

PGE₂ and dibutyl cyclic adenosine monophosphate prolong eosinophil survival in vitro

Craig D. Peacock, PhD, Neil L. A. Misso, PhD, D. Neil Watkins, PhD, FRACP, and Philip J. Thompson, FRACP *Perth, Australia*

Background: Apoptosis represents a mechanism by which the accumulation and inflammatory potential of eosinophils in asthma might be limited. Mediators derived from the airway epithelium may influence the rate of eosinophil apoptosis.

Objective: We have investigated the effects on eosinophil apoptosis of 3 mediators that are likely to be produced by the airway epithelium, namely PGE₂, TNF- α , and nitric oxide.

Methods: Peripheral blood eosinophils from healthy adult volunteers were purified by density gradient centrifugation and negative immunomagnetic selection. Eosinophils were cultured for 16 or 40 hours with PGE₂ (10 nmol/L), dibutyl cyclic adenosine monophosphate (AMP; 100 μ mol/L), TNF- α (500 U/mL), the nitric oxide donors, S-nitroso-N-acetylpenicillamine (100 μ mol/L), and 2,2'-(hydroxynitrosohydrazono)bis-ethanamine (1 mmol/L), or dibutyl cyclic guanosine monophosphate (100 μ mol/L). Control cultures consisted of untreated, IL-5-treated (100 U/mL), and anti-Fas-treated (400 ng/mL) cells. Eosinophil apoptosis was assessed by flow cytometric analysis of annexin V-FITC binding to externalized phosphatidylserine, by electrophoresis of phosphorus 32 end-labeled DNA fragments, and by flow cytometric assessment of hypodiploid DNA with propidium iodide.

Results: PGE₂ and cyclic AMP inhibited spontaneous eosinophil apoptosis at both 16 and 40 hours as did the PGE₂ receptor agonist, 11-deoxy PGE₁, at 40 hours, but these effects were not inhibited by a protein kinase A antagonist. TNF- α delayed apoptosis in eosinophil cultures at 16 hours, whereas S-nitroso-N-acetylpenicillamine, 2,2'-(hydroxynitrosohydrazono)bis-ethanamine, and cyclic guanosine monophosphate had little effect. Anti-Fas had little effect on spontaneous eosinophil apoptosis but significantly reduced the inhibitory effects of PGE₂, cyclic AMP, and TNF- α . Assessments of apoptosis by DNA fragmentation gave similar but quantitatively less sensitive results.

Conclusion: Inhibition of spontaneous eosinophil apoptosis by PGE₂ appears to be mediated by EP₂ receptors but is not protein kinase A dependent. By enhancing eosinophil survival, PGE₂ may increase the proinflammatory potential of these cells in chronic asthma. (*J Allergy Clin Immunol* 1999;104:153-62.)

Key words: Eosinophil, apoptosis, asthma, IL-5, Fas, TNF- α , PGE₂, annexin V

Airway inflammation is a characteristic feature of asthma and is associated with airway obstruction and hyperreactivity.¹ There is significant evidence attesting to a central role for eosinophils and their secreted mediators in the underlying airway inflammation in asthma.² Increased numbers of eosinophils are found in the bronchoalveolar lavage fluid, sputum, and peripheral blood of patients with asthma,^{3,4} and these are positively correlated with the severity of airway hyperreactivity and disease.^{2,5} Eosinophil activation results in the release of granule-derived proteins, including eosinophil cationic protein and major basic protein (which, as well as being cytotoxic for bronchial epithelium, increase nonspecific bronchial hyperreactivity and impair ciliary function).^{6,7}

Eosinophils are terminally differentiated cells that do not proliferate in culture but spontaneously undergo programmed cell death. This can be delayed by cytokines such as GM-CSF, IL-3, and, most notably, IL-5.^{8,9} This cell death process exhibits features characteristic of apoptosis, including cleavage of DNA into approximately 200-bp oligonucleosome length fragments. Importantly, apoptotic eosinophils are recognized and phagocytosed by macrophages,⁹ allowing their intact removal from the tissues and avoiding the undesirable proinflammatory response that would occur with necrosis. Hence, eosinophil apoptosis may represent an important mechanism by which the contribution of eosinophils to airway inflammation can be regulated. Corticosteroids have been reported to accelerate eosinophil apoptosis, an effect that may be particularly relevant to their use in asthma therapy.¹⁰ Furthermore, the Fas receptor transduces apoptotic signals in a wide variety of cells through interaction with its natural ligand or agonistic anti-Fas mAbs,¹¹ and a number of studies suggest Fas has a similar role in eosinophil apoptosis.¹²⁻¹⁵

The epithelium has an important homeostatic role in mammalian airway physiologic features.¹⁶ PGE₂ is the predominant prostanoid product of the airway epithelium and has important inhibitory effects on inflammatory cell activation, including inhibition of eosinophil chemotaxis, aggregation, degranulation, and IL-5-induced survival.¹⁷⁻¹⁹ The respiratory epithelium is also a major source of airway nitric oxide (NO).^{20,21} Elevated concentrations of NO have been described in asthma and have been hypothesized to contribute to the generation of a preferential T_{H2}-like inflammatory response in the respi-

From the Asthma and Allergy Research Institute and Department of Medicine, University of Western Australia, Perth.

Supported by grants from the Asthma Foundation of Western Australia and Sir Charles Gairdner Hospital Research Foundation.

Received for publication June 2, 1998; revised Mar 12, 1999; accepted for publication Mar 12, 1999.

Reprint requests: Philip J. Thompson, FRACP, Asthma and Allergy Research Institute Inc, Ground Floor, E Block, Sir Charles Gairdner Hospital, Nedlands, W.A. 6009, Australia.

Copyright © 1999 by Mosby, Inc.

0091-6749/99 \$8.00 + 0 1/1/98641

Abbreviations used

db-cAMP:	Dibutyryl adenosine 3',5'-cyclic monophosphate
db-cGMP:	Dibutyryl guanosine 3',5'-cyclic monophosphate
DETANONOate:	2,2'-(Hydroxynitrosohydrazono)bis-ethanamine
MAP:	Mitogen-activated protein
MB:	Methylene blue
NO:	Nitric oxide
PI:	Propidium iodide
SNAP:	S-Nitroso-N-acetylpenicillamine
TE:	Tris-HCl and EDTA

ratory tract.²² This may have implications for eosinophil activity and survival. Eosinophils themselves are a source of NO,²³ and NO appears to play a role in eosinophil migration²⁴ and their production of TNF- α .²⁵ TNF- α , an important proinflammatory mediator in asthma, enhances both cytotoxic and chemotactic responses of eosinophils in vitro,^{26,27} whereas depletion of TNF- α in a rat model of airway inflammation diminished eosinophil recruitment into the lung in response to antigen.²⁸ Furthermore, TNF- α is a member of the TNF family of proteins to which Fas ligand also belongs and induces apoptosis in a variety of cell types.¹¹ However, despite important regulatory roles in the recruitment and activation of eosinophils, the effects of PGE₂, NO, and TNF- α on eosinophil survival through the regulation of apoptosis remain to be fully elucidated.

Eosinophil apoptosis has previously been quantified by a variety of different techniques that may be measuring different events in the apoptotic pathway. The internucleosomal DNA degradation that is a hallmark feature of apoptosis can be identified by the characteristic ladder pattern seen on agarose gel electrophoresis. Chromatin degradation can also be assessed by propidium iodide staining of DNA, and apoptosis can be quantified by flow cytometry as the proportion of cells within the hypodiploid region.²⁹ In contrast to these assays, which measure nuclear events in apoptosis, a more recent technique involves the use of the aminophospholipid-binding protein, annexin V. This protein binds to phosphatidylserine, which is normally confined to the internal leaflet of the plasma membrane but is externalized during apoptosis. This cell surface event, which is thought to occur early in the apoptotic process,³⁰ can be quantified by flow cytometry as the proportion of cells binding annexin V.

The aim of our study was to investigate the effects of PGE₂, NO, and TNF- α on spontaneous eosinophil apoptosis and to determine whether PGE₂ modulates IL-5-induced eosinophil survival or Fas-mediated apoptosis. Because the correlation between various methods for assessing eosinophil apoptosis has not been explored, we used techniques that assess both cell surface changes (annexin V binding) and nuclear events (DNA fragmentation) in eosinophil apoptosis.

METHODS**Subjects**

Heparinized peripheral venous blood was obtained from 12 healthy adult donors (7 men and 5 women; 20 to 45 years of age). Atopic status was tested by skin prick sensitivity to a panel of 10 common allergens, and atopy was assigned on the basis of 1 or more positive (≥ 3 mm diameter) responses. Informed consent was obtained from all donors, and the study was approved by the Committee for Human Rights at the University of Western Australia. Because limited numbers of eosinophils were isolated from a single blood sample, the effects of all the various mediators could not be examined with cells from every individual. The number of individual donor blood samples used for each experiment is indicated where appropriate. Spontaneous eosinophil apoptosis and its modulation by the various mediators were not significantly different in atopic subjects ($n = 6$) compared with nonatopic subjects ($n = 6$). Therefore the results represent pooled data from both atopic and nonatopic subjects.

Mediators, cytokines, and antibodies

S-nitroso-N-acetylpenicillamine (SNAP), dibutyryl cyclic adenosine monophosphate (db-cAMP), dibutyryl cyclic guanosine monophosphate (db-cGMP), methylene blue (MB; Sigma, St Louis, Mo), recombinant human IL-5 (Genzyme, Cambridge, Mass), murine anti-human Fas IgM mAb (clone CH-11; Upstate Biotechnology, Lake Placid, NY), TNF- α (Boehringer, Mannheim, Germany), and DETANONOate (Cayman Chemical Co, Ann Arbor, Mich) were diluted in PBS (pH 7.4) before use. PGE₂, 11-deoxy PGE₁, and 17-phenyl trinor PGE₂ (all from Cayman Chemical Co) were dissolved in ethanol (1 mmol/L) and further diluted in PBS. H-89 and SC-51089 (Biomol, Plymouth Meeting, Pa) were dissolved in dimethyl sulfoxide (10 mmol/L and 1 mmol/L, respectively) and further diluted in RPMI-1640.

Purification and culture of eosinophils

Peripheral blood eosinophils were isolated by density gradient centrifugation (Percoll, density 1.088; Pharmacia Biotech, Uppsala, Sweden) followed by hypotonic lysis of erythrocytes and negative immunomagnetic selection of eosinophils with CD16 microbeads (MACS; Miltenyi Biotec, Germany), as previously described.³¹ This technique consistently provided eosinophil preparations with a purity greater than 97%, as determined by differential counts on cyto-spin preparations stained with Giemsa-type stain (Diff-Quik). Purified eosinophils were resuspended at a concentration of 4×10^5 cells/mL in RPMI-1640 medium supplemented with 10% FCS (CSL, Melbourne, Australia), 2 mmol/L L-glutamine, 20 mmol/L HEPES, and 50 μ g/mL gentamicin. Eosinophils (2×10^5 cells, 0.5 mL) were added to 24-well tissue culture plates precoated with FCS to inhibit adherence. After 16 or 40 hours incubation (37°C; 5% carbon dioxide) with and without various mediators, eosinophils were harvested and apoptosis was assessed.

Assessment of eosinophil apoptosis by flow cytometry

Analysis of annexin V binding. Differential analysis of apoptotic and necrotic cells was performed by incubating the cultured eosinophils (2×10^5 cells/tube) for 15 minutes at room temperature in 100 μ L of labeling buffer (10 mmol/L HEPES, 140 mmol/L NaCl, 5 mmol/L CaCl₂; pH 7.4) containing 20 μ L/mL of annexin V-FITC (Boehringer Mannheim) and 1 μ g/mL of propidium iodide (PI; Sigma Chemical Co). Eosinophils were washed in labeling buffer, resuspended in 500 μ L of the same, and immediately analysed on a FACScan flow cytometer with CellQuest software (Becton Dickinson, San Jose, Calif). Five thousand cells were

acquired, and apoptotic cells were quantified as the percentage of the total population that were annexin V–FITC positive but PI negative. Necrotic cells were double-positive (FITC⁺/PI⁺), and although the numbers of necrotic eosinophils tended to increase with time in culture, they represented less than 5% of the total cell number in all experiments (data not shown).

Analysis of PI binding to DNA. Labeling solution consisting of 40 μ L of 50 μ g/mL PI was added to the eosinophils, which were then permeabilized by the addition of an equal volume of 0.1% Triton X-100 (Sigma Chemical Co) in PBS (pH 7.4), permitting access of PI into all eosinophils and not just necrotic cells. The proportion of cells within the hypodiploid DNA region was assessed by flow cytometry.²⁹

Assessment of eosinophil apoptosis by analysis of DNA fragmentation

DNA was extracted from eosinophils, as described previously.⁹ Briefly, eosinophils (6×10^5 total) were harvested, washed, and lysed overnight at 37°C in 0.5 mL lysis buffer (10 mmol/L Tris-HCl, pH 8.2; 0.1 mol/L NaCl; 1 mmol/L EDTA) containing 1% SDS and 0.2 mg/mL proteinase K). DNA was precipitated with ethanol (–20°C), washed in 70% ethanol, resuspended in TE buffer (20 mmol/L Tris-HCl, pH 7.6; 5 mmol/L EDTA), and quantified by spectrophotometry at 260 nm. End labeling of DNA fragments with ³²P at the 3'-end was performed, as described previously.³² Briefly, DNA (1 μ g in 29.5 μ L of water) was incubated at 37°C for 60 minutes with 10 μ L 5 \times reaction buffer (1 mol/L potassium cacodylate, 0.125 mol/L Tris-HCl, 1.25 mg/mL BSA; pH 6.6), 5 μ L CoCl₂ solution (25 mmol/L), 0.5 μ L (12.5 U) terminal transferase, 14.9 fmol ddATP (all from Boehringer Mannheim), and 0.63 μ Ci [α -³²P]ddATP (Amersham, Sydney, Australia). The reaction was terminated with 5 μ L 0.25 mol/L EDTA and 2 μ L bacterial rRNA solution (25 mg/mL; Boehringer Mannheim) was added as carrier. DNA was precipitated with 12 μ L of 10 mol/L ammonium acetate and 180 μ L of cold ethanol (–70°C; 60 minutes); the pellet was resuspended in TE buffer (55 μ L), and the precipitation was repeated. The DNA pellet was resuspended in TE buffer and electrophoresed through 2% agarose in Tris-acetic acid–EDTA buffer. The gel was dried, and autoradiography was performed on a PhosphorImager SI with ImagerSI Version 4.0 and ImageQuaNT Version 4.2a software (Molecular Dynamics, Sunnyvale, Calif). Eosinophil apoptosis was calculated as the percentage of the total radioactive signal in the lane of interest that was contributed by fragmented DNA.

Statistical analysis

Data are presented as means \pm SEM. Differences between treatments were assessed for statistical significance by 1-way, repeated measures ANOVA and the Tukey–Kramer multiple comparison test. An unpaired, 2-tailed Student *t* test was used to compare results from atopic and nonatopic subjects. Probability (*P*) values of less than .05 were considered significant. All statistical analyses were performed with GraphPad Prism Version 2.01 (GraphPad Software Inc, San Diego, Calif).

RESULTS

IL-5 inhibits externalization of plasma membrane phosphatidylserine in eosinophils

Using annexin V, we confirmed that spontaneous eosinophil apoptosis is significantly decreased at 16 and 40 hours in the presence of 100 U/mL of IL-5 (*n* = 12; *P* < .001; Fig 1). In contrast, stimulation of the eosinophil Fas receptor with anti-Fas mAb (400 ng/mL)

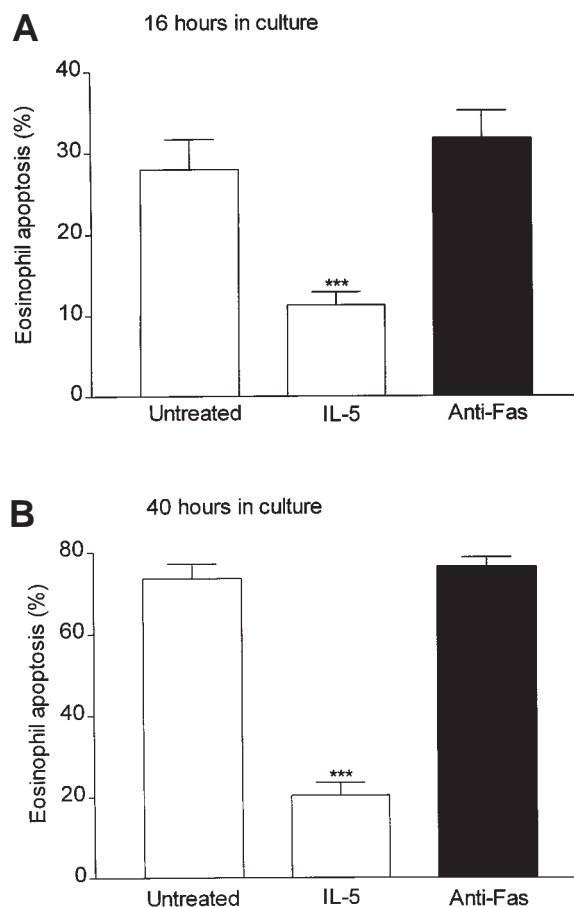


FIG 1. Comparison of apoptosis in untreated eosinophils and after treatment with IL-5 or anti-Fas. Purified eosinophils were cultured for 16 (**A**) or 40 (**B**) hours either untreated or in the presence of IL-5 (100 U/mL) or anti-Fas mAb (CH-11, 400 ng/mL); apoptosis was assessed by flow cytometry as the percentage of cells binding annexin V (*n* = 12; ****P* < .001, compared with untreated, ANOVA).

induced only a small increase in apoptosis (*n* = 12; *P* > .05).

PGE₂ inhibits spontaneous eosinophil apoptosis

PGE₂ reduced eosinophil apoptosis at 16 hours, with significant effects being observed at concentrations of 1 and 10 nmol/L (*n* = 7; *P* < .05 and < .01, respectively; Fig 2, *A*). Similarly, eosinophils cultured for 40 hours with 1 or 10 nmol/L PGE₂ were significantly less apoptotic compared with untreated cells (*n* = 8; *P* < .05 and < .001, respectively; Fig 2, *B*). There was a trend toward increasing the inhibition of apoptosis with increasing PGE₂ concentration, although differences for the 3 concentrations of PGE₂ (0.1, 1, and 10 nmol/L) did not reach significance. These concentrations of PGE₂ are likely to be biologically relevant as the affinity constants of PGE₂ for EP receptors are 1 to 10 nmol/L, depending on the receptor subtype and tissue.³³

To further investigate the role of PGE₂ in eosinophil apoptosis, specific PGE₁ and PGE₂ receptor agonists

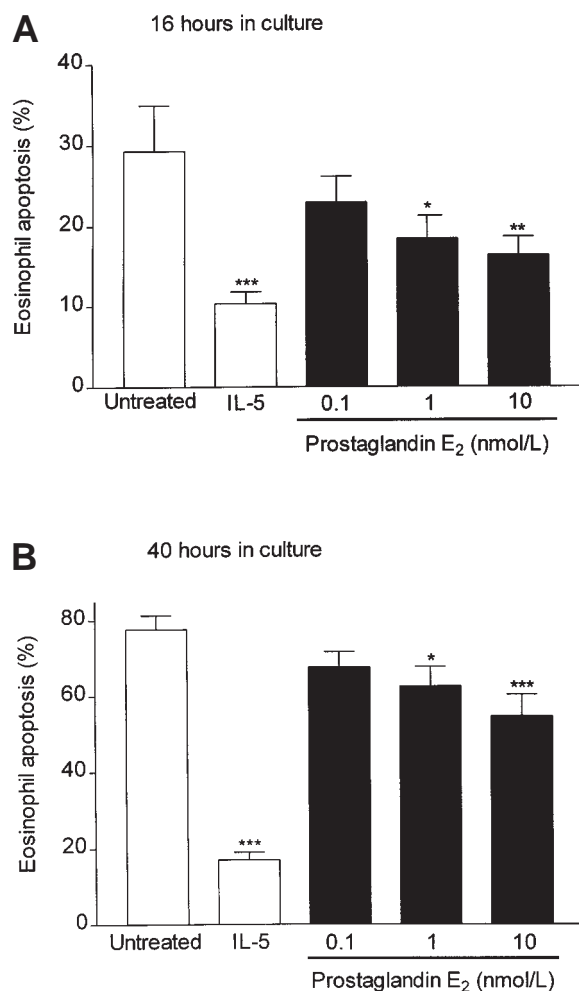


FIG 2. The effect of PGE₂ on eosinophil apoptosis. Purified eosinophils were cultured for 16 (**A**; *n* = 7) or 40 (**B**; *n* = 8) hours in the presence of PGE₂ (0.1, 1, and 10 nmol/L); apoptosis was assessed by flow cytometry as the percentage of cells binding annexin V. For comparison, data for untreated and IL-5-treated (100 U/mL) cultures is shown (**P* < .05, ***P* < .01, ****P* < .001, compared with untreated, ANOVA).

were added to eosinophil cultures for 40 hours. Inhibition of apoptosis by the EP₂ receptor agonist, 11-deoxy PGE₁ (100 nmol/L; 71.8% ± 8% vs 90.1% ± 3.3%; *n* = 5; *P* < .001) was similar to that observed with PGE₂. In contrast, the EP₁ receptor agonist 17-phenyl trinor PGE₂ did not significantly inhibit spontaneous eosinophil apoptosis (84.0% ± 4.9% vs 90.1% ± 3.3%; *n* = 5; *P* > .05). Furthermore, the EP₁-specific receptor antagonist SC-51089 did not block the enhanced eosinophil survival induced by PGE₂ or 11-deoxy PGE₁.

Cyclic AMP inhibits spontaneous eosinophil apoptosis

These results suggested that the antiapoptotic effect of PGE₂ on eosinophils is mediated by way of the EP₂ receptor subtype, which stimulates cAMP generation by

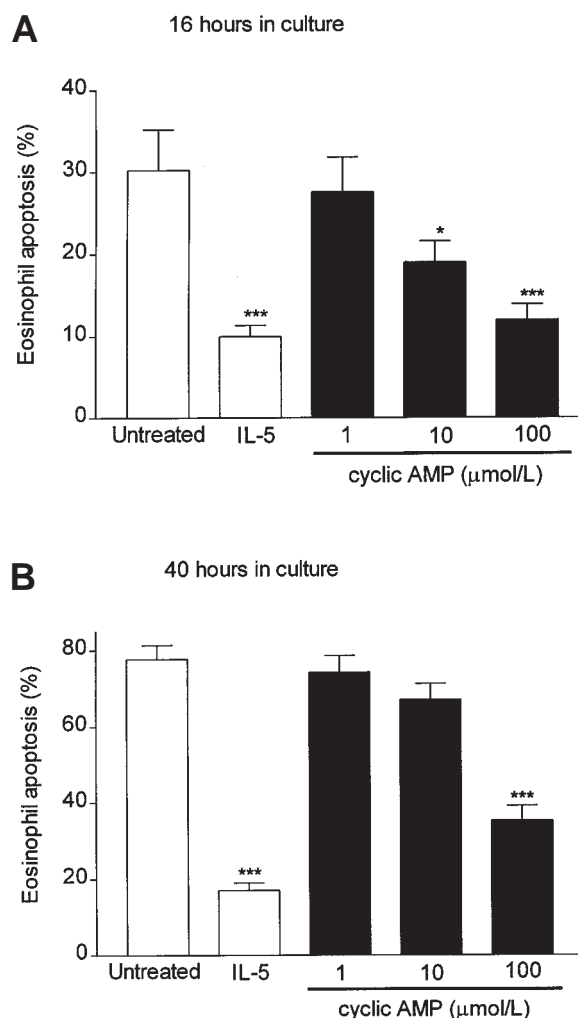


FIG 3. The effect of db-cAMP on eosinophil apoptosis. Purified eosinophils (*n* = 8) were cultured for 16 (**A**) or 40 (**B**) hours in the presence of db-cAMP (1, 10, and 100 μmol/L); apoptosis was assessed by flow cytometry as the percentage of cells binding annexin V. For comparison, data for untreated and IL-5 (100 U/mL) treated cultures is shown (**P* < .05, ****P* < .001, compared with untreated, ANOVA).

activation of a G_s protein and adenylate cyclase.^{18,33} Hence we determined the effect of a stable cyclic AMP analog (db-cAMP) on eosinophil apoptosis. Spontaneous eosinophil apoptosis at 16 hours was significantly inhibited by 10 and 100 μmol/L db-cAMP (*n* = 8; *P* < .05 and < .001, respectively; Fig 3, A). The efficacy of 100 μmol/L db-cAMP at this time was comparable to that of 100 U/mL IL-5. Eosinophil apoptosis after 40 hours of culture was also decreased by db-cAMP, but only at the 100 μmol/L concentration (*n* = 8; *P* < .001; Fig 3, B). As with PGE₂, there was a trend toward increased inhibition of apoptosis with increasing db-cAMP concentration, but again differences for the 3 concentrations (1, 10, and 100 μmol/L) did not reach significance. These concentrations of db-cAMP have also been shown to inhibit neutrophil apoptosis.³⁴

TNF- α delays spontaneous eosinophil apoptosis

In the presence of TNF- α (50 or 500 U/mL) eosinophil apoptosis was reduced at 16 hours ($n = 8$; $P < .05$; Fig 4, A), but by 40 hours this effect was less evident (Fig 4, B), suggesting only a transient role for this cytokine in modulating eosinophil survival. However, similar concentrations of TNF- α were previously shown to inhibit neutrophil apoptosis.³⁵

Spontaneous eosinophil apoptosis is unaffected by NO donor mediators

The effects of NO are mediated by activation of guanylate cyclase and increased production of the second messenger cGMP.³⁶ Cultured eosinophils were exposed to the NO donor SNAP (1 and 100 μ mol/L) and DETANONOate (1 mmol/L), a stable cGMP analog (db-cGMP, 1, 10, and 100 μ mol/L), and the guanylate cyclase inhibitor MB (50 μ mol/L). None of these mediators significantly altered the percentage of apoptotic eosinophils at either 16 or 40 hours ($n = 7$; $P > .05$; Table I). However, these concentrations of SNAP, DETANONOate, and db-cGMP have previously been shown to induce biologic effects including inhibition of Fas-mediated apoptosis in eosinophils.¹⁵

The effects of PGE₂, db-cAMP, and TNF- α on IL-5-mediated eosinophil survival

Eosinophils were cultured with combinations of these mediators for 40 hours. PGE₂ or db-cAMP in combination with IL-5 did not alter the percentage of apoptotic cells compared with that observed with IL-5 alone. However, apoptosis of eosinophils cultured with IL-5 in combination with TNF- α was significantly greater compared with cells cultured with IL-5 alone ($21.3\% \pm 2.3\%$ vs $13.9\% \pm 1.9\%$; $n = 5$; $P < .01$).

The effects of protein kinase A inhibition on eosinophil apoptosis

The possible mechanism of action of PGE₂ and db-cAMP was investigated by use of the specific protein kinase A antagonist, H-89. At a concentration of 1 μ mol/L, H-89 had no effect on spontaneous eosinophil apoptosis nor did it modulate the effects of IL-5, PGE₂, db-cAMP, or TNF- α . However, at a concentration of 10 μ mol/L, H-89 significantly inhibited spontaneous eosinophil apoptosis ($55.9\% \pm 5.4\%$ vs $90.1\% \pm 3.3\%$; $n = 4$; $P < .001$). At this concentration, H-89 also substantially enhanced the apoptosis of eosinophils cultured with IL-5 ($41.1\% \pm 5.2\%$ vs $13.9\% \pm 1.9\%$; $n = 4$; $P < .001$) but did not significantly alter the effects of PGE₂ or db-cAMP.

Inhibition of eosinophil apoptosis by PGE₂, cAMP, and TNF- α is reduced by anti-Fas

Addition of anti-Fas mAb (400 ng/mL) at the start of 16-hour cultures diminished the antiapoptotic effects of PGE₂ (10 nmol/L), db-cAMP (100 μ mol/L), and TNF- α

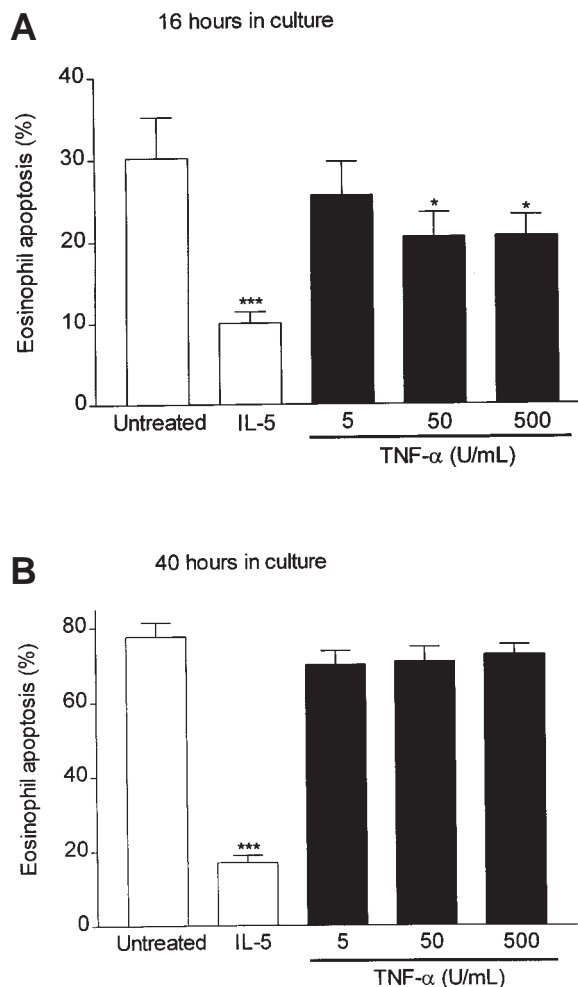


FIG 4. The effect of TNF- α on eosinophil apoptosis. Purified eosinophils ($n = 8$) were cultured for 16 (A) or 40 (B) hours in the presence of TNF- α (5, 50, and 500 U/mL); apoptosis was assessed by flow cytometry as the percentage of cells binding annexin V. For comparison, data for untreated and IL-5 (100 U/mL) treated cultures is shown (* $P < .05$, *** $P < .001$, compared with untreated, ANOVA).

(500 U/mL) relative to untreated eosinophils, although PGE₂ and db-cAMP were still significantly inhibitory ($n = 7$; $P < .05$ and $< .01$, respectively; Table II). However, at 40 hours PGE₂ did not significantly inhibit the apoptosis of eosinophils treated with anti-Fas mAb ($n = 8$; $P > .05$), and although db-cAMP inhibited apoptosis in the presence of anti-Fas mAb ($P < .001$), the effect was significantly less than that found in the absence of anti-Fas mAb ($P < .001$; Table II).

Comparison of annexin V binding and DNA fragmentation in apoptotic eosinophils

Analysis of DNA fragmentation by 3'-end labeling with ³²P revealed reduced eosinophil apoptosis at 0 hours and after IL-5 treatment, compared with untreated eosinophils at 16 and 40 hours (Fig 5). However the magnitude of these effects was less than that assessed by

TABLE I. Spontaneous eosinophil apoptosis after treatment with NO donor mediators*

Treatment	Time in culture	
	16 h	40 h
None	29.9 ± 5.7	78.0 ± 4.2
DETANONOate (1 mmol/L)	31.5 ± 4.0	81.9 ± 3.9
SNAP (1 µmol/L)	27.2 ± 4.5	77.1 ± 3.7
SNAP (100 µmol/L)	22.7 ± 2.9	75.6 ± 3.1
SNAP (100 µmol/L) + MB (50 µmol/L)	24.0 ± 3.1	79.4 ± 3.3
db-cGMP (100 µmol/L)	23.7 ± 3.9	70.2 ± 4.3

*Purified eosinophils were cultured for 16 or 40 hours in the presence of DETANONOate, SNAP (with and without MB), and db-cGMP. The percentage of apoptotic cells was determined by flow cytometric analysis of annexin V binding and compared statistically with untreated eosinophil cultures (n = 7; ANOVA).

annexin V binding (Fig 1). Quantitative differences between untreated eosinophils and those treated with PGE₂ (10 nmol/L), db-cAMP (100 µmol/L), or TNF-α (500 U/mL) were even less discernible at both 40 (Fig 5) and 16 hours (data not shown). Both annexin V binding and PI staining revealed similar trends with respect to the effects of the various mediators on eosinophil apoptosis at 16 and 40 hours, and there were no significant differences in the values obtained by these 2 methods (n = 7 and 8, respectively; *P* > .05; Table III). However, with PI it was not possible to discern statistically significant inhibition of eosinophil apoptosis with IL-5 or db-cAMP at 16 hours and with PGE₂ or TNF-α at either 16 or 40 hours (*P* > .05; Table III).

DISCUSSION

Eosinophils play a significant role in the pathogenesis of asthma, and changes in numbers and degree of activation of lung eosinophils probably influence disease severity. As such, an increase in viable eosinophil numbers through inhibition of apoptosis is likely to enhance the potential for further airway inflammation. We have confirmed earlier reports of a potent inhibition of eosinophil apoptosis in the presence of IL-5, a cytokine important in the differentiation and activation of eosinophils and strongly associated with the inflammatory response in asthma.³⁷ Treatment of eosinophils with TNF-α, another important proinflammatory cytokine also resulted in delayed apoptosis. In support of this, TNF-α has been demonstrated to inhibit Fas-induced acceleration of neutrophil apoptosis³⁸ and to play a role in the enhancement of eosinophil survival by mast cells *in vitro*.³⁹

Our experiments demonstrated that PGE₂ suppresses eosinophil apoptosis and that this is likely to be mediated by interaction with the EP₂ receptor subtype on eosinophils. We also demonstrated that db-cAMP inhibits spontaneous eosinophil apoptosis, which is consistent with 1 previous study⁴⁰ assessing the interaction of cAMP and GM-CSF. These data together with previous work that shows that EP₂ receptor-specific suppression of thymocyte apoptosis by

PGE₂ is mediated by cAMP⁴¹ and that other cAMP-increasing agents, such as rolipram, inhibit eosinophil apoptosis¹⁴ support the concept that cAMP mediates the effects of PGE₂ on eosinophils. The principal signal transduced by cAMP is activation of protein kinase A and phosphorylation of protein substrates, such as Raf-1.⁴² However, the specific protein kinase A antagonist H-89 at a concentration of 1 µmol/L had no effect on spontaneous apoptosis or on the enhanced survival mediated by PGE₂ and cAMP. These results confirm the observations of Hallsworth et al⁴⁰ and suggest that PGE₂ and cAMP mediated enhancement of eosinophil survival is protein kinase A independent.

At a concentration of 10 µmol/L, H-89 not only significantly inhibited spontaneous eosinophil apoptosis but also inhibited the antiapoptotic effect of IL-5. This concentration of H-89, although higher than that used in a previous study of eosinophil apoptosis,⁴⁰ is lower than the 25 to 100 µmol/L concentrations used in previous studies on neutrophils, which showed that H-89 did not affect spontaneous apoptosis.^{34,43} Therefore one interpretation of our results is that in eosinophils, unlike neutrophils,⁴³ basal protein kinase A activity contributes to the proapoptotic pathway and that this can be inhibited by 10 µmol/L but not 1 µmol/L H-89. However, such involvement of protein kinase A is not supported by our data that shows that the enhancement of eosinophil survival by db-cAMP or PGE₂ was not affected even by 10 µmol/L H-89. This contrasts with neutrophils, where inhibition of apoptosis by cAMP is blocked by H-89, suggesting a protein kinase A dependent mechanism.^{34,43} Furthermore, the mechanism of inhibition of IL-5-induced survival by 10 µmol/L H-89 is unclear because protein kinase A is not known to have a role in transducing the IL-5 signal.^{44,45} It is possible that at this concentration H-89 may be having a nonspecific effect on eosinophils.

In contrast to previous studies^{17,40} that showed inhibition of IL-5- and GM-CSF-induced eosinophil survival by the PGE₂ analog, misoprostol, and db-cAMP, respectively, we did not observe any inhibition of IL-5-induced survival by PGE₂ or db-cAMP, although in both previous studies the effects on eosinophil survival were assessed considerably later (4 and 7 days) than in our study. Because the IL-5 survival signal may be transduced by a Ras-Raf-1-MAP kinase pathway⁴⁶ and because this pathway could potentially be inhibited by protein kinase A-dependent phosphorylation of Raf-1,⁴⁷ the lack of any effect of PGE₂ or cAMP on IL-5-induced survival is further evidence that PGE₂ and cAMP are not acting through protein kinase A.

Although inhibition of eosinophil apoptosis is in keeping with the proinflammatory roles of IL-5 and TNF-α, our observation that eosinophil apoptosis was also inhibited by PGE₂ is in apparent conflict with other reports that suggest that PGE₂ has anti-inflammatory effects on eosinophils.¹⁷⁻¹⁹ However, the inhibition of spontaneous eosinophil apoptosis by physiologic concentrations of PGE₂, as identified for the first time in our study, raises

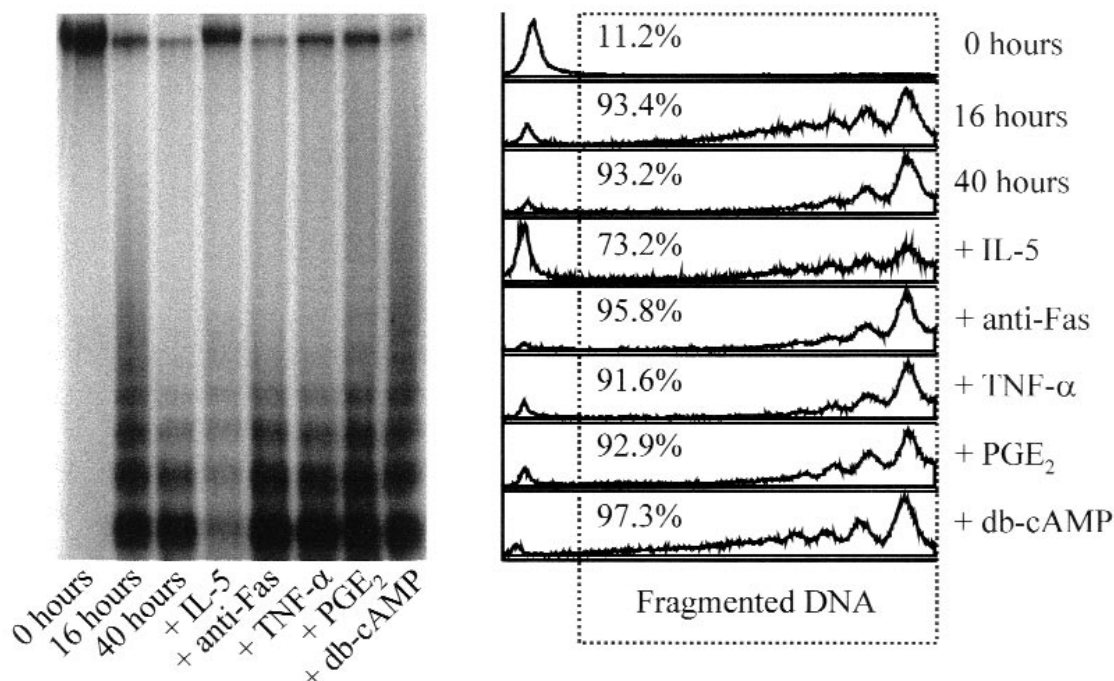


FIG 5. Quantitative assessment of eosinophil DNA fragmentation as a measure of apoptosis. DNA from eosinophils cultured for 40 hours with or without IL-5 (100 U/mL), anti-Fas (400 ng/mL), TNF- α (500 U/mL), PGE₂ (10 nmol/L), or db-cAMP (100 μ mol/L) was purified, ³²P 3'-end labeled, and electrophoresed on an agarose gel. Results from untreated eosinophils at 0 and 16 hours are also shown. **A**, Phosphorimager scan of the agarose gel shows DNA fragmentation. **B**, The relative radioactive signal from individual bands was quantified with ImageQuant analysis software. Apoptosis was calculated as the percentage of the total radioactive signal that was contributed by fragmented DNA.

TABLE II. Anti-Fas reduces the inhibitory effects of PGE₂, db-cAMP, and TNF- α on eosinophil apoptosis

Treatment	16 h in culture		40 h in culture	
	(-) Anti-Fas	(+) Anti-Fas	(-) Anti-Fas	(+) Anti-Fas
None	29.3 \pm 5.7	33.3 \pm 4.3	77.6 \pm 3.7	76.7 \pm 2.8
PGE ₂ (10 nmol/L)	16.3 \pm 2.3*	22.5 \pm 2.9†	54.6 \pm 5.7‡	66.0 \pm 2.8
cAMP (100 μ mol/L)	12.7 \pm 2.0‡	20.7 \pm 3.4*	35.2 \pm 3.9‡	56.7 \pm 4.4‡§
TNF- α (500 U/mL)	20.1 \pm 2.6†	27.7 \pm 3.9	72.5 \pm 2.7	74.4 \pm 3.5

Purified eosinophils were cultured for 16 (n = 7) or 40 hours (n = 8) with or without anti-Fas mAb (CH-11, 400 ng/mL) and the effects of PGE₂, db-cAMP, or TNF- α on apoptosis were assessed by flow cytometry as the percentage of cells binding annexin V. Percentage apoptosis for each treatment was compared statistically with untreated eosinophil cultures (*P < .01, †P < .05, ‡P < .001; ANOVA). Anti-Fas-containing cultures were also compared with similarly treated cultures not containing anti-Fas (§P < .001; ANOVA).

TABLE III. Comparison of eosinophil apoptosis as assessed by annexin V or PI

Treatment	16 h in culture		40 h in culture	
	Annexin V	PI	Annexin V	PI
None	29.3 \pm 5.7	17.7 \pm 3.0	77.6 \pm 3.7	63.9 \pm 7.4
IL-5 (100 U/mL)	10.3 \pm 1.5*	9.2 \pm 2.1	17.0 \pm 2.0*	17.1 \pm 3.4*
α -Fas (400 ng/mL)	33.3 \pm 4.3	20.0 \pm 4.1	76.7 \pm 2.8	67.2 \pm 6.9
PGE ₂ (10 nmol/L)	16.3 \pm 2.3†	12.5 \pm 2.6	54.6 \pm 5.7*	46.7 \pm 6.4
cAMP (100 μ mol/L)	12.0 \pm 1.9*	11.0 \pm 2.0	35.2 \pm 3.9*	27.8 \pm 4.6*
TNF- α (500 U/mL)	20.6 \pm 2.6‡	14.0 \pm 3.8	72.5 \pm 2.7	50.5 \pm 6.5

Purified eosinophils were cultured for 16 (n = 7) or 40 hours (n = 8) in the presence of IL-5, anti-Fas mAb (CH-11), PGE₂, db-cAMP, or TNF- α . The percentage of apoptotic cells was determined by flow cytometric analysis of both annexin V binding and PI staining of DNA. Percentage apoptosis for each treatment was compared statistically with untreated eosinophil cultures assayed by the same method (*P < .001, †P < .01, ‡P < .05, ANOVA). For each treatment, values obtained by the 2 methods were not significantly different (P > .05; ANOVA).

questions regarding the role of this quantitatively important epithelial-derived mediator in the airways. Endogenous PGE₂ has been thought to play a mainly bronchoprotective and anti-inflammatory role in the airways,⁴⁸ but whether the enhancement of eosinophil survival *in vivo* by PGE₂ results in increased activation and release of proinflammatory mediators requires further investigation.

Increased NO production is associated with airway inflammation, which would appear consistent with studies that demonstrate inhibition of spontaneous eosinophil apoptosis by the NO donor mediators, azide and hydroxylamine, and stable analogs of the NO second messenger cGMP.⁴⁹ In contrast, our studies revealed that neither db-cGMP nor the NO donors SNAP and DETANONOate significantly altered rates of spontaneous eosinophil apoptosis. Supporting this, Hebestreit et al¹⁵ also found that NO donor mediators had little effect on spontaneous eosinophil apoptosis but did inhibit acceleration of apoptosis induced by agonistic anti-Fas mAb. Thus the effect of NO on eosinophil survival may be dependent on the preexisting cytokine/mediator milieu.

Several investigators have reported significantly enhanced eosinophil apoptosis in response to treatment with the anti-Fas mAb, CH-11.¹²⁻¹⁵ In our study, apoptosis was not enhanced in eosinophils treated with anti-Fas for 16 hours. The response to anti-Fas depends on the concentration of antibody used¹³ and may also depend on experimental conditions such as the density at which eosinophils are cultured⁵⁰ and the pH of the medium. One study that used RPMI1640 medium buffered with HEPES found little enhancement of apoptosis in eosinophils treated with 100 ng/mL anti-Fas.³⁸ In our study anti-Fas was used at an intermediate concentration (400 ng/mL) relative to those used in previous studies, and although anti-Fas did not enhance spontaneous eosinophil apoptosis, it did significantly diminish the antiapoptotic effects of PGE₂ and db-cAMP. Therefore it is possible that spontaneous eosinophil apoptosis in our culture system was already maximally induced. As such, an additional proapoptotic signal in the form of anti-Fas may have been redundant. However, our results that show that anti-Fas reduces the antiapoptotic effect of PGE₂ suggest that the Fas pathway may be important in counteracting the delay in apoptosis caused by some cytokines or mediators.

The method used to detect apoptosis represents a further source of variation between different investigations. Although we were able to qualitatively demonstrate apoptosis by using 3 different techniques, quantitative analysis of DNA fragmentation did not identify the inhibitory effects of PGE₂, db-cAMP, and TNF- α on eosinophil apoptosis that were quite clearly demonstrated by flow cytometric analysis of annexin V binding. DNA fragmentation is a very specific qualitative indicator of apoptosis, but we observed that, when used quantitatively, the technique showed little sensitivity in the earlier stages of apoptosis or to changes in the extent of apoptosis. Thus quantitative differences in the levels of

spontaneous eosinophil apoptosis at 16 or 40 hours (Fig 1) are not readily apparent from the DNA fragmentation analysis (Fig 5). ³²P end labeling of DNA fragments permits detection of small amounts of fragmented DNA with much greater sensitivity than is possible on ethidium bromide-stained gels. However, because of a disproportionate increase in the number of 3'-ends when DNA is cleaved into smaller fragments, quantitation based on this technique may be biased toward major DNA fragmentation that occurs relatively late.⁵¹ Therefore more subtle quantitative variations in apoptosis, particularly early in the process, may be obscured. In contrast, the annexin V method appeared to be sensitive to concentration-dependent changes in the level of apoptosis, as observed with PGE₂ and db-cAMP.

We also compared annexin V binding with another flow cytometric technique based on the staining of DNA with PI and found support for the concept that nuclear events, including DNA fragmentation, are delayed relative to externalization of phosphatidylserine and other morphologic events.^{30,51} The externalization of phosphatidylserine early in the apoptotic process probably accounts for the greater sensitivity of annexin V in detecting apoptosis relative to PI assessment of DNA fragmentation. This latter method also has the disadvantage of failing to distinguish between necrosis and apoptosis. However, by differential annexin V/PI analysis, levels of necrosis were low (<5%) at all stages of eosinophil culture, and therefore this is unlikely to account for the different results obtained with the 2 techniques. Furthermore, it seems likely that annexin V binding measures a more functionally relevant end point because externalized phosphatidylserine appears to be a marker for macrophage recognition and phagocytosis of apoptotic cells.³⁰ It would appear from our study that flow cytometric analysis of annexin V binding is a more accurate and sensitive technique for identifying and quantifying changes in eosinophil apoptosis compared with techniques based on the analysis of DNA fragmentation. In addition, DNA-independent assays, such as annexin V binding, measure the actual proportion of a cell population undergoing apoptosis under given conditions.

In conclusion, this study of *in vitro* eosinophil apoptosis has demonstrated that inflammatory mediators that are likely to be released by the airway epithelium in asthma can influence eosinophil survival. The factors influencing eosinophil apoptosis at sites of inflammation in allergic diseases are complex,⁵² and the relative importance of the effects of individual mediators such as PGE₂ are difficult to assess. Nevertheless, PGE₂ is quantitatively one of the most important mediators produced by the bronchial epithelium,¹⁶ and therefore the influence of PGE₂ on eosinophil survival, particularly in the airways, is likely to be quite important. The observation that both PGE₂ and TNF- α inhibit eosinophil apoptosis despite having opposite effects on eosinophil activation suggests fundamental questions of whether activation and degranulation of eosinophils commits them to apoptosis or survival or, conversely, whether eosinophils committed to apoptosis

remain responsive to activation signals; these questions need to be addressed.

We thank Mr Jason Lenzo, Professor Arun Dharmarajan, and Dr Darryl Knight for their assistance.

REFERENCES

1. Reed CE. New therapeutic approaches in asthma. *J Allergy Clin Immunol* 1986;77:537-43.
2. Bousquet J, Chanez P, Lacoste JY, Barneon G, Ghavanian N, Enander I, et al. Eosinophilic inflammation in asthma. *N Engl J Med* 1990;323:1033-9.
3. Baigelman W, Chodosh S, Pizzuto D, Cupples LA. Sputum and blood eosinophils during corticosteroid treatment of acute exacerbations of asthma. *Am J Med* 1983;75:929-36.
4. Diaz P, Galleguillos FR, Gonzalez MC, Pantin CF, Kay AB. Bronchoalveolar lavage in asthma: the effect of disodium cromoglycate (cromolyn) on leukocyte counts, immunoglobulins, and complement. *J Allergy Clin Immunol* 1984;74:41-8.
5. Taylor KA, Luksha PR. Peripheral blood eosinophil counts and bronchial hyperresponsiveness. *Thorax* 1987;42:452-6.
6. Flavahan NA, Slifman NR, Gleich GJ, Vanhoutte PM. Human eosinophil major basic protein causes hyperreactivity of respiratory smooth muscle: role of the epithelium. *Am Rev Respir Dis* 1988;138:685-8.
7. Hastie AT, Loegering DA, Gleich GJ, Kueppers F. The effect of purified human eosinophil major basic protein on mammalian ciliary activity. *Am Rev Respir Dis* 1987;135:848-53.
8. Tai PC, Sun L, Spry CJF. 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.
9. 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.
10. Meagher LC, Cousin JM, Seckl JR, Haslett C. Opposing effects of glucocorticoids on the rate of apoptosis in neutrophilic and eosinophilic granulocytes. *J Immunol* 1996;156:4422-8.
11. Nagata S. Apoptosis by death factor. *Cell* 1997;88:355-65.
12. Matsumoto K, Schleimer RP, Saito H, Iikura Y, Bochner BS. Induction of apoptosis in human eosinophils by anti-Fas antibody treatment in vitro. *Blood* 1995;86:1437-43.
13. Druilhe A, Cai Z, Haile S, Chouaib S, Pretolani M. Fas-mediated apoptosis in cultured human eosinophils. *Blood* 1996;87:2822-30.
14. Yasui K, Hu B, Nakazawa T, Agetatsu K, Komiyama A. Theophylline accelerates human granulocyte apoptosis not via phosphodiesterase inhibition. *J Clin Invest* 1997;100:1677-84.
15. Hebestreit H, Dibbert B, Balatti I, Braun D, Schapowal A, Blaser K, et al. Disruption of Fas receptor signaling by nitric oxide in eosinophils. *J Exp Med* 1998;187:415-25.
16. Knight DA, Stewart GA, Thompson PJ. The respiratory epithelium and airway smooth muscle homeostasis. *Clin Exp Allergy* 1994;24:698-706.
17. Alam R, Dejamatt A, Stafford S, Forsythe PA, Kumar D, Grant JA. Selective inhibition of the cutaneous late but not immediate allergic response to antigens by misoprostol, a PGE analog: results of a double-blind, placebo-controlled randomized study. *Am Rev Respir Dis* 1993;148:1066-70.
18. Teixeira MM, al-Rashed S, Rossi AG, Hellewell PG. Characterization of the prostanoid receptors mediating inhibition of PAF-induced aggregation of guinea-pig eosinophils. *Br J Pharmacol* 1997;121:77-82.
19. Kita H, Abu-Ghazaleh RI, Gleich GJ, Abraham RT. Regulation of Ig-induced eosinophil degranulation by adenosine 3'-cyclic monophosphate. *J Immunol* 1991;146:2712-8.
20. Gaston B, Drazen JM, Loscalzo J, Stamler JS. The biology of nitrogen oxides in the airways. *Am Rev Respir Dis* 1994;149:538-51.
21. Watkins DN, Peroni DJ, Basclain KA, Garlepp MJ, Thompson PJ. Expression and activity of nitric oxide synthases in human airway epithelium. *Am J Respir Cell Mol Biol* 1997;16:629-39.
22. Barnes PJ, Liew FY. Nitric oxide and asthmatic inflammation. *Immunol Today* 1995;16:128-30.
23. del Pozo V, de Arruda-Chaves E, de Andres B, Cardaba B, Lopez-Farre A, Gallardo S, et al. Eosinophils transcribe and translate messenger RNA for inducible nitric oxide synthase. *J Immunol* 1997;158:859-64.
24. Ferreira HH, Medeiros MV, Lima CS, Flores CA, Sannomiya P, Autunes E, et al. Inhibition of eosinophil chemotaxis by chronic blockade of nitric oxide biosynthesis. *Eur J Pharmacol* 1996;310:201-7.
25. Arock M, Le Goff L, Becherel PA, Dugas B, Debre P, Mossalayi MD. Involvement of Fc epsilon RII/CD23 and L-arginine dependent pathway in IgE-mediated activation of human eosinophils. *Biochem Biophys Res Commun* 1994;30:265-71.
26. Silberstein DS, David JR. Tumor necrosis factor enhances eosinophil toxicity to *Schistosoma mansoni* larvae. *Proc Natl Acad Sci USA* 1986;83:1055-9.
27. Nagata M, Fukuda T, Numao T, Makino S, Dohi Y. Tumor necrosis factor alpha induces migration of human eosinophils. *Arerugi* 1993;42:549-55.
28. Lukacs NW, Strieter RM, Chensue SW, Widmer M, Kunkel SL. TNF-alpha mediates recruitment of neutrophils and eosinophils during airway inflammation. *J Immunol* 1995;154:5411-7.
29. 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.
30. Martin SJ, Reutelingsperger CPM, McGahon AJ, Rader JA, Schie RCAA, LaFace DM, et al. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *J Exp Med* 1995;182:1545-56.
31. Misso NLA, Peroni DJ, Watkins DN, Stewart GA, Thompson PJ. Glutathione peroxidase activity and mRNA expression in eosinophils and neutrophils of asthmatic and non-asthmatic subjects. *J Leukocyte Biol* 1998;63:124-30.
32. Tilly JL, Hsueh AJ. Microscale autoradiographic method for the qualitative and quantitative analysis of apoptotic DNA fragmentation. *J Cell Physiol* 1993;154:519-26.
33. Coleman RA, Smith WL, Narumiya S. Classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. *Pharmacol Rev* 1994;46:205-29.
34. Rossi AG, Cousin JM, Dransfield I, Lawson MF, Chilvers ER, Haslett C. Agents that elevate cAMP inhibit human neutrophil apoptosis. *Biochem Biophys Res Commun* 1995;217:892-9.
35. Colotta F, Re F, Polentarutti N, Sozzani S, Mantovani A. Modulation of granulocyte survival and programmed cell death by cytokines and bacterial products. *Blood* 1992;80:2012-20.
36. Schmidt HH, Lohmann SM, Walter U. The nitric oxide and cGMP signal transduction system: regulation and mechanism of action. *Biochim Biophys Acta* 1993;1178:153-75.
37. Sanderson CJ. Interleukin-5, eosinophils, and disease. *Blood* 1992;79:3101-19.
38. Liles WC, Kiener PA, Ledbetter JA, Aruffo A, Klebanoff SJ. Differential expression of Fas (CD95) and fas ligand on normal human phagocytes: implications for the regulation of apoptosis in neutrophils. *J Exp Med* 1996;184:429-40.
39. Levi-Schaffer F, Temkin V, Malamud V, Feld S, Zilberman Y. Mast cells enhance eosinophil survival in vitro: role of TNF-alpha and granulocyte-macrophage colony-stimulating factor. *J Immunol* 1998;160:5554-62.
40. Hallsworth MP, Gienbycz 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.
41. Goetzel EJ, An SZ, Zeng L. Specific suppression by prostaglandin E2 of activation-induced apoptosis of human CD4+CD8+ T lymphoblasts. *J Immunol* 1995;154:1041-7.
42. Mischak H, Seitz T, Janosch P, Eulitz M, Steen H, Schellerer M, et al. Negative regulation of Raf-1 by phosphorylation of serine 621. *Mol Cell Biol* 1996;16:5409-18.
43. Parvathani LK, Buescher ES, Chacon-Cruz E, Beebe SJ. Type I cAMP-dependent protein kinase delays apoptosis in human neutrophils at a site upstream of caspase-3. *J Biol Chem* 1998;273:6736-43.
44. Pazdrak K, Olszewska-Pazdrak B, Stafford S, Garofalo RP, Alam R. Lyn, Jak2, and Raf-1 kinases are critical for the antiapoptotic effect of interleukin-5, whereas only Raf-1 kinase is essential for eosinophil activation and degranulation. *J Exp Med* 1998;188:421-9.
45. Hiraguri M, Miike S, Sano H, Kurasawa K, Saito Y, Iwamoto I. Granulocyte-macrophage colony-stimulating factor and IL-5 activate mitogen-activated protein kinase through Jak2 kinase and phosphatidylinositol 3-kinase in human eosinophils. *J Allergy Clin Immunol* 1997;100:S45-S51.

46. Pazdrak K, Schreiber D, Forsythe P, Justement L, Alam R. The intracellular signal transduction mechanism of interleukin 5 in eosinophils: the involvement of Lyn tyrosine kinase and the Ras-Raf-1-MEK-microtubule-associated protein kinase pathway. *J Exp Med* 1995;181:1827-34.
47. Hafner S, Adler HS, Mischak H, Janosch P, Heidecker G, Wolfman A, et al. Mechanism of inhibition of Raf-1 by protein kinase A. *Mol Cell Biol* 1994;14:6696-703.
48. Pavord ID, Tattersfield AE. Bronchoprotective role for endogenous prostaglandin E₂. *Lancet* 1994;344:436-8.
49. Beauvais F, Michel L, Dubertret L. The nitric oxide donors, azide and hydroxylamine, inhibit the programmed cell death of cytokine-deprived human eosinophils. *FEBS Lett* 1995;361:229-32.
50. Hannah S, Nadra I, Dransfield I, Pryde JG, Rossi AG, Haslett C. Constitutive neutrophil apoptosis in culture is modulated by cell density independently of β 2 integrin-mediated adhesion. *FEBS Lett* 1998;421:141-6.
51. Collins JA, Schandl CA, Young KK, Vesely J, Willingham MC. Major DNA fragmentation is a late event in apoptosis. *J Histochem Cytochem* 1997;45:923-34.
52. Simon H-U. Eosinophil apoptosis in allergic diseases: an emerging new issue. *Clin Exp Allergy* 1998;28:1321-4.

Correction

The following correction applies to an abstract that appeared in volume 103, number 1, part 2, p S253, 1999, of the Journal.

970 Comparative Pharmacokinetic and Pharmacodynamic Crossover Study of Seldane® Tablets and Allegra™ Capsules. A G Harris,¹ D G Iezzoni,¹ J P Hubbell,² R Chen,¹ P Cornet¹, ¹ITGI, ²SPRI, Kenilworth, NJ

A bioequivalence study compared Seldane® (terfenadine) Tablets (S) with Allegra™ (fexofenadine HCl) Capsules (A). The double-blind, two-way crossover study of 24 healthy volunteers randomly assigned to receive each medication—S (60 mg bid), A (60 mg bid) (7 AM/7 PM) for 7d separated by a 14d washout—assessed PK and histamine-induced skin wheal and flare reactions.

Fasting blood was collected prior to AM dosing on days 1 and 7 and 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 16 hrs post AM dosing, at 7 AM on day 2, and 24 and 36 hrs post final dosing. Skin tests with intracutaneous injected histamine were done on day 0 at 7 AM and 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 13, 16 hrs post testing. AM skin tests following blood draw and after dosing were done at the same time on days 0, 1, 7. On days 2 and 8, AM skin tests were post blood draw.

Plasma concentrations were assayed by LC/MS/MS (LOQ: terfenadine, 0.050 ng/mL; fexofenadine [F], 0.500 ng/mL).

Male subjects (23), 19-44 y, 136-207 lbs, completed; 1 withdrew.

F was significantly less bioavailable and with significantly shorter T_{1/2} after dosing with A versus S.

PK (Mean): F Following:

Day	1		7	
	A	S	A	S
AUC ₍₀₋₁₂₎ (hr•ng/mL)	914 .62	113 3.69*	1114. 23	17 02.32*
C _{max} (ng/mL)	197 .66	210 .24	218.2 3	26 2.55
T _{max} (hr)	2.1 1	1.7 0	2.05	2.3 5
T _{1/2} (hr)	3.3 1	3.6 3	11.36	14. 78*

*P≤0.001 A vs S

Wheal suppression on day 7 was significantly less after A.

Mean % Suppression (0-12 hrs)

Wheal	Day 1		Day 7	
	A	S	A	S
Size (area)	29.17	35.40*	48.76	59.23†
AUC ₀₋₁₂	42.41	47.02‡	51.43	62.09†

*P=0.028 A vs S

†P≤0.001 A vs S

‡P=0.066 A vs S

Conclusion: Fexofenadine is less bioavailable after Allegra™ than after Seldane® and the T_{1/2} is shorter, which is consistent with the lesser inhibition of the histamine-induced skin wheal reaction. Calculated equivalent bioavailability is: Allegra™ (80 mg) = Seldane® (60 mg).