitment was assessed into discrete sites of cutaneous inflammation following challenge with antigen.

MATERIALS AND METHODS

Animals

Female CBA/Ca mice (18–20 g) were obtained from Harlan (Bicester, UK) and René Rachou Reseach Institute—Fiocruz (Belo Horizonte, Brazil). CBA/Ca mice overexpresssing the murine IL-5 gene (Tg1 mice) were a gift of Glaxo Wellcome (Stevenage, UK) and were bred in the Bioscience Unit of the National Heart and Lung Institute, Imperial College of Science Technology and Medicine (London, UK).

Reagents

Methylated bovine serum albumin (MBSA), concavalin A (Con A), Dextran, Percoll, and Freund's complete adjuvant (FCA) were purchased from Sigma Chemical Co. (Poole, UK); Dulbeccos's phosphate-buffered saline (PBS; calcium- and magnesium-free, pH 7.4) was from Life Technologies (Paisley, UK); LTB_4 was from Cascade (Reading, UK); and *Schistosoma mansoni* eggs and antigen were kindly provided by Dr. Corrêa-Oliveira (René Rachou Research Institute).

Purification and radiolabeling of murine eosinophils

Eosinophils (>97% purity and viability) were purified from the blood of IL-5 transgenic mice, labeled with ^{111}In, and injected into nontransgenic CBA/CA mice as previously described [21, 22]. Briefly, blood was obtained by cardiac puncture (three–four donor mice/experiment), and red cells were sedimented using Dextran (T500, one part blood to four parts Dextran 1.25%). The leukocyte-rich supernatant was removed, centrifuged (300 *g*, 7 min), and layered onto a discontinuous, four-layer, Percoll gradient (densities: 1.070, 1.075, 1.080, and 1.085 g/ml). The gradients were centrifuged at 1500 *g* for 25 min at 20°C, and eosinophils and lymphocytes were collected from the 1.080/1.085 interface. Lymphocytes were removed by using negative immunoselection with rat anti-mouse CD2 and B220 mAbs (Pharmingen, San Diego, CA). Purified eosinophils were then labeled with ^{111}In chelated to 2-mercaptopyridine-*N*-oxine.

DTH reactions

A Th1-predominant, DTH reaction was prepared by immunizing animals with MBSA in FCA, as previously described [22, 23]. Briefly, mice received two intradermal (i.d.) injections in the abdominal skin of 50 μl MBSA (5 mg/ml) that had been emulsified in FCA. For the elicitation of the reaction, animals were injected i.d. with MBSA (0.1–10.0 μg/site, dissolved in PBS) 6 or 7 days after immunization. A Th2-predominant was elicited by immunizing mice with 1000 eggs of *S. mansoni* [intraperitoneally (i.p.) in 100 μl PBS], as previously described [24]. After immunization (6 or 7 days), animals were challenged i.d. with *Schistosoma* egg antigen (SEA, 0.1–10 μg/site, dissolved in PBS).

Measurement of ^{111}In-eosinophil recruitment in murine skin

The purified, ^{111}In-labeled eosinophils were injected intravenously (i.v.; 1×10^6 cells/animal) into recipient mice that had been anesthetized previously with a mixture of Hypnorm/Hypnovel/distilled water (1/1/4). After 10 min to allow the circulation of the radiolabeled cells, i.d. injections of MBSA, SEA, or LTB_4 were given in 50 μl vol into the shaved dorsal skin of the immunized mice (up to four injections/animal). ^{111}In-labeled eosinophil accumulation was assessed at various 4-h periods (0–4, 4–8, and 20–24 h) following the i.d. injections. A blood sample was then obtained via cardiac puncture, the animals were sacrificed with an overdose of sodium pentobarbitone, the dorsal skin was removed, cleaned free of excess blood, and the sites were punched out. The samples were counted in an automatic 5-head gamma-counter (Canberra Packard Ltd., Pangbourne, Berks, UK). Eosinophil numbers in the skin sites were expressed as the number ^{111}In-eosinophils per skin site.

Pretreatment with antibodies

For the neutralization of IL-4, animals were treated with the anti-IL-4 monoclonal antibody (mAb) 11B11 (2 mg/mouse) i.p. 1 h prior to challenge with antigen. For the neutralization of interferon-γ (IFN-γ), animals were pretreated i.p. with the anti-IFN-γ mAb R4-6A2 (1 mg/mouse) 1 h prior to challenge. Control animals received a similar dose of rat immunoglobulin G (IgG). For the depletion of $CD4^+$ lymphocytes, animals were pretreated with the mAb GK1-5 (1 mg/mouse, i.p.) 2 h prior to challenge. $CD8^+$ lymphocyte depletion was carried out by pretreating animals with mAb 2–43 (2 mg/mouse, i.p.) 2 h prior to challenge. The depletion of CD4 or CD8 was confirmed by performing fluorescein-activated cell sorter (FACS) analysis (B&D FACScan) of the lymphocyte population in blood and spleen using anti-CD4 or anti-CD8 fluorescein isothiocyanate (FITC)-labeled antibodies (Sigma). To evaluate the possible role of eotaxin as a mediator of eosinophil recruitment, skin sites were injected with an anti-eotaxin polyclonal antibody (20% dilution in saline) 15 min prior to the i.v. injection of ^{111}In-eosinophils. Control sites in the same animal received an i.d. injection of pre-immune rabbit serum (20% dilution in saline). This dose of anti-eotaxin polyclonal antibody has been shown previously to abrogate the migration of eosinophils induced by 150 pmoles of eotaxin [21].

Activation of spleen lymphocytes and detection of cytokines

Spleens were collected aseptically from naïve animals and animals immunized with FCA/MBSA or *Schistosoma* eggs and teased into single-cell suspension. A pool of spleens from three animals in each group was used for each experiment. Red blood cells were then removed by spinning the cell suspension (15 min, 800 *g*) over a Ficoll-Paque gradient (Pharmacia, Upsala, Sweden; 1.077). Cells obtained at the top of the gradient were collected, washed thrice, and resuspended in a final solution of 3×10^6 cells/ml in RPMI containing 10% fetal bovine serum (FBS). Cell suspension (500 μl) was added to each well of 24-well plates and incubated with buffer, SEA (1, 5, and 25 μg/ml), MBSA (1–100 μg/ml), or Con A (5 μg/ml). Samples were collected and stored 48 or 72 h later at −70°C until the measurement of cytokines. IL-4, IL-5, IL-10 (72 h cultures), and IFN-γ (48 h cultures) were measured using sandwich enzyme-linked immunosorbent assay (ELISA) with antibody pairs purchased from Pharmingen and according to the protocol of the supplier.

Reverse transcriptase-polymerase chain reaction (RT-PCR) assay for measuring *in vivo* expression of cytokine mRNA

To investigate the expression of cytokine/chemokine mRNA, immunized or naive mice were challenged with specific antigen, and skin sites were removed

1, 3, 6, and 24 h later. Total RNA was extracted from each sample by the acid guanidinium, thiocyanate-phenol-chloroform extraction method. Total RNA (1 μg) was reverse-transcribed by the addition of 2.5 U RNAsin (Promega Corp., Madison, WI), 2.5 mM deoxynucleotides (dNTPs) (Boehringer Mannheim Biochemicals, Mannheim, Germany), 0.1 M dithiothreitol (Gibco BRL Life Technologies, Inc., Grand Island, NY), IX Moloney murine leukemia virus RNAase H^- RT buffer (Life Technologies), 25 ng Random hexamer oligonucleotides (Boehringer Mannheim), and 200 U Moloney murine leukemia virus RNAse H^- RT (Life Technologies) in 20 μl total vol. The reaction proceeded for 1 h at 37°C and was terminated by boiling for 5 min after the addition of 175 μl H_2O. The cDNA obtained (5 μL) was used for amplification in a 30 μl PCR reaction containing 2.5 mM dNTPs (Pharmacia), a 0.2 mM concentration of the 3′ and 5′ external primers, 1.5 mM $MgCl_2$, IX GeneAmp PCR buffer, and 5 U Taq DNA polymerase (Promega). PCR conditions were executed as follows: denaturation (95°C for 3 min and 94°C for 1 min), annealing (52°C for 1 min), and extension (72°C for 2 min). The number of PCR cycles is given after each sequence below, and this was followed by a final extension of 7 min at 72°C. PCR products and molecular weight markers were run on 6% polyacrylamide gel and stained with silver nitrate. Computer images of gels were obtained for semiquantitation using a densitometer. The primers (sense and anti-sense) were selected from the published cDNA sequences [25] and commercially synthesized (MWG Oligo Synthesis, London, UK). Eotaxin, CAC-GAA-GCT-TTA-GGT-AAG-CAG-TAA-CTT-CCA-TCT-GTC-TC//GCG-GCT-AGC-TGA-CTA-AAT-CAA-GCA-GTT-CTT-AGG-CTC-TG (35 cycles); IL-4, CTC-AGT-ACT-ACG-AGT-AAT-CCA//GAA-TGT-ACC-AGG-AGC-CAT-ATC (35 cycles); IFN-γ, AAC-GCT-ACA-CAC-TGC-ATC-TTG-G//GAC-TTC-AAA-GAC-TCT-GAG-G (30 cycles); and hipoxanthine phosphorybosyl transferase, GTT-GGA-TAC-AGG-CCA-GAC-TTT-GTT-G//GAT-TCA-ACT-TGC-GCT-CAT-CTT-AGG-C (30 cycles).

Quantitation of bone marrow eosinophils

The number of eosinophils in the bone marrow of mice was quantified from bone marrow cell suspension of femurs as previously described [26, 27]. Left femurs from immunized or naïve mice were isolated and flushed with 5 ml Hank's buffered salt solution containing 0.25% BSA. Samples were spun and cell numbers determined in a Neubauer haemocytometer after dilution of samples in Turk's stain. The percentage of mature eosinophils was determined using standard morphologic criteria in cytospin preparations of bone marrow samples.

Statistical analysis

Experiments were analyzed by using two-way analysis of variance (ANOVA) on normally distributed data. P values were assigned using Newman-Keuls procedure and values of $P < 0.05$ were considered statistically significant. Percentage inhibition was calculated subtracting background values. Results are presented as the mean ± SE.

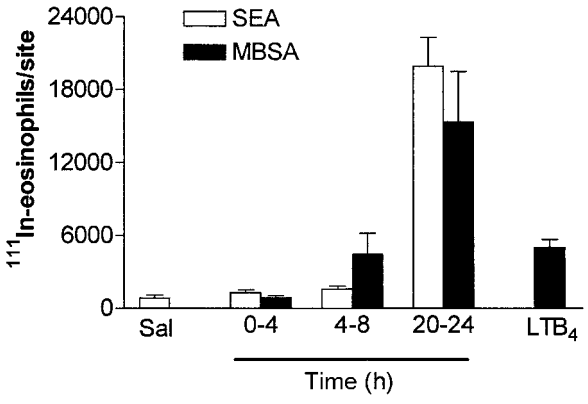

Fig. 1. Kinetics of the recruitment of ^{111}In-eosinophils in Th1- and Th2-predominant, DTH reactions. Animals that had been immunized with MBSA/FCA (Th1) or *S. mansoni* eggs (Th2) received i.d. injections of antigen (MBSA, 10 μg/site; SEA, 1 μg/site) at 20 h, 4 h, and immediately before the i.v. administration of ^{111}In-eosinophils. LTB$_4$ (150 pmol/site) was given immediately before the ^{111}In-eosinophils. The number of ^{111}In-eosinophils that migrated to skin sites was assessed 4 h after their i.v. administration. Results are the mean ± SE of six animals in each group.

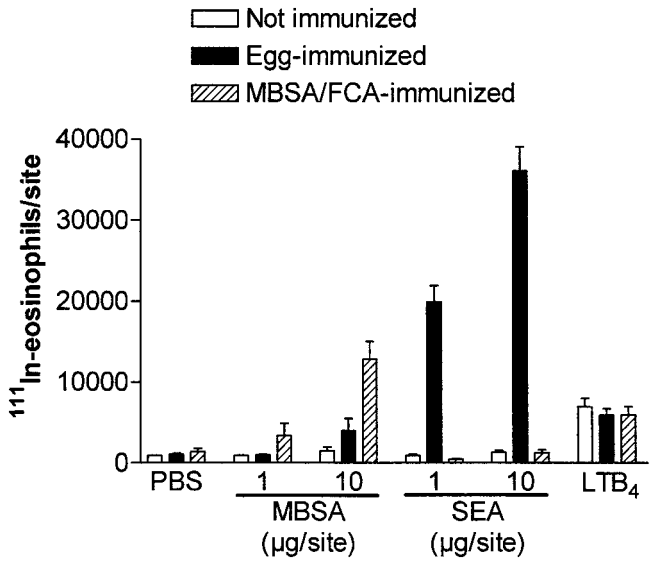

Fig. 2. Dose- and antigen-dependent recruitment of ^{111}In-eosinophils in Th1- and Th2-predominant, DTH reactions. Animals that were not immunized (open bars) or that had been immunized with MBSA/FCA (Th1, hatched bars) or *S. mansoni* eggs (Th2, closed bars) received i.d. injections of antigen (MBSA, 1 or 10 μg/site; SEA, 1 or 10 μg/site) 20 h before the i.v. administration of ^{111}In-eosinophils. The effects of the i.d. injection of PBS and LTB$_4$ (150 pmol/site) immediately before the i.v. administration of ^{111}In-eosinophils are shown for comparison. The number of ^{111}In-eosinophils that migrated to skin sites was assessed 4 h after their i.v. administration. Results are the mean ± SE of six animals in each group.

RESULTS

Recruitment of ^{111}In-labeled eosinophils in sites of DTH reactions

The i.d. administration of SEA in animals immunized with *S. mansoni* eggs induced a time- and dose-dependent recruitment of ^{111}In-labeled eosinophils (**Figs. 1** and **2**). There was no significant ^{111}In-eosinophil recruitment from 0–4 or 4–8 h, but a large number of cells were recruited at the late time point of 20–24 h (Fig. 1). In a similar manner, the i.d. administration of MBSA in animals immunized with MBSA in FCA (MBSA/FCA) resulted in a time- and dose-dependent recruitment of ^{111}In-labeled eosinophils (Figs. 1 and 2). In this reaction, ^{111}In-eosinophil recruitment was already noticeable from 4–8 h, but a significantly greater number of cells were recruited in the 20–24 h measurement period (Fig. 1). The specificity of both reactions was assessed by the i.d. injection of the relevant antigen in naive, *Schistosoma* egg- or MBSA/FCA-immunized animals. As clearly demonstrated in Figure 2, the sensitization procedure imparted a great degree of specificity to the reactions. A large number of ^{111}In-eosinophils migrated to sites injected with antigen in the relevant immunized animal but not in naive animals or those immunized with the other irrelevant antigen (Fig. 2). For comparison, the i.d. administration of LTB$_4$, an effective eosinophil chemoattractant [7, 21], induced

a similar recruitment of ^{111}In-eosinophils in naive and immunized animals, although the response was substantially smaller (Fig. 2).

Histological studies of skin sites in naïve mice

The administration of SEA to *S. mansoni*, egg-immunized animals induced a marked inflammatory infiltrate at 24 h in the injected skin sites. The majority of the migrating cells was mononuclear, but eosinophils comprised about 20–30% of the inflammatory infiltrate (**Fig. 3, C** and **D**). The administration of MBSA to animals immunized with MBSA/FCA also induced a marked mononuclear cell infiltrate in the injected skin sites (Fig. 3, A and B). Nevertheless, and in contrast to the ability of ^{111}In-labeled eosinophils to migrate to sites of MBSA challenge, only occasional endogenous eosinophils were noticed (Fig. 3B).

Cytokine profile of spleen cells and challenged tissue of immunized animals

Spleen cells were obtained from naive, *S. mansoni*, egg- and MBSA/FCA-immunized animals and stimulated with Con A, MBSA, or SEA. Con A-stimulated spleen cells from naive animals secreted a significant amount of IFN-γ and IL-10 but not IL-5 or IL-4 (**Fig. 4**). Neither SEA nor MBSA induced significant cytokine secretion from splenocytes of naive animals (Fig. 4). Upon stimulation with specific antigen (SEA) or Con A, splenocytes from *S. mansoni*, egg-immunized mice secreted significant amounts of IFN-γ, IL-10, and IL-5 (Fig. 4). Splenocytes from MBSA/FCA-immunized animals produced a large amount of IFN-γ spontaneously, but this was enhanced further in the presence of Con A or specific antigen (MBSA; Fig. 4). In splenocyte cultures, little IL-4, IL-5, or IL-10 was produced in the presence of MBSA (Fig. 4).

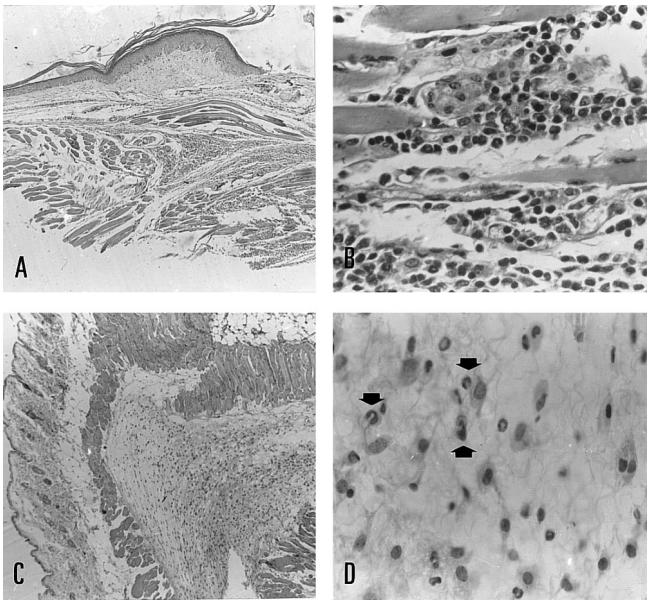

Fig. 3. Histological sections of skin sites of Th1- and Th2-predominant, DTH reactions. Animals that had been immunized with MBSA/FCA (A and B) or *S. mansoni* eggs (C and D) received i.d. injections of antigen (MBSA, 10 μg/site; SEA, 1 μg/site, respectively) 24 h prior to sacrifice. Note the marked inflammatory infiltrate seen in both reactions at 24 h (A and C; original size, ×100). In greater magnification (original size, ×400), note the large number of infiltrating eosinophils in Th2 predominant, DTH reactions (D) but not in Th1-predominant, DTH reactions (B).

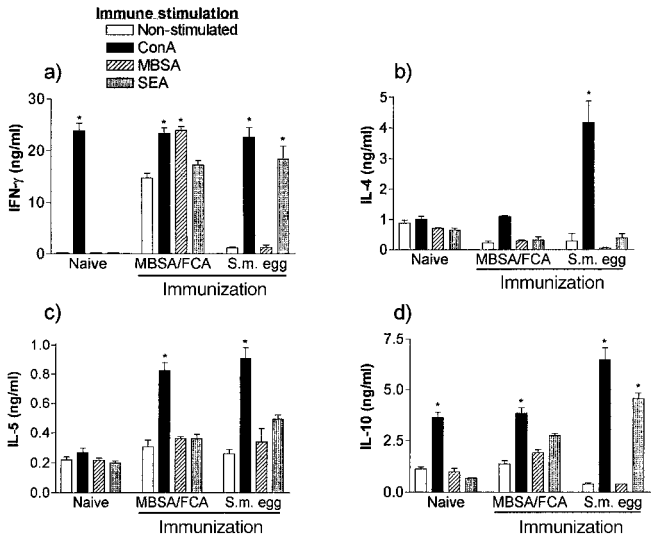

Fig. 4. Profile of the production of (a) IFN-γ, (b) IL-4, (c) IL-5, and (d) IL-10 by splenocytes obtained from naïve animals or animals immunized with MBSA/FCA or *S. mansoni* eggs. Splenocytes from naïve or immunized animals were stimulated with vehicle (open bars), Con A (5 μg/ml, closed bars), MBSA (10 μg/ml, hatched bars), or SEA (5 μg/ml, stippled bars). Supernatants were collected for cytokine measurements by ELISA 48 h (IL-4, IL-5, and IL-10) or 72 h (IFN-γ) later. Results are representative of two similar experiments and are the mean of triplicates from pooled cells of three mice in each group.

The kinetics of the expression of mRNA for the Th2 cytokine IL-4 and the Th1 cytokine IFN-γ in skin sites of MBSA/FCA-immunized and *S. mansoni*, egg-immunized and -challenged animals are shown in **Figure 5**. In MBSA/FCA-immunized animals, the challenge with specific antigen induced a significant expression of IFN-γ early (from 1–6 h) in the course of the reaction and low-level expression of IL-4 at 3 and 24 h (Fig. 5a). The challenge of egg-immunized animals with SEA induced low-level expression of IFN-γ from 1–6 h and high levels of IL-4 expression from 6–24 h after challenge (Fig. 5b). There was no detectable expression of IL-4 or IFN-γ in immunized animals challenged with PBS, and injection of antigen in naive animals had little effect on cytokine mRNA expression (unpublished results).

Effects of the depletion of CD4+ and CD8+ cells

Pretreatment of animals with the anti-CD4 mAb GK1.3 resulted in virtually complete depletion of circulating and spleen CD4+ cells in *S. mansoni*, egg- and MBSA/FCA-immunized animals, as assessed by flow cytometry (unpublished results). As seen in **Figure 6**, anti-CD4 treatment abrogated the recruitment of ^{111}In-eosinophils in SEA- and MBSA-induced DTH reactions. In contrast, pretreatment with the anti-CD8 mAb R4-6A2, which depleted CD8+ cells (unpublished results), had no significant effect on the numbers of infiltrating ^{111}In-eosinophils in both reactions (Fig. 6). For comparison, neither antibody treatment had any significant effect on LTB_4-induced, ^{111}In-eosinophil recruitment (Fig. 6c).

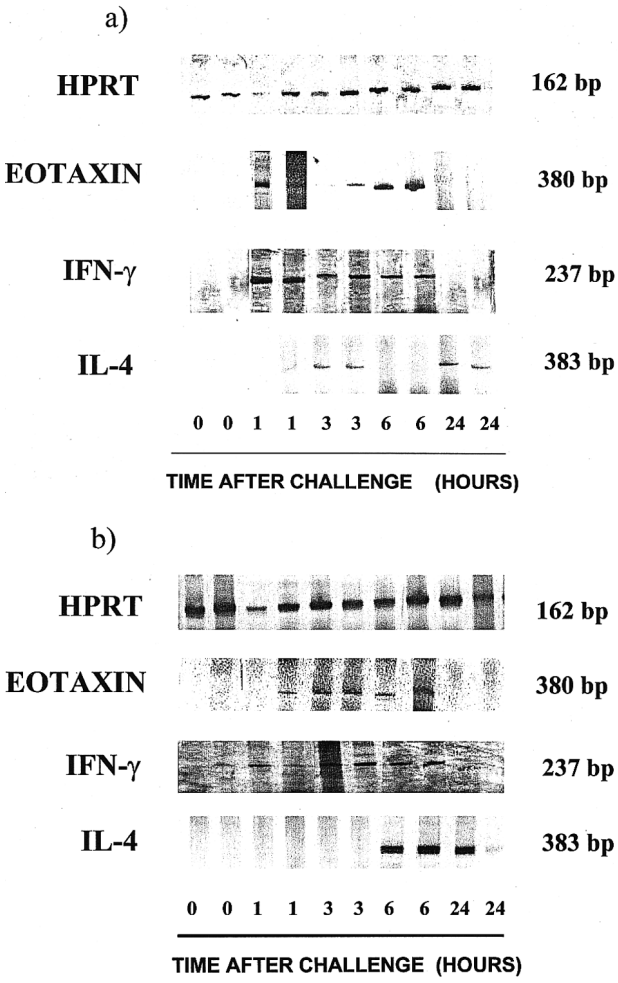

Fig. 5. Expression of mRNA for eotaxin, IL-4, and IFN-γ in (a) Th1- and (b) Th2-predominant, DTH reactions. Mice were immunized with MBSA/FCA (Th1) or *S. mansoni* eggs (Th2) and challenged i.d. with the relevant antigen (MBSA, 10 μg/site; SEA, 1 μg/site, respectively). Before (time 0) and at various times after challenge (1, 3, 6, and 24 h), skin sites from three animals were removed, pooled, mRNA-extracted, and reverse-transcribed using specific primers for HPRT, eotaxin, IFN-γ, and IL-4. The data are representative of two similar experiments.

Effects of the neutralization of IL-4 and IFN-γ

The next series of experiments assessed whether pretreatment with blocking antibodies against IL-4 and IFN-γ would affect the recruitment of ^{111}In-eosinophils in sites of late-onset allergic reactions. The antibodies were given after the immunization procedure and 1 h prior to challenge. As demonstrated in **Figure 7a**, pretreatment with anti-IL-4, but not anti-IFN-γ, partially inhibited ^{111}In-eosinophil recruitment into sites of DTH reactions induced by SEA in *S. mansoni*, egg-immunized animals. Surprisingly, anti-IL-4 and anti-IFN-γ abrogated the recruitment of ^{111}In-eosinophils in sites of DTH reactions induced by MBSA in MBSA/FCA-immunized animals (Fig. 7b). Neither antibody treatment affected the recruitment of ^{111}In-eosinophils induced by the i.d. injection of LTB_4 (Fig. 7c).

Effects of the neutralization of eotaxin

We have shown previously that the chemokine eotaxin induced significant ^{111}In-eosinophil recruitment in murine skin and was released endogenously in sites of allergic inflammation [21]. When the expression of eotaxin was assessed by RT-PCR, significant message was detected early (from 1–6 h) in the course of both reactions (Fig. 5). Moreover and in agreement with the significant expression of eotaxin, an anti-eotaxin polyclonal antibody used at a dilution (20% in PBS) previously shown to abrogate the effects of eotaxin [21], inhibited ^{111}In-eosinophil recruitment in MBSA/FCA and egg/SEA DTH reactions (**Fig. 8**).

Bone marrow eosinophils in *S. mansoni*, egg- and MBSA/FCA-immunized animals

The results above demonstrate that endogenous eosinophils fail to migrate into sites of Th1 predominant, MBSA/FCA reactions, whereas these cells are found in abundance in *S. mansoni*, egg-immunized and SEA-challenged animals (Fig. 3). In contrast, exogenously added ^{111}In-eosinophils appear to migrate to both reactions upon challenge with specific antigen. To gain further insights into the mechanisms explaining the recruitment of endogenous eosinophils, we evaluated the total number of leukocytes and mature eosinophils present in the bone marrow of MBSA/FCA and *S. mansoni*, egg-immunized mice. As seen in **Figure 9**, immunization with *S. mansoni* eggs induced a significant increase in the total number of mature eosinophils in the bone marrow of mice, whereas MBSA/FCA immunization enhanced the total number of cells but failed to affect eosinophil numbers. The increase in total leukocytes in the bone marrow of MBSA/FCA mice was explained to some extent by an increase in the number of mature neutrophils (unpublished results).

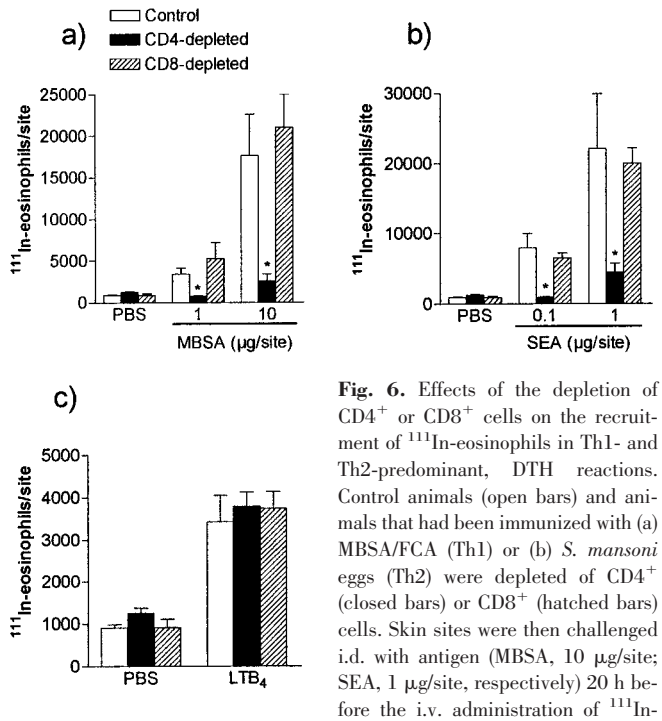

Fig. 6. Effects of the depletion of $CD4^+$ or $CD8^+$ cells on the recruitment of ^{111}In-eosinophils in Th1- and Th2-predominant, DTH reactions. Control animals (open bars) and animals that had been immunized with (a) MBSA/FCA (Th1) or (b) *S. mansoni* eggs (Th2) were depleted of $CD4^+$ (closed bars) or $CD8^+$ (hatched bars) cells. Skin sites were then challenged i.d. with antigen (MBSA, 10 μg/site; SEA, 1 μg/site, respectively) 20 h before the i.v. administration of ^{111}In-eosinophils. The effects of the i.d. injection of PBS and (c) LTB_4 (150 pmol/site) immediately before the i.v. administration of ^{111}In-eosinophils into control and $CD4^+$ or $CD8^+$ cell-depleted animals are shown for comparison. The number of ^{111}In-eosinophils that migrated to skin sites was assessed 4 h after their i.v. administration. Results are the mean ± SE of six animals in each group. *$p < 0.01$ when compared with control animals.

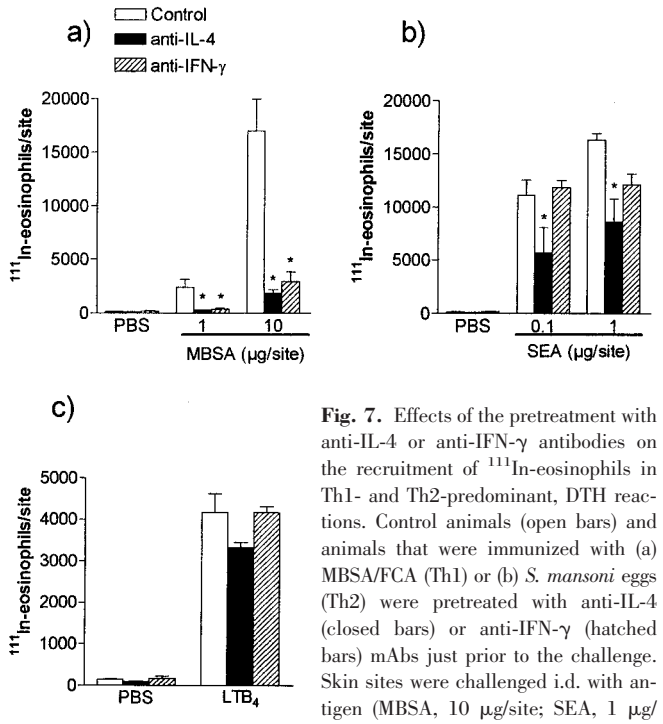

Fig. 7. Effects of the pretreatment with anti-IL-4 or anti-IFN-γ antibodies on the recruitment of ^{111}In-eosinophils in Th1- and Th2-predominant, DTH reactions. Control animals (open bars) and animals that were immunized with (a) MBSA/FCA (Th1) or (b) *S. mansoni* eggs (Th2) were pretreated with anti-IL-4 (closed bars) or anti-IFN-γ (hatched bars) mAbs just prior to the challenge. Skin sites were challenged i.d. with antigen (MBSA, 10 μg/site; SEA, 1 μg/site, respectively) 20 h before the i.v. administration of ^{111}In-eosinophils. The effects of the i.d. injection of PBS and (c) LTB_4 (150 pmol/site) immediately before the i.v. administration of ^{111}In-eosinophils into control and antibody-treated animals are shown for comparison. The number of ^{111}In-eosinophils that migrated to skin sites was assessed 4 h after their i.v. administration. Results are the mean ± SE of six animals in each group. $*p < 0.05$, and $**P < 0.01$ when compared with control animals.

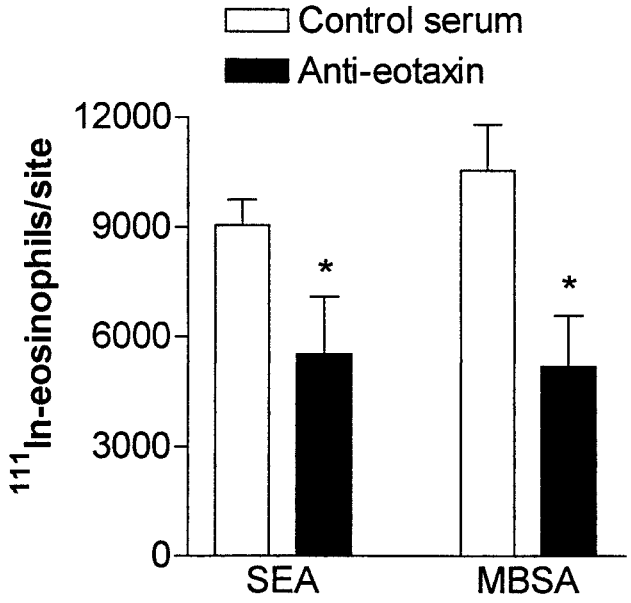

Fig. 8. Effects of the local pretreatment with anti-eotaxin antibody on the recruitment of ^{111}In-eosinophils in Th1- and Th2-predominant, DTH reactions. Animals that had been immunized with MBSA/FCA (Th1) or *S. mansoni* eggs (Th2) were challenged i.d. with antigen (MBSA, 10 μg/site; SEA, 1 μg/site, respectively) 20 h before the i.v. administration of ^{111}In-eosinophils. Control, pre-immune serum (20% dilution in saline, open bars) or anti-eotaxin polyclonal antibody (20% dilution in saline, closed bars) was injected i.d. just prior to the i.v. administration of ^{111}In-eosinophils. The number of ^{111}In-eosinophils that migrated to skin sites was assessed 4 h after their i.v. administration. Results are the mean ± SE of seven–eight animals in each group. $*p < 0.05$ when compared with sites treated with control serum.

DISCUSSION

The recruitment of activated eosinophils into skin sites appears to play a major role in the pathophysiology of allergic disorders [1–3]. The recruitment of eosinophils to sites of inflammation *in vivo* is largely dependent on the local expression of chemoattractant molecules (e.g., chemokines and LTB_4) and CAMs [4]. Nevertheless, there is some evidence to support a role for IL-5-driven blood eosinophilia in determining eosinophil recruitment in sites of inflammation [8–10, 28, 29]. In the present study, we have investigated whether the IL-5-driven blood eosinophilia plays a determinant role for the recruitment of eosinophils *in vivo*. To evaluate this central role of primed blood eosinophils for eosinophil recruitment *in vivo*, radiolabeled eosinophils purified from the blood of IL-5 transgenic mice were injected into nontransgenic, immunized mice, and their recruitment was assessed into discrete sites of cutaneous inflammation following challenge with antigen.

Two immunization procedures were used in the experiments described herein. A set of animals was immunized with *S. mansoni* egg and challenged with SEA, and another set of animals was immunized with MBSA in CFA and challenged with MBSA [22–24]. In the former reaction, a significant proportion of endogenous migrating cells were eosinophils (20–30%), and splenocytes cultured in the presence of specific antigen produced significant amounts of IL-4, IL-5, IL-10, and IFN-γ, characterizing a Th0/2 phenotype. It is unclear whether the production of IFN-γ, in addition to Th2-type cytokines, by SEA-activated lymphocytes is important for the eosinophil migration observed or just represents the great ability of parasite antigens to stimulate the immune system. However, these results are in marked agreement with inflammatory reactions induced in other tissues by a similar immunization and challenge procedure [24, 30]. In the MBSA-induced, DTH reaction, a marked inflammatory infiltrate characterized by the infiltra-

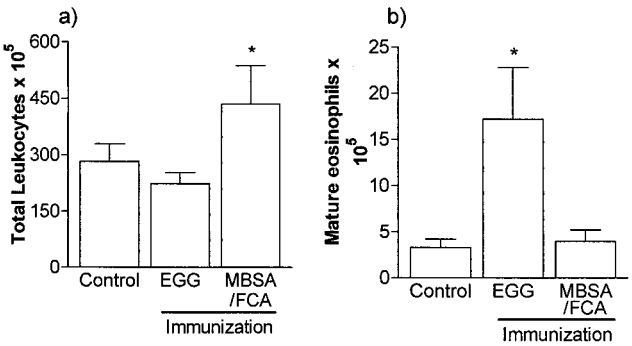

Fig. 9. Number of total cells and mature eosinophils in bone marrow obtained from naïve animals or animals immunized with MBSA/FCA or *S. mansoni* eggs. Left femurs were obtained from naïve mice or mice that had been immunized with MBSA/FCA or *S. mansoni* eggs six days before the experiment and prior to challenge. Bone marrows were flushed with RPMI as described in Materials and Methods, and the number of (a) total leukocytes and (b) mature eosinophils was evaluated. Results are the mean ± SE of five animals in each group. $*p <$ 0.05 when compared with control.

tion of mononuclear cells was observed, and very rare eosinophils or neutrophils were seen throughout the lesions at 24 h. Splenocytes from these animals produced a significant amount of IFN-γ spontaneously, and this was increased in response to specific antigen.

In contrast to the findings for endogenous eosinophils described above, ^{111}In-eosinophils migrated into sites of MBSA/FCA, delayed-onset, hypersensitivity reactions in an antigen- and CD4-dependent manner. Similarly, in animals immunized with *S. mansoni* eggs and challenged with SEA, ^{111}In-eosinophils were also recruited in an antigen- and CD4-dependent manner. Depletion of $CD8^+$ cells was not accompanied by any significant change of ^{111}In-eosinophil migration. These data are in good agreement with other studies demonstrating a fundamental role for $CD4^+$ T cells in the control of eosinophil migration in sites of late-onset, allergic inflammation [18, 31, 32] and demonstrate that, when present in sufficient amounts in blood, eosinophils derived from IL-5-transgenic animals migrate to sites of both Th1 and Th2-predominant reactions.

The challenge of *S. mansoni*, egg-immunized animals with SEA induced the local expression of IL-4 mRNA. In agreement with this finding, pretreatment of these animals with anti-IL-4 mAb was accompanied by a significant inhibition of ^{111}In-eosinophil recruitment after challenge. In contrast, little IFN-γ was expressed after i.d. challenge with SEA, and treatment with anti-IFN-γ mAb had little effect on ^{111}In-eosinophil migration. A central role for IL-4 in mediating eosinophil influx into the lung or skin in Th2-predominant, allergic inflammation has also been described previously [33, 34]. In MBSA/FCA-immunized animals, there was a significant expression of IFN-γ early in the course of the reaction, and treatment with anti-IFN-γ mAb abrogated ^{111}In-eosinophil recruitment into skin sites of challenged animals. Moreover, pretreatment with anti-IL-4 also abrogated ^{111}In-eosinophil recruitment in MBSA/FCA animals challenged with specific antigen. This was an unexpected finding, inasmuch as splenocytes from these animals produced little IL-4 upon stimulation with specific antigen, and previous studies have demonstrated the dependence of this Th1-predominant reaction on IL-12 production [23]. Nevertheless, it is worth noting that, albeit in low levels, IL-4 mRNA was detected following challenge of immunized animals with MBSA. Furthermore, a role for IL-4 in mediating eosinophil influx in sites of contact hypersensitivity reactions has been described previously [35]. Overall, these studies argue for an essential role for IL-4 in mediating eosinophil recruitment following antigen challenge in cutaneous hypersensitivity reactions. Moreover, they imply that mechanisms, in addition to the local expression of IL-4, drive the specific recruitment of endogenous eosinophils observed in both hypersensitivity reactions. The means by which IL-4 drives eosinophil recruitment in our model has not been investigated in detail. Nevertheless, our previous studies suggest that the modulation by IL-4 of the expression of very late antigen (VLA)-4 ligands, such as vascular cell adhesion molecule 1 (VCAM-1), may underlie the ability of this cytokine to regulate eosinophil recruitment in mouse skin [22]. Alternatively, IL-4 may be driving the local production of eosinophil chemoattractants, specially eotaxin [17, 36, 37], by skin cells (e.g., fibroblasts), which may then drive local eosinophil recruitment.

We and others have demonstrated previously the important role of the chemokine eotaxin for the recruitment of eosinophils into sites of allergic inflammation *in vivo* [21, 38–40]. Here, we show that eotaxin mRNA is expressed in late-onset hypersensitivity reactions and, more importantly, that an anti-eotaxin polyclonal antibody partially inhibits eosinophil recruitment when administered just prior to antigen challenge. These experiments are in agreement with previous studies demonstrating the expression of eotaxin by Th1 and Th2 lymphocytes [41]. Overall, these results argue that the local expression of the chemokine eotaxin cannot explain the differential recruitment of endogenous eosinophils observed in MBSA/FCA- and *S. mansoni*-immunized and -challenged mice.

Next, we investigated whether the availability of mature eosinophils in bone marrow and blood could provide an explanation for the specificity of the migration of endogenous eosinophils to sites of Th2-, but not Th1-predominant, DTH reactions. Our results clearly show that, in mice immunized with *S. mansoni* eggs, there is an increase in the number of eosinophils in the bone marrow. In contrast, in MBSA/FCA-immunized mice, neutrophil, but not eosinophil, numbers increased. These results demonstrate a correlation between availability of eosinophils in bone marrow and the ability of endogenous eosinophils to migrate into sites of DTH reactions. When exogenous, IL-5-derived eosinophils are added, these cells are readily available for migration *in vivo*. In support of this hypotheses, Nagai *et al.* [42] have shown previously that the overproduction of IL-5 enhanced eosinophil migration in a model of dinitrofluorobenzene-induced contact hypersensitivity. Together, these results corroborate the idea that the bone marrow may play an active role in the recruitment of eosinophils *in vivo* [43].

In conclusion, we demonstrate the ability of exogenously added, IL-5-primed eosinophils to migrate into sites of late onset Th1- or Th2-predominant reactions *in vivo*. Exogenous eosinophil migration was $CD4^+$ T cell- and IL-4-dependent and partially blocked by an anti-eotaxin antibody. Nevertheless, endogenous eosinophils only migrated when found in increased numbers in the bone marrow [present data; 42], suggesting an important role for the bone marrow in controlling eosinophil migration *in vivo*. Strategies aimed at modulating eosinophil production and/or release from the bone marrow may be of benefit in the therapy of eosinophil-associated diseases, such as asthma and atopic dermatitis.

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