

# Delayed eosinophil apoptosis in asthma

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**Background:** Eosinophilic inflammation of the airways is a key characteristic of asthma. A defect in apoptosis might contribute to the chronic tissue eosinophilia associated with asthma.

**Objective:** Our purpose was to examine whether the rate of apoptosis differs between peripheral blood eosinophils from asthmatic patients and healthy volunteers.

**Methods:** Peripheral blood was obtained from volunteers with asthma and from control volunteers. Eosinophils were isolated by CD16-negative selection to >99% purity and were cultured for 48 hours. The number of apoptotic eosinophils in the culture was assessed by flow cytometric analysis of relative DNA content in propidium iodide-stained cells. Eosinophil apoptosis is expressed as apoptosis index (number of apoptotic cells/total number of cells).

**Results:** Eosinophils from asthmatic patients not taking steroid medication survived longer (apoptosis index 0.25) than those of healthy control subjects (apoptosis index 0.40,  $P < .05$ ). In contrast, the rate of apoptosis in eosinophils from patients concurrently taking steroids (apoptosis index 0.46) is higher than that of those not using steroids ( $P < .01$ ) and not different from that of healthy subjects. To assess whether endogenous IL-3, IL-5, and GM-CSF production contributes to the delayed eosinophil apoptosis, the effects of the corresponding neutralizing antibodies were studied on eosinophil longevity. Neutralization of GM-CSF, but not of IL-3 or IL-5, increased slightly but significantly ( $P < .01$ ) the rate of apoptosis in eosinophils obtained from patients with asthma. To assess whether  $\beta_2$ -agonist medication could contribute to the observed differences, we determined the *in vitro* effects of albuterol, fenoterol, and salmeterol on eosinophil apoptosis. All  $\beta_2$ -agonists inhibited eosinophil apoptosis by 12% to 19%. A possibility existed that a prior *in vivo* exposure to IL-5, GM-CSF, or  $\beta_2$ -agonists would explain the observed difference. To study this,

eosinophils were incubated with GM-CSF, IL-5, and albuterol for 2 to 3 hours, followed by washout of the added compounds, and were subsequently cultured for 48 hours. However, an exposure to GM-CSF (7 pmol/L) or IL-5 (10 pmol/L) for 15 to 180 minutes was not a sufficient signal to prevent eosinophil apoptosis. In contrast, exposure to albuterol (100 nmol/L) for 120 minutes was sufficient to induce a significant ( $P < .05$ ) decrease in eosinophil apoptosis.

**Conclusions:** The results suggest that eosinophil apoptosis is delayed in asthma. This delay may be partly explained by production of GM-CSF. The *in vitro* effects of  $\beta_2$ -agonists suggest that  $\beta_2$ -agonist use might contribute to the prolonged eosinophil survival through inhibition of apoptosis and thus may worsen eosinophilia in asthmatic patients. Use of inhaled glucocorticoids seems to totally reverse the delayed eosinophil apoptosis in asthma. (*J Allergy Clin Immunol* 2000;106:77-83.)

**Key words:** Albuterol, apoptosis, asthma, eosinophil, fenoterol, GM-CSF, IL-5, salmeterol

Eosinophilic inflammation of the airways is a key characteristic of asthma. Normally eosinophils undergo apoptosis (programmed cell death) and are subsequently recognized and cleared by macrophages without an inflammatory response.<sup>1</sup> A defect in apoptosis might explain the persistent airway eosinophilia associated with asthma. Although cytokines such as IL-3 and IL-5 and GM-CSF are known to regulate eosinophil apoptosis *in vitro*<sup>2</sup> or *ex vivo*,<sup>3</sup> the *in vivo* mechanism of regulation of eosinophil apoptosis in asthmatic patients remains unknown. This study was performed to determine whether apoptosis differs between human peripheral blood eosinophils obtained from healthy volunteers and steroid-naïve or steroid-treated asthmatic subjects.

## METHODS

### Material

The reagents were from the following sources: anti-CD16 microbeads and magnetic cell separation system (Miltenyi Biotec, Bergisch Gladbach, Germany), Ficoll-Paque (Pharmacia AB, Uppsala, Sweden), recombinant human IL-3, IL-5, and GM-CSF and neutralizing antibodies for human IL-3, IL-5, and GM-CSF and IgG<sub>1</sub> isotype control (R&D Systems, Abingdon, UK), albuterol, dibutyl- $\gamma$ -cyclic AMP, fenoterol, propidium iodide, theophylline, and Triton X-100 (Sigma Chemical, Poole, UK), and salmeterol (Glaxo Wellcome, Durham, UK). FCS and RPMI 1640 culture media were purchased from Gibco BRL (Paisley, UK).

### Subjects

We included 16 healthy individuals (11 men, ages  $33 \pm 2$  years) and 34 patients with asthma (27 men, ages  $34 \pm 2$  years). Of the asthmatic patients, 21 were not using corticosteroids and 13 were using regular inhaled corticosteroids. All patients with asthma not

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**Abbreviation used**EC<sub>50</sub>: Median effective concentration

on inhaled corticosteroids used intermittent  $\beta_2$ -agonist medication (albuterol 100  $\mu$ g 1-2 puffs as needed). They were asked to refrain from  $\beta_2$ -agonist use overnight (ie, for 10 hours) before sampling. Patients on regular inhaled corticosteroids used beclomethasone ( $933 \pm 353$   $\mu$ g,  $n = 3$ ), budesonide ( $866 \pm 256$   $\mu$ g,  $n = 7$ ), or fluticasone ( $667 \pm 167$   $\mu$ g,  $n = 3$ ). One patient on fluticasone used also oral prednisolone (5 mg daily). Of those patients using inhaled corticosteroids, 3 also used regular inhaled salmeterol (50  $\mu$ g twice daily) and 2 regular formoterol (12  $\mu$ g twice daily). Patients on regular inhaled glucocorticoids were allowed to take their regular medication (both steroids and  $\beta_2$ -agonists) 1 hour before sampling. All patients not using steroids had either mild intermittent or mild persistent asthma and of those patients on inhaled steroids 5 were classified as having mild persistent, 7 as having moderate persistent, and 1 as having severe persistent asthma according to the 1997 classification.<sup>4</sup> In experiments to evaluate the effects of  $\beta_2$ -agonists, theophylline, and dibutyl-AMP and in the washout experiments with GM-CSF, IL-5, and albuterol, eosinophils from healthy volunteers were used. Subjects gave informed consent to a study protocol approved by the ethical committees of Tampere University Hospital (Tampere, Finland) and Royal Brompton Hospital Ethics Committee (London, UK).

### Eosinophil separation

Eosinophils were separated with the method of Hansel et al.<sup>5</sup> Briefly, venous blood (50 to 100 mL) from healthy or asthmatic individuals was collected into 10 to 20 mL of acid-citrate-dextrose anticoagulant. White blood cells were obtained by sedimentation with 3% hydroxyethyl starch, layered onto Ficoll, and centrifuged at 500g for 30 minutes at 18°C. The mononuclear cell layer was discarded and the pellet containing granulocytes and red blood cells was washed in HBSS. Contaminating red blood cells were lysed by hypotonic lysis. The remaining granulocytes were washed, counted, and resuspended in 300  $\mu$ L RPMI 1640 containing 2% FCS and 5 mmol/L EDTA (RPMI/FCS/EDTA). Eosinophils were purified from neutrophils with use of immunomagnetic anti-CD16 antibody-conjugated beads (1  $\mu$ L of beads per  $2 \times 10^6$  neutrophils). After the addition of beads, cells were incubated at 4°C for 40 minutes before resuspension in 6 mL of RPMI/FCS/EDTA. The mixture was loaded onto a separation column positioned within a magnetic field and eluted with 40 mL of RPMI/FCS/EDTA. The CD16<sup>+</sup> cells (ie, neutrophils) were retained by the column, whereas the eluted eosinophils were collected, washed in RPMI 1640, counted, and then resuspended at  $10^6$  cells per milliliter. Eosinophil purity was >99% as assessed by microscopic examination in Kimura stain. The cells were cultured for 48 hours (+37°C, 5% carbon dioxide) in RPMI 1640 medium with 10% FCS plus antibiotics.

### Apoptosis

The percentage of apoptotic cells was assessed by measuring the relative DNA content by flow cytometry of propidium iodide-stained cells.<sup>6,7</sup> Activation of endonucleases nicking DNA preferentially at the internucleosomal linker regions and resulting in DNA fragmentation is considered to be a very specific feature of apoptosis.<sup>7,8</sup> Membrane permeabilization with detergent (Triton X-100) results in extrusion of the degraded low-molecular-weight DNA from the apoptotic cells. Therefore less DNA is stained in apoptotic cells with the intercalating dye propidium iodide. The reduced DNA content of apoptotic nuclei results in a hypodiploid DNA peak

that can be discriminated from the narrow peak of cells with normal diploid DNA ( $G_0/G_1$  peak) content in the red fluorescence channels.<sup>6-8</sup> In a separate set of samples the percentage of apoptotic cells obtained with this method correlated well with the appearance of morphologic features (chromatin condensation and disappearance of normal bilobular nucleus, cell shrinkage and overall condensation of the cytoplasmic material) typical of apoptosis.<sup>1,3,9,10</sup> In eosinophils during 48 hours of culture ( $r = 0.95$  for correlation between the percentage of apoptotic cells obtained with morphologic analysis and by measuring the relative DNA content by flow cytometry,  $P < .0001$ ,  $n = 20$ ). The presence or absence of apoptotic cells in the culture as assessed by flow cytometric analysis was also confirmed by the appearance or lack of the typical ladder pattern in the DNA fragmentation assay by agarose gel electrophoresis (data not shown).<sup>6,7</sup> Eosinophil apoptosis was expressed as apoptosis index (Number of apoptotic cells/Total number of cells). No significant disintegration of the cells was observed. To validate the assay further, the eosinophil apoptosis index was measured from 11 volunteers twice over a period of 11 to 35 weeks; a "between-day" coefficient of variation of 8.1% was obtained.

### Statistical analysis

All data are expressed as the mean  $\pm$  SEM of ( $n$ ) determinations. ANOVA supported by Student-Newman-Keuls or Dunn's multiple comparisons test was used when the differences between groups were analyzed. The null hypothesis was rejected when  $P < .05$ .

## RESULTS

### Apoptosis

The eosinophil apoptosis index in asthmatic patients not using steroids was lower ( $0.25 \pm 0.04$ ,  $n = 21$ ) than that of healthy control subjects ( $0.40 \pm 0.03$ ,  $n = 16$ ,  $P < .05$ ). In contrast, use of corticosteroids normalized the eosinophil apoptosis index ( $0.46 \pm 0.06$  in patients concurrently using steroids,  $n = 13$ ,  $P < .01$  compared with patients without steroids and not significantly different from normal subjects; Fig 1). The peripheral blood eosinophil counts were  $0.10 \pm 0.02 \times 10^9/L$  for healthy control subjects,  $0.19 \pm 0.02 \times 10^9/L$  for asthmatic patients not using steroids, and  $0.23 \pm 0.04 \times 10^9/L$  for patients concurrently using steroids. There was no correlation between the apoptosis index and the peripheral blood eosinophil count in any of the study groups ( $r = -0.076$ ,  $r = 0.124$ , and  $r = 0.327$ , for steroid-naïve asthmatic patients, asthmatics on inhaled steroids, and healthy control subjects, respectively) or when the groups were combined ( $r = 0.08$ ).

### Effect of IL-3, IL-5, and GM-CSF neutralization on eosinophil apoptosis

IL-3, IL-5, and GM-CSF have been shown to prolong eosinophil survival in vitro by delaying apoptosis.<sup>11-13</sup> To study whether production of the above-mentioned cytokines by eosinophils explains the delayed apoptosis found in asthmatic patients, the effects of neutralizing antibodies to IL-3, IL-5, and GM-CSF (5  $\mu$ g/mL) were studied on apoptosis in cytokine-deprived eosinophils. To confirm that the neutralizing antibodies are functionally active, their ability to reverse the effect of the added cytokine was first studied to justify their use. All the neutralizing antibodies reversed 36% to 62% of the effect of

the corresponding survival-prolonging cytokine added at a concentration previously shown to produce maximal antiapoptotic effect (IL-3, 100 pmol/L; IL-5, 10 pmol/L; GM-CSF, 7 pmol/L; Table I). Neutralizing antibody directed to GM-CSF increased slightly but significantly (increase in apoptotic index  $0.05 \pm 0.01$ ,  $n = 8$ ,  $P < .01$ ) the rate of eosinophil apoptosis compared with the IgG<sub>1</sub> isotype control (Fig 2). In contrast, neutralizing antibodies to IL-3 or IL-5 had no effect (increase in apoptotic index  $0.01 \pm 0.01$  and  $-0.01 \pm 0.01$ , respectively,  $n = 8$ ). No additive effect could be obtained when all three neutralizing antibodies were added together (increase in apoptotic index  $0.04 \pm 0.01$ ,  $n = 8$ ) compared with the IgG<sub>1</sub> isotype control ( $P < .01$ ). However, the magnitude of the effect of GM-CSF neutralizing antibody seems not to be enough to explain the difference between healthy control subjects and patients with asthma not using steroids (difference in apoptotic index 0.15). Another possibility is that circulating eosinophils, before isolation in vivo or even during the isolation process, would be in contact with the survival-prolonging cytokines. However, such cytokines are effectively washed out during the isolation process. To test the possibility that a prior in vivo or ex vivo contact with survival-prolonging cytokines would explain the observed difference, the effect of preincubation (15-180 minutes) of the eosinophils with IL-5 (10 pmol/L) or GM-CSF (7 pmol/L) at concentrations previously shown to produce maximal antiapoptotic effect was studied. After preincubation, the cytokine was washed out and the cells were subsequently cultured for 48 hours. The majority of eosinophils ( $87\% \pm 0.6\%$  and  $80\% \pm 1\%$ ) incubated for 48 hours with IL-5 (10 pmol/L) and GM-CSF (7 pmol/L), respectively, were not apoptotic ( $n = 3$ ). In contrast, brief exposure of eosinophils to IL-5 (10 pmol/L) or GM-CSF (7 pmol/L) for 15, 30, 60, or 180 minutes before culture inhibited apoptosis maximally by 21% compared with vehicle-treated, time-matched control samples (Table II). Thus the eosinophils require continuous exposure to IL-5<sup>6</sup> or GM-CSF for the survival-prolonging action to be maintained. Thus a short ( $\leq 3$  hours) prior in vivo contact with these cytokines is not likely to explain the observed difference.

### Effect of $\beta_2$ -agonists on eosinophil apoptosis in vitro

Albuterol, fenoterol, and salmeterol inhibited apoptosis in eosinophils in vitro in a concentration-dependent manner (Fig 3). However, albuterol and fenoterol had a biphasic effect on eosinophil apoptosis. On a molar basis, fenoterol was a more potent inhibitor of apoptosis than albuterol or salmeterol (Fig 3). The median effective concentration ( $EC_{50}$ ) values were  $6.5 \pm 5.0$ ,  $0.7 \pm 0.5$ , and  $94 \pm 58$  nmol/L for albuterol, fenoterol, and salmeterol, respectively. In contrast, salmeterol at high concentrations seemed to result in a greater degree of inhibition than fenoterol or albuterol. To find out whether prior in vivo contact between eosinophils and  $\beta_2$ -agonists could contribute to the prolonged eosinophil survival, the effect of preincubation (30-120 minutes) of eosinophils with

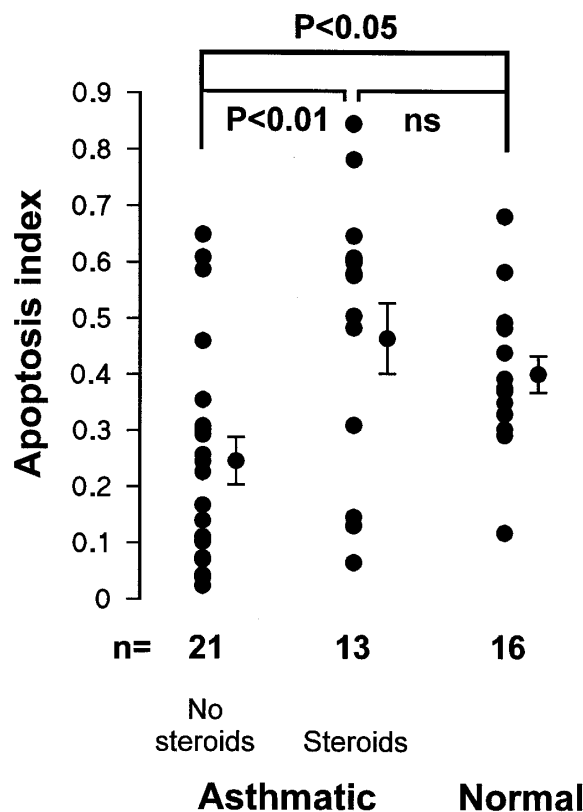


**FIG 1.** Apoptosis index after 48 hours of culture in eosinophils from healthy subjects and patients with asthma either using or not using corticosteroids. Error bars indicate mean  $\pm$  SEM. ns, Not significant.

**TABLE I.** The reversal by neutralizing antibodies (mAb-anti-) of the antiapoptotic effects of IL-3 (100 pmol/L), IL-5 (10 pmol/L), and GM-CSF (7 pmol/L) in human eosinophils

| Treatment                 | Apoptosis index     | Percentage neutralization of the effect of added cytokine |
|---------------------------|---------------------|-----------------------------------------------------------|
| Medium + IgG <sub>1</sub> | 0.40 $\pm$ 0.05 (3) |                                                           |
| IL-3 + IgG <sub>1</sub>   | 0.07 $\pm$ 0.01 (3) |                                                           |
| IL-3 + mAb-anti-IL-3      | 0.19 $\pm$ 0.05 (3) | 33 $\pm$ 10                                               |
| Medium + IgG <sub>1</sub> | 0.35 $\pm$ 0.04 (5) |                                                           |
| IL-5 + IgG <sub>1</sub>   | 0.03 $\pm$ 0.01 (5) |                                                           |
| IL-5 + mAb-anti-IL-5      | 0.24 $\pm$ 0.04 (5) | 62 $\pm$ 8                                                |
| GM-CSF + IgG <sub>1</sub> | 0.05 $\pm$ 0.01 (5) |                                                           |
| GM-CSF + mAb-anti-GM-CSF  | 0.20 $\pm$ 0.04 (5) | 50 $\pm$ 10                                               |

Eosinophils were preincubated with the neutralizing antibodies (5  $\mu$ g/mL) or the IgG<sub>1</sub> isotype control (5  $\mu$ g/mL) for 15 minutes, whereafter the cytokines (or medium in control) were added. Eosinophils were subsequently cultured for 48 hours. Apoptosis was assessed by flow cytometric analysis of propidium iodide-stained DNA content. Shown are the apoptosis index and the percentage inhibition of the response of the added cytokine (ie, neutralization). Data represent mean  $\pm$  SEM of (n) independent determinations from different cell preparations.

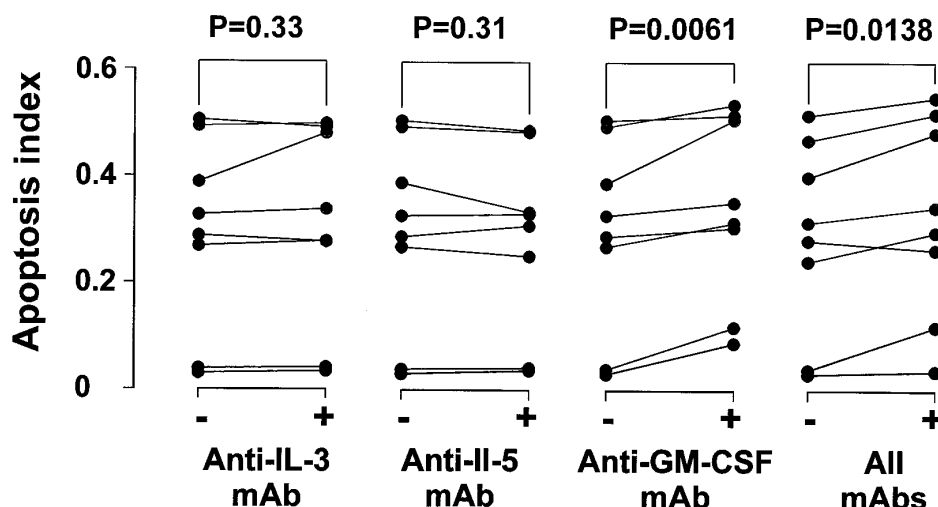


**FIG 2.** The effects of neutralization of IL-3, IL-5, and GM-CSF on eosinophil apoptosis index. Eosinophils from patients with asthma not using inhaled steroids were cultured with IgG<sub>1</sub> isotype control (5  $\mu$ g/mL) or with neutralizing mAbs to IL-3, IL-5, or GM-CSF (5  $\mu$ g/mL) for 48 hours. When all neutralizing antibodies (at 5  $\mu$ g/mL each) were added to the same culture, the isotype control IgG<sub>1</sub> was used at 15  $\mu$ g/mL. Each data point represents eosinophils from a single donor. For details see Methods.

**TABLE II.** The effect of preincubation of eosinophils with IL-5 and GM-CSF on eosinophil apoptosis

| Preincubation time (min) | Inhibition of apoptosis (%) |            |
|--------------------------|-----------------------------|------------|
|                          | IL-5                        | GM-CSF     |
| 15                       | 9 $\pm$ 3                   | -5 $\pm$ 7 |
| 30                       | 10 $\pm$ 7                  | 0 $\pm$ 4  |
| 60                       | 10 $\pm$ 1                  | 5 $\pm$ 7  |
| 180                      | 20 $\pm$ 4                  | 21 $\pm$ 2 |
| Continuously present     | 87 $\pm$ 1                  | 80 $\pm$ 1 |

Eosinophils were preincubated with IL-5 (10 pmol/L) or GM-CSF (7 pmol/L) for the indicated time, washed, and resuspended in fresh medium containing no IL-5 or GM-CSF and subsequently cultured for 48 hours. Apoptosis was assessed by flow cytometric analysis of propidium iodide-stained DNA content. Shown is the percentage inhibition of eosinophil apoptosis compared with vehicle-treated, time-matched control samples. After 48 hours of culture the apoptotic index in the absence of any cytokines was  $0.37 \pm 0.07$  and in the continuous presence of IL-5 (10 pmol/L) or GM-CSF (7 pmol/L) the apoptosis index values were  $0.05 \pm 0.00$  and  $0.08 \pm 0.02$ , respectively. Data represent the mean  $\pm$  SEM of 3 independent determinations from different cell preparations. Data for IL-5 from reference 6. See Methods for further details.

albuterol (100 nmol/L) was studied. After preincubation, albuterol was washed out and the cells were subsequently cultured for 48 hours. When continuously present, albuterol (100 nmol/L), produced  $14.3\% \pm 3.4\%$  inhibition of apoptosis ( $n = 5$ ,  $P < .01$ ). In contrast, an exposure of eosinophils to albuterol (100 nmol/L) for 120 minutes before culture produced 45% of the maximal antiapoptotic effect of albuterol (Table III).

Previously, cyclic AMP-elevating agents such as PGE<sub>2</sub><sup>14</sup> and forskolin<sup>15</sup> have been reported to prolong eosinophil survival.  $\beta_2$ -Agonists have been described to increase the concentrations of cyclic AMP in eosinophils.<sup>16</sup> To evaluate whether cyclic AMP could be involved, the

effects of other intracellular cyclic AMP-elevating agents such as theophylline, a nonselective phosphodiesterase inhibitor, and dibutyryl-cyclic AMP, a cell permeable analog of cyclic AMP, were studied. Both theophylline and dibutyryl-cyclic AMP inhibited eosinophil apoptosis in a concentration-dependent manner (Table IV).

## DISCUSSION

Our study shows for the first time that peripheral blood eosinophil apoptosis is delayed in asthmatic patients compared with healthy control subjects and that this defect can be reversed by inhaled glucocorticoids. A prolonged peripheral blood eosinophil survival, probably by delayed programmed cell death, has recently been reported to be a feature of inhalant allergy and extrinsic and intrinsic atopic dermatitis compared with nonatopic subjects.<sup>10</sup> Furthermore, it has been reported from our laboratory that in mild asthmatic patients demonstrating a late asthmatic response, the survival of peripheral blood eosinophils is prolonged after allergen challenge.<sup>17</sup> Recently, it was reported by Druilhe et al<sup>18</sup> and Vignola et al<sup>19</sup> that in bronchial biopsy specimens from patients with asthma there are more eosinophils and fewer apoptotic eosinophils compared with healthy controls or patients with chronic bronchitis, respectively. Their results suggest that asthmatic inflammation is associated with an enhanced survival of eosinophils caused by reduced apoptosis. Our study extends the published data by showing that there is a functional defect in the apoptotic death of peripheral blood eosinophils in asthma. This also suggests that the reduced number of apoptotic eosinophils found in the asthmatic lung may not simply result from the survival-prolonging effects of IL-5<sup>3</sup> or GM-CSF<sup>19</sup> as suggested. It may also partly be the result of the defect in the

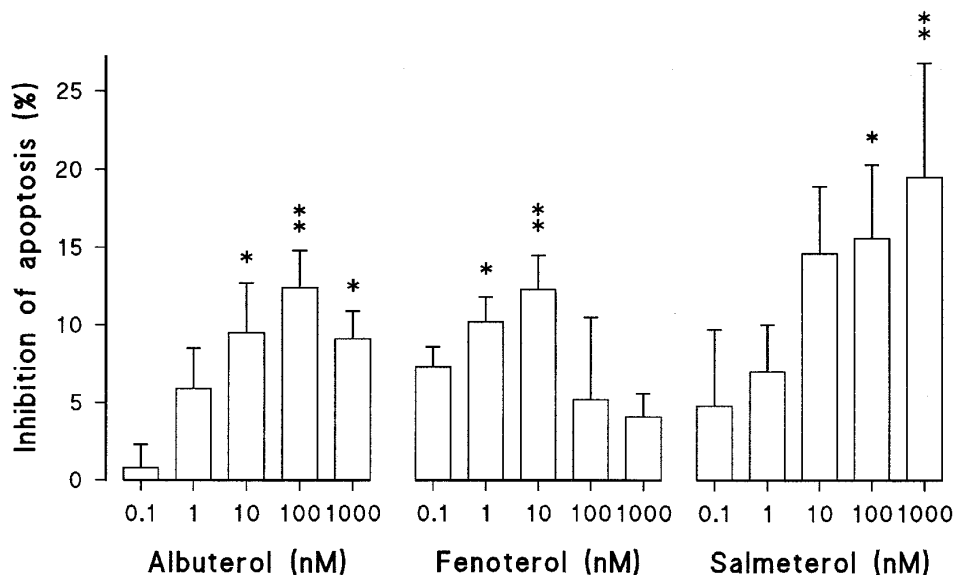


**FIG 3.** The inhibitory effect (percentage inhibition) of albuterol, fenoterol, and salmeterol on apoptosis during 48 hours of culture in eosinophils isolated from the blood of healthy volunteers. Mean  $\pm$  SEM,  $n = 5$ . Asterisk,  $P < .05$ ; two asterisks,  $P < .01$  compared with the respective solvent control.

**TABLE III.** The effect of preincubation of eosinophils with albuterol on apoptosis

| Preincubation time (min) | Inhibition of apoptosis (%)                           |                                                          |
|--------------------------|-------------------------------------------------------|----------------------------------------------------------|
|                          | Inhibition compared with time-matched solvent control | Percentage of albuterol effect when continuously present |
| 30                       | $-1.1 \pm 3.2$                                        | -7.5                                                     |
| 60                       | $5.1 \pm 5.9$                                         | 35.5                                                     |
| 120                      | $6.5 \pm 2.5^*$                                       | 45.3                                                     |
| Continuously present     | $14.3 \pm 3.4^\dagger$                                | 100                                                      |

Eosinophils were preincubated with albuterol (100 nmol/L) for the indicated time, washed, and resuspended in fresh medium containing no albuterol and subsequently cultured for 48 hours. Apoptosis was assessed by flow cytometric analysis of propidium iodide-stained DNA content. Shown is the percentage inhibition of eosinophil apoptosis compared with vehicle-treated, time-matched control samples. After 48 hours of culture the apoptosis index in the absence of any additions was  $0.48 \pm 0.06$  and in the continuous presence of albuterol the apoptosis index was  $0.42 \pm 0.06$ . Data represent the mean  $\pm$  SEM of 5 independent determinations from different cell preparations. See Methods for further details.

\* $P < .05$  compared with the corresponding control with no added albuterol.

$^\dagger P < .01$  compared with the corresponding control with no added albuterol.

apoptotic cell death process demonstrated in the current study in peripheral blood eosinophils.

We have recently demonstrated that the constitutive rate of apoptosis was reproducible within individuals over consecutive blood samples many weeks apart, indicating that the basis of the difference is intrinsic to each donor.<sup>6</sup> The delayed eosinophil apoptosis found in the current study may result from several factors. One explanation for the difference between individuals or between healthy and asthmatic donors could be the differential release of autocrine factors that are either proapoptotic or antiapoptotic. The most probable candidates would be the survival-prolonging cytokines IL-3, IL-5, and GM-CSF.<sup>11-13</sup> To elucidate this possibility, the effect of neutralization of the cytokines by specific antibodies was studied. Neutralization of GM-CSF increased the rate of apoptosis slightly. In contrast, neutralization of IL-3 or

**TABLE IV.** The effects of theophylline and dibutylcyclic AMP on apoptosis of cytokine-deprived human eosinophils isolated from healthy volunteers

| Treatment                               | Apoptotic index |
|-----------------------------------------|-----------------|
| Control                                 | $0.55 \pm 0.02$ |
| Theophylline ( $\mu\text{mol/L}$ )      |                 |
| 10                                      | $0.56 \pm 0.05$ |
| 100                                     | $0.55 \pm 0.04$ |
| 1000                                    | $0.36 \pm 0.03$ |
| Dibutylcyclic AMP ( $\mu\text{mol/L}$ ) |                 |
| 10                                      | $0.49 \pm 0.01$ |
| 100                                     | $0.27 \pm 0.06$ |
| 1000                                    | $0.19 \pm 0.01$ |

Eosinophils were cultured for 48 hours and apoptosis was assessed by flow cytometric analysis of propidium iodide-stained DNA content. Data represent the mean  $\pm$  SEM of 3 independent determinations from different cell preparations. See Methods for further details.

IL-5 had no effect. However, the increase in apoptosis index induced by GM-CSF neutralization was relatively small compared with the difference between asthmatic patients and healthy volunteers. Thus production of GM-CSF by the eosinophils may not completely explain the delay in apoptosis. Another attractive explanation is that in vivo eosinophils may be exposed to survival-prolonging cytokines such as IL-5 or GM-CSF. However, a brief exposure (15 to 180 minutes) of eosinophils to IL-5<sup>6</sup> or GM-CSF was not sufficient to inhibit apoptosis in subsequent culture. Thus eosinophils require continuous exposure to IL-5<sup>6</sup> or GM-CSF for the survival-prolonging action to be maintained. Furthermore, the prolonged eosinophil survival in the spontaneously surviving eosinophils (ie, those having a low apoptosis index) is effectively reversed by SB 203580, an inhibitor of p38 mitogen-activated protein kinase.<sup>6</sup> In contrast, the survival-prolonging effects of IL-5 or GM-CSF are not affected by this inhibitor.<sup>6</sup> Thus it is unlikely that the in vivo contact with survival-prolonging cytokines IL-3, IL-5, or GM-CSF accounts for the difference between donors or patients with asthma and healthy controls.

Excessive use of  $\beta_2$ -adrenoceptor agonists has been associated with increased risk of death or near death from asthma.<sup>20</sup> One possible mechanism for this could be prevention of eosinophil clearance by inhibition of apoptosis resulting in a more severe inflammatory response and asthmatic symptoms. In fact, use of albuterol has been associated with increased numbers of eosinophils in lung.<sup>21</sup> To assess whether  $\beta_2$ -agonist medication could contribute to the observed differences, we determined the in vitro effects of albuterol, fenoterol, and salmeterol on eosinophil apoptosis and showed that  $\beta_2$ -agonists inhibited apoptosis in eosinophils. Our results are supported by the recent report of Nielson and Hadjokas<sup>22</sup> showing that isoproterenol tended to decrease spontaneous eosinophil apoptosis. Furthermore, we and others have demonstrated previously<sup>14,15</sup> and in the current study that other cyclic AMP-elevating agents such as forskolin, PGE<sub>2</sub>, and theophylline and the cell-permeable cyclic AMP analog dibutyryl cyclic AMP increase eosinophil survival by inhibiting apoptosis. A significant inhibition of eosinophil apoptosis by albuterol was obtained at 10 to 1000 nmol/L concentrations (EC<sub>50</sub> 6.5 nmol/L), with fenoterol at 1 to 10 nmol/L concentrations (EC<sub>50</sub> 0.7 nmol/L), and with salmeterol at 100 to 1000 nmol/L concentrations (EC<sub>50</sub> 94 nmol/L). The mean maximum plasma concentration of albuterol after a single 4-mg tablet is 30 to 60 nmol/L.<sup>23,24</sup> After inhalation by metered-dose inhaler or nebulizer the peak plasma concentrations of albuterol have been reported to be 7.5 to 23 nmol/L.<sup>25</sup> For inhaled fenoterol the corresponding peak plasma concentrations are 0.5 to 1.2 nmol/L.<sup>25</sup> Thus the inhibition of eosinophil apoptosis by albuterol and fenoterol in vitro occurred at clinically relevant drug concentrations. Long-term administration of salmeterol 50  $\mu$ g twice daily by inhalation to patients with asthma resulted in a peak plasma concentration of 0.36 nmol/L.<sup>26</sup> Salmeterol is a lipophilic substance. Inhaled salmeterol binds to the

bronchial tissue and acts locally in the lung. Thus plasma concentrations are not predictive of therapeutic efficacy.<sup>26</sup> Therefore, although the inhibition of apoptosis by salmeterol in vitro required higher concentrations than those obtained in plasma after inhalation, we cannot exclude the possibility that salmeterol might inhibit eosinophil apoptosis locally in the lung.

To find out whether prior in vivo contact between eosinophils and  $\beta_2$ -agonists could contribute to the prolonged eosinophil survival, the effect of preincubation of eosinophils with albuterol was studied. An exposure of eosinophils to albuterol for 120 minutes before culture produced 45% of its maximal antiapoptotic effect. This indicates that an in vivo exposure to  $\beta_2$ -agonists might have a minor contribution to the delayed eosinophil apoptosis in asthma. However, the inhibition of eosinophil apoptosis by  $\beta_2$ -agonist was maximally 19%. The apoptosis index of asthmatic patients (0.25) was approximately 38% lower than that of healthy controls (0.40). Thus the effect of  $\beta_2$ -agonists in vitro is not enough to explain the observed difference between the groups. Taken together, it appears that although the inhibition of eosinophil apoptosis by albuterol and fenoterol occurred at clinically relevant concentrations, it does not indicate that use of  $\beta_2$ -agonists would cause the delayed eosinophil apoptosis in asthma found in the current study. However, on the basis of the in vitro data,  $\beta_2$ -agonists may contribute to or worsen this defect in the absence of glucocorticoids.

The maximal inhibitory effect of  $\beta_2$ -agonists on eosinophil apoptosis was 19%. It could be argued that such a modest effect is not clinically relevant in asymptomatic or mild asthmatic patients with relatively low numbers of eosinophils in the airways. However, in situations with severe airway eosinophilia, a 10% to 19% increase in eosinophil survival might result in a significant cumulative increase in the absolute numbers of eosinophils in the airways. However, even in patients with mild asthma regular treatment with inhaled albuterol resulted in a significant increase in eosinophil numbers in sputum concomitantly with an enhanced late allergen airway response.<sup>21</sup> Compared with the inhibitory effect (up to 90% inhibition) of cytokines IL-3, IL-5, and GM-CSF on eosinophil apoptosis,<sup>1,2,6,11-13</sup> the effects of  $\beta_2$ -agonists are modest. Because  $\beta_2$ -agonists are successfully used to treat asthmatic symptoms, it would be unrealistic to expect them to inhibit eosinophil apoptosis to a similar extent than these inflammatory cytokines. However, this does not exclude the possibility that regular long-term  $\beta_2$ -agonist use might augment eosinophilic inflammation. Thus the results of our study suggest that regular  $\beta_2$ -agonist use in the absence of steroids might prolong eosinophil survival through inhibition of apoptosis and thus worsen eosinophilia in asthmatic patients.

Glucocorticoids have been reported to induce eosinophil apoptosis in vitro.<sup>1,2,16,27</sup> Furthermore, a decrease in the numbers of sputum<sup>9</sup> and bronchial<sup>18</sup> eosinophils associated with an increased apoptosis index after steroid treatment has been reported in patients with asthma. Because only 1 of 13 patients used oral steroids in

addition to inhaled corticosteroids, the current results suggest that inhaled glucocorticoids affect not only those in the lung but also circulating eosinophils and reduce their numbers by induction of apoptosis. In addition to systemic effects, inhaled glucocorticoids might suppress pulmonary production of survival-prolonging cytokines such as IL-5 or GM-CSF that also affect circulating eosinophils. However, in the case of IL-5 or GM-CSF this seems to be an unlikely explanation for the effect of glucocorticoids seen in the current study. A brief contact (for 15 to 180 minutes) with IL-5<sup>6</sup> or GM-CSF is not sufficient to prolong eosinophil survival (ie, eosinophils require continuous exposure to IL-5 or GM-CSF for the survival-prolonging action to be maintained). In contrast, part of the delayed eosinophil apoptosis seemed to be due to production of GM-CSF by the eosinophils. A possibility thus exists that glucocorticoids reverse the prolonged survival at least partly by inhibiting the production of GM-CSF. Normalization of the prolonged survival of circulating eosinophils by inhaled glucocorticoids further supports their role as first-line anti-inflammatory therapy in asthma.

Interestingly, Wedi et al<sup>10</sup> reported that eosinophil survival in inhalant allergy or atopy did not correlate with peripheral blood eosinophil counts or total IgE levels. Similarly, we did not find any significant correlation between the apoptosis index and the peripheral blood eosinophil count. Blood eosinophil counts reflect a complex relationship between the number of cells leaving the bone marrow, marginating, and then leaving the blood. Eosinophils circulate usually less than 24 hours (half-life 18 hours) before migrating to the tissues where they can survive for several days.<sup>28</sup> Thus we would not expect a defect in eosinophil apoptosis to result in peripheral blood eosinophilia but an increase in the number of tissue eosinophils.

Taken together, the findings suggest that eosinophil apoptosis is delayed in asthma. This delay may be partly explained by production of GM-CSF in eosinophils. It seems not to be caused by  $\beta_2$ -agonists, but the in vitro effects of  $\beta_2$ -agonists suggest that in the absence of steroids,  $\beta_2$ -agonist use might contribute to the prolonged eosinophil survival through inhibition of apoptosis and thus may worsen eosinophilia in asthmatic patients. Use of inhaled glucocorticoids totally reverses the delayed eosinophil apoptosis in asthma.

## REFERENCES

- Haslett C. Granulocyte apoptosis and inflammatory disease. *Br Med Bull* 1997;53:669-83.
- Walsh GM. Mechanisms of human eosinophil survival and apoptosis. *Clin Exp Allergy* 1997;27:482-7.
- Simon HU, Yousefi S, Schranz C, Schapowal A, Bachert C, Blaser K. Direct demonstration of delayed eosinophil apoptosis as a mechanism causing tissue eosinophilia. *J Immunol* 1997;158:3902-8.
- Murphy S, Bleeker ER, Boushey H, Buist AS, Busse W, Clark NM, et al. Expert Panel Report 2: guidelines for the diagnosis and management of asthma. Bethesda (MD): National Institutes of Health; 1997. NIH publication No.: 97-4051. Also available at <http://www.nhlbi.nih.gov/guidelines/asthma/asthgdln.htm>.
- Hansel TT, Pound JD, Pilling D, Kitas GD, Salmon M, Gentle TA, et al. Purification of human blood eosinophils by negative selection using immunomagnetic beads. *J Immunol Methods* 1989;122:97-103.
- Kankaanranta H, De Souza PM, Barnes PJ, Salmon M, Giembycz MA, Lindsay MA. SB 203580, an inhibitor of p38 mitogen-activated protein kinase, enhances constitutive apoptosis of cytokine-deprived human eosinophils. *J Pharm Exp Ther* 1999;290:621-8.
- Kankaanranta H, De Souza PM, Giembycz MA, Lindsay MA. Human eosinophil isolation and the measurement of apoptosis. In: Chung KF, Adcock IM, editors. *Methods in molecular medicine: asthma*. Totowa: Humana Press. In press.
- Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J Immunol Methods* 1991;139:271-9.
- Woolley KL, Gibson PG, Carty K, Wilson AJ, Twaddell SH, Woolley MJ. Eosinophil apoptosis and the resolution of airway inflammation in asthma. *Am J Respir Crit Care Med* 1996;154:237-43.
- Wedi B, Raap U, Lewrick H, Kapp A. Delayed eosinophil programmed cell death in vitro: a common feature of inhalant allergy and extrinsic and intrinsic atopic dermatitis. *J Allergy Clin Immunol* 1997;100:536-43.
- Tai PC, Sun L, Spry CJ. Effects of IL-5, granulocyte/macrophage colony-stimulating factor (GM-CSF) and IL-3 on the survival of human blood eosinophils in vitro. *Clin Exp Immunol* 1991;85:312-6.
- Yamaguchi Y, Suda T, Ohta S, Tominaga K, Miura Y, Kasahara T. Analysis of the survival of mature human eosinophils: interleukin-5 prevents apoptosis in mature human eosinophils. *Blood* 1991;78:2542-7.
- Stern M, Meagher L, Savill J, Haslett C. Apoptosis in human eosinophils: programmed cell death in the eosinophil leads to phagocytosis by macrophages and is modulated by IL-5. *J Immunol* 1992;148:3543-9.
- Peacock CD, Misso NL, Watkins DN, Thompson PJ. PGE<sub>2</sub> and dibutyryl cyclic adenosine monophosphate prolong eosinophil survival in vitro. *J Allergy Clin Immunol* 1999;104:153-62.
- Hallsworth MP, Giembycz MA, Barnes PJ, Lee TH. Cyclic AMP-elevating agents prolong or inhibit eosinophil survival depending on prior exposure to GM-CSF. *Br J Pharmacol* 1996;117:79-86.
- Giembycz MA, Lindsay MA. Pharmacology of the eosinophil. *Pharmacol Rev* 1999;51:213-340.
- Evans DJ, Lindsay MA, O'Connor BJ, Barnes PJ. Priming of circulating human eosinophils following late response to allergen challenge. *Eur Respir J* 1996;9:703-8.
- Druihe A, Wallaert B, Tsicopoulos A, Lupe e Silva JR, Tillie-Leblond I, Tonnel AB, et al. Apoptosis, proliferation, and expression of Bcl-2, Fas, and Fas ligand in bronchial biopsies from asthmatics. *Am J Respir Cell Mol Biol* 1998;19:747-57.
- Vignola AM, Chanez P, Chiappara G, Siena L, Merendino A, Reina C, et al. Evaluation of apoptosis of eosinophils, macrophages, and T lymphocytes in mucosal biopsy specimens of patients with asthma and chronic bronchitis. *J Allergy Clin Immunol* 1999;103:563-73.
- Spitzer WO, Suissa S, Ernst P, Horwitz RJ, Habbick B, Cockcroft D, et al. The use of  $\beta$ -agonists and the risk of death or near death from asthma. *N Engl J Med* 1992;326:501-6.
- Gauvreau GM, Jordana M, Watson RM, Cockcroft DW, O'Byrne PM. Effect of regular inhaled albuterol on allergen-induced late responses and sputum eosinophils in asthmatic subjects. *Am J Respir Crit Care Med* 1997;156:1738-45.
- Nielson CP, Hadjokas NE. Beta-adrenoceptor agonists block corticosteroid inhibition in eosinophils. *Am J Respir Crit Care Med* 1998;157:184-91.
- Jonkman JHG, Freie HMP, van der Boon WJ, Grasmeijer G. Single dose absorption profiles and bioavailability of two different salbutamol tablets. *Arzneimittel Forschung* 1986;36:1133-5.
- Powell ML, Chung M, Weisberger M, Gural R, Radwanski E, Symchowicz S, et al. Multiple-dose albuterol kinetics. *J Clin Pharmacol* 1986;26:643-6.
- Hochhaus G, Möllmann H. Pharmacokinetic/pharmacodynamic characteristics of the  $\beta_2$ -agonists terbutaline, salbutamol and fenoterol. *Int J Clin Pharmacol* 1992;30:342-62.
- Adkins JC, McTavish D. Salmeterol: a review of its pharmacological properties and clinical efficacy in the management of children with asthma. *Drugs* 1997;54:331-54.
- Schleimer RP, Bochner BS. The effects of glucocorticoids on human eosinophils. *J Allergy Clin Immunol* 1994;94:1202-13.
- Spry CJF. The natural history of eosinophils. In: Smith H, Cook RM, editors. *Immunopharmacology of eosinophils*. London: Academic Press; 1993. p 1-9.