

Delayed eosinophil programmed cell death in vitro: A common feature of inhalant allergy and extrinsic and intrinsic atopic dermatitis

Bettina Wedi, MD, Ulrike Raap, Heitje Lewrick, and Alexander Kapp, MD

Hannover, Germany

The present studies were undertaken to characterize the potential role of eosinophil programmed cell death (PCD) in atopic diseases. Peripheral blood eosinophil PCD was found to be delayed in inhalant allergy ($p < 0.05$) and delayed to an even greater extent in atopic dermatitis (AD) ($p < 0.0001$) when compared to nonatopic subjects. There was no difference in the occurrence of PCD between the extrinsic and the intrinsic type of AD, pointing to a secondary role of specific sensitization. Blockade of eosinophil PCD was not responsible for peripheral blood eosinophilia, because we found no obvious relationship of eosinophil survival to blood eosinophil count. Eosinophil supernatants of more patients with AD than of patients with inhalant allergy dose-dependently inhibited PCD in nonatopic eosinophils, and it was shown that this effect was possibly due to autocrine production of granulocyte-macrophage-colony stimulating factor, probably IL-5. Eosinophil expression of CD95 (Fas antigen) did not change over time in culture and was not modulated by cytokines prolonging eosinophil survival. In contrast, IL-3, IL-5, and granulocyte-macrophage-colony stimulating factor caused an upregulated expression of CD69. However, in AD, CD69 on eosinophils was upregulated without the need of exogenous growth factor or factors over time in culture, thus confirming an autocrine production of proeosinophilic cytokines. In conclusion, our data clearly indicate that eosinophil PCD is markedly delayed in the so-called atopic diseases irrespective of allergen sensitization and suggest that this effect is mediated by the autocrine production of growth factors by eosinophils. (*J Allergy Clin Immunol* 1997;100:536-43.)

Key words: Atopic diseases, atopic dermatitis, eosinophils, programmed cell death, cytokines

Atopic diseases such as allergic rhinitis, asthma, and atopic dermatitis (AD) represent chronic inflammatory disorders characterized by an exacerbating and remitting course with explosive worldwide increase.¹ In general, atopy is defined by specific sensitization to allergens. Nevertheless, at present there is scant evidence that

Abbreviations used

AD:	Atopic dermatitis
ADe:	Extrinsic atopic dermatitis
ADi:	Intrinsic atopic dermatitis
GM-CSF:	Granulocyte-macrophage-colony stimulating factor
IA:	Inhalant allergy
mAb:	Monoclonal antibody
PCD:	Programmed cell death
TGF:	Transforming growth factor

allergy is central to the development of AD.² Despite their atopic constitution, many patients with mild to moderate AD do not have clinically relevant food or aeroallergen sensitivities; therefore, controversy has surrounded the contribution of IgE-mediated hypersensitivity to the pathogenesis of AD classically belonging to the atopic diseases. Several lines of evidence suggest an immunologic pathogenesis of atopic disorders such as altered monocyte and T cell functions resulting in an imbalance of the complex cytokine network.³⁻⁵ On the other hand, recent studies point to a major role of eosinophils as potent effector cells contributing to inflammation by release of chemical mediators and cytotoxic granule proteins such as eosinophil cationic protein, eosinophil peroxidase, major basic protein, and eosinophil-derived neurotoxin/eosinophil protein X.⁶⁻⁹

Patients with exacerbated AD, nasal allergy, and bronchial asthma often show increased numbers of circulating and tissue eosinophils.¹⁰⁻¹³ Serum eosinophil cationic protein levels are significantly increased in patients with atopic disorders, and clinical improvement is associated with its decrease.¹⁴⁻²¹ Peripheral blood eosinophilia in early childhood is associated with subsequent development of atopic disease, which further points to an important role of eosinophil activation.^{22, 23} Because mature circulating eosinophils are terminally differentiated cells, incapable of proliferation and self-renewal, regulation of eosinophil survival may represent a major mechanism through which functional eosinophils accumulate at inflammatory sites.

Previous in vitro studies have demonstrated enhanced survival rates of normal peripheral blood eosinophils mediated by granulocyte-macrophage-colony stimulating factor (GM-CSF), IL-3 and IL-5.²⁴⁻²⁷ Clinically it is

From the Department of Dermatology, Hannover Medical School, Hannover.

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Reprint requests: Bettina Wedi, MD, Department of Dermatology, Hannover Medical School, Ricklinger Str. 5, D-30449 Hannover, Germany.

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obvious that systemic glucocorticosteroids significantly improve the inflammatory process in atopic diseases while causing a significant eosinopenia, which may be mediated by steroid-induced programmed cell death (PCD) in proinflammatory eosinophils.²⁸ Apoptosis facilitates ingestion of intact granulocytes without disorganization of their histotoxic contents. Any delay in the onset of eosinophil apoptosis will therefore postpone eosinophil engulfment, delay resolution, and thus perpetuate chronic inflammation.²⁹

To clarify the role of eosinophil accumulation and PCD in atopic disorders, we compared survival rates and occurrence of apoptosis in peripheral blood eosinophils of nonatopic (NA) subjects, subjects with inhalant allergy (IA), subjects with extrinsic atopic dermatitis (ADe), and subjects with intrinsic atopic dermatitis (ADi).

METHODS

Reagents

Human recombinant GM-CSF (6.85×10^8 U/mg), IL-3 (6.9×10^7 U/mg), and IL-5 (4×10^7 U/mg) were obtained from Genzyme (Cambridge, Mass.). Before testing, all stimulating reagents were diluted in control medium (Hanks balanced salt solution [HBSS] containing 1 mg/ml bovine serum albumin). Dynabeads M-450 sheep anti-mouse IgG were purchased from Dynal A.S. (Oslo, Norway). All monoclonal antibodies were purchased from Immunotech (Marseille, France) except for CD69, which was from Becton Dickinson (San Jose, Calif.). Mouse anti-human GM-CSF monoclonal antibody (mAb) (1 μ g neutralizes 50 units of GM-CSF bioactivity) was obtained from Genzyme.

The Cell Death Detection ELISA^{PLUS} was obtained from Boehringer Mannheim, Mannheim, Germany. Ficoll-Paque solution was purchased from Kabi Pharmacia Diagnostics AB (Uppsala, Sweden). The following enzyme immunoassay kits were purchased from R & D Systems, Minneapolis, Minn.: human TGF- β -1 (range, 31.2 to 200 pg/ml); human IL-5 (Quantikine; range, 7.8 to 500 pg/ml); GM-CSF (Quantikine HS; range, 7.8 to 500 pg/ml); and IL-3 (range, 0.5 to 32 pg/ml). Ribonuclease A and proteinase K were purchased from Merck (Darmstadt, Germany). DNA standard was made by treating lambda DNA with the restriction enzyme *Hind*III (Boehringer Mannheim). RPMI-1640, fetal calf serum, penicillin, and streptomycin were obtained from Life Technologies (Eggenstein, Germany). Cell culture medium was RPMI-1640 with 10% fetal calf serum, 1% penicillin/streptomycin. All other chemicals and reagents were obtained from Sigma Chemical (Deisenhofen, Germany).

Subjects

Peripheral venous blood was obtained after informed consent from 21 subjects with AD (mean age, 39 ± 17 years), 13 subjects with IA during season without a history of AD (mean age, 32 ± 8 years), and 25 NA controls (mean age, 46 ± 21 years) subjected to the Department of Dermatology. Subjects were defined as having IA on the basis of (1) positive personal history of seasonal asthma or allergic rhinitis, (2) detection of specific IgE antibodies (multi-RAST for at least 7 frequent allergens = Sx1), and (3) one or more positive skin prick test responses to a panel of 10 common aeroallergens. In NA controls, atopy was excluded by negative history, negative skin test responses, and

laboratory findings. AD was defined according to the criteria of Hanifin and Rajka.³⁰ Only patients with active AD, defined by the presence of erythema, papulation, and excoriation involving more than 20% of skin surface area were included in the study. On the basis of the presence of specific sensitization, the 21 patients with AD were subdivided into those with ADe ($n = 12$) and those with ADi ($n = 9$).³¹ NA subjects were healthy or had diagnoses such as scabies or drug eruption. None of the subjects received systemic corticosteroids or antihistamines at least 1 week before the venipuncture.

Purification of eosinophils

Peripheral blood eosinophils were separated by Ficoll density gradient centrifugation and immunomagnetic negative selection by using anti-CD16 antibody-coated Dynabeads as described in detail.³²⁻³⁴ Purity was 95% or greater after isolation, as assessed by Kimura staining, and viability was 100%, as determined by trypan blue dye exclusion. Neutrophils were the most frequent contaminating cells, and the preparation contained virtually no monocytes.

Eosinophil survival in vitro

Eosinophils were resuspended in culture medium to 1×10^6 /ml and 1 ml aliquots were added to 24 well flat-bottomed tissue culture plates and stimulated with an equal volume of control medium, GM-CSF, IL-3, or IL-5 (100 U/ml each) at 37°C, 5% CO₂. Cells were removed from each well by gentle pipetting. To confirm that all the cells had been removed, the wells were examined directly with an inverted microscope. Viability of cultured eosinophils was assessed at different time points by trypan blue dye exclusion. Those eosinophils that excluded trypan blue after a 5-minute incubation were considered viable cells. At least 100 eosinophils were counted under the light microscope. Thereafter, cultured eosinophils were pelleted by a 5-minute centrifugation at 1000 rpm at 4°C, and the supernatants were carefully removed and immediately frozen at -80°C.

For coculture experiments, supernatants of cultured eosinophils from patients with AD or IA or NA subjects were added to fresh eosinophils of NA donors (1×10^6 /ml). Moreover, with eosinophils from AD patients, some blocking experiments with antihuman GM-CSF mAbs were performed. In detail, 1×10^6 eosinophils from AD patients were incubated with or without anti-GM-CSF mAbs neutralizing 1000 U/ml GM-CSF to determine whether prolonged eosinophil survival may be abrogated.

Quantitative determination of cell death by ELISA

The Cell Death Detection ELISA^{PLUS}, a commercially available photometric enzyme immunoassay, was used according to the instructions of the manufacturer (Boehringer Mannheim, Germany) for the quantitative in vitro determination of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) in the eosinophil culture supernatants. The test uses mouse mAbs directed against DNA and histones.³⁵ A DNA-histone complex served as the positive control. The eosinophil culture supernatants were diluted 1:50 in RPMI before testing. In previous experiments it was demonstrated that supernatants of freshly isolated eosinophils as well as culture medium alone did not give positive absorbance values (A_{450nm}) compared to background control.

DNA fragmentation assay

Pelleted eosinophils were lysed overnight by the addition of 250 μ l lysis buffer (10 mmol/L ethylenediaminetetraacetic acid [pH 8.0], 50 mmol/L Tris [pH 8.0], 0.5% N-lauroylsarcosine, 0.5

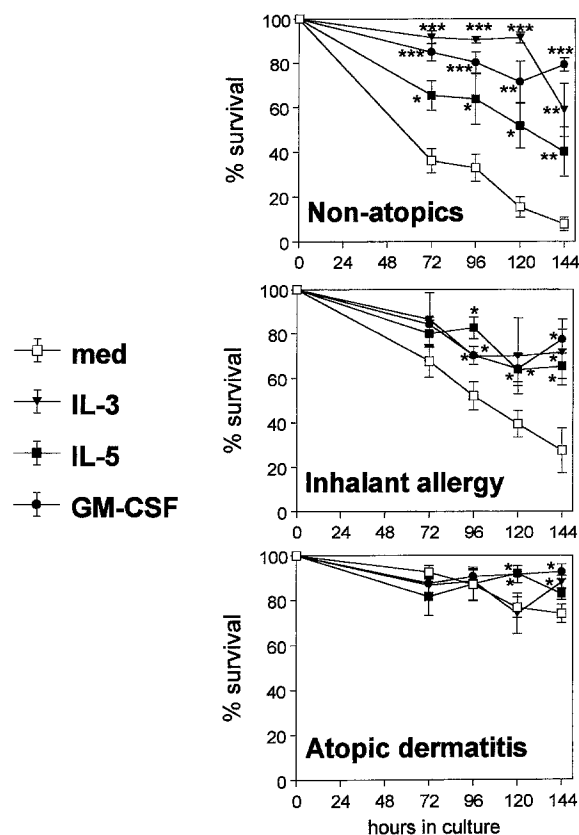


FIG. 1. Comparison of survival over time of eosinophils from subjects with AD, subjects with IA, and NA subjects. Culture was in medium or with cytokines (100 U/ml each). The data are given as mean $\pm$ SEM values of 4 to 11 separate experiments with eosinophils of different patients. Survival of unstimulated eosinophils (*med*) was significantly increased at all time-points studied in IA compared to NA subjects ($p < 0.05$), in AD compared to NA subjects ($p < 0.0001$), and in AD compared to IA ($p < 0.05$) (not indicated). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.0001$ (stimulus versus medium).

mg/ml proteinase K) at 50° C. After digestion with RNase A (250 μ g/ml) for more than 1 hour, 50 μ l 5 mol/L NaCl was added, and the proteins were sedimented by centrifugation at 13,000 g for 15 minutes at 4° C. The supernatants were transferred to fresh tubes and nucleosomal DNA fragments were precipitated by adding a 2.5-fold volume of ice-cold ethanol (100%) and incubating at -80° C overnight. After centrifugation at 13,000 g for 60 minutes, nucleosomal DNA pellets were resuspended in Tris-ethylenediamine tetraacetic acid buffer (pH 8.0) and applied to the slots of a 1.8% agarose gel. Electrophoresis was carried out in 0.5x Tris-borate/ethylenediamine tetraacetic acid buffer containing 0.1 μ g/ml ethidium bromide. After electrophoresis, fragments were visualized by UV light and photographed.

Flow cytometric analysis

To characterize the surface expression pattern of eosinophil Fas antigen over time, single-color immunofluorescence was performed as described previously.³⁶ Eosinophil granulocytes were stained directly with fluorescein isothiocyanate-conjugated mAbs against Fas/CD95 (clone UB2; isotype IgG1) and the isotypic control IgG1 (mouse; clone 679.1Mc7). Moreover

mAbs against the eosinophil marker p24/CD9 (clone ALB6; isotype IgG1), the activation marker anti-Leu-23/CD69 (clone L78; isotype IgG1), and Fc γ RIII/CD16 (clone 3G8; isotype IgG1) were used as controls. In brief, 2×10^5 eosinophils in a volume of 20 μ l were incubated with 2 μ g of the mAb or the equivalent amount of a mouse isotype control antibody (diluted 1:3) for 30 minutes at 4° C and washed twice with ice-cold phosphate-buffered saline (containing 0.1 mmol/L ethylene diamine tetra acetic acid and 0.02% NaN₃). The cells were resuspended in phosphate-buffered saline before analysis with a FACScan (Becton Dickinson, San Jose, Calif.). Nonviable cells were gated out of the window and at least 5000 events were analyzed. Surface antigen expression was determined over time in culture in unstimulated eosinophils as well as in eosinophils stimulated by GM-CSF, IL-3, and IL-5 (100 U/ml each).

Statistical Analysis

All data are presented as means $\pm$ SEM. Depending on the type of distribution the statistical significance between groups was determined by Student's *t* test, Mann-Whitney rank sum test, or Pearson product moment correlation by using a statistical software package (SigmaStat for Windows, Jandel Scientific, Erkrath, Germany).

RESULTS

Eosinophil count and total IgE level

Patients with AD (mean eosinophil count $\pm$ SEM was $16.7 \pm 1.7\%$ in ADe type and $10.4 \pm 2.5\%$ in ADi) and IA (eosinophil count, $17.6 \pm 2.7\%$) did not show increased absolute eosinophil numbers when compared to NA volunteers (eosinophil count, $14.2 \pm 1.6\%$).

In contrast, total IgE levels were significantly higher in patients with ADe (geometric mean, 2989.5 kU/L) as compared to NA subjects (15.5 kU/L) ($p < 0.0001$), patients with IA (286.6 kU/L) ($p < 0.05$), and patients with ADi (22.2 kU/L) ($p < 0.005$).

Effect of culture on eosinophil viability

With increasing time in culture, there was a progressive increase in the proportion of apoptotic eosinophils. However, we found consistent differences between subjects with IA, subjects with AD, and NA volunteers (Fig. 1). Eosinophils from patients with AD and IA showed higher survival rates when cultured with culture medium alone than did eosinophils from NA volunteers. This effect was statistically significant at each time point from 72 hours to 144 hours and most obvious for eosinophils from patients with AD ($p < 0.0001$). Eosinophils from patients with AD even showed increased survival rates compared to eosinophils from subjects with IA ($p < 0.05$). Survival rates did not differ between subjects with ADe or ADi, and there was no relationship between eosinophil survival rates and peripheral blood eosinophil counts or total IgE levels (data not shown). Stimulation with IL-3 resulted in comparable survival rates in all groups studied. However, exposure of AD eosinophils to IL-5 and GM-CSF further prolonged survival at 120 hours and 144 hours ($p < 0.05$ versus NA subjects; $p < 0.05$ versus patients with IA). In NA subjects IL-3, GM-CSF, and IL-5 (in increasing order) increased viability of cultured eosinophils (see Fig. 1). This was

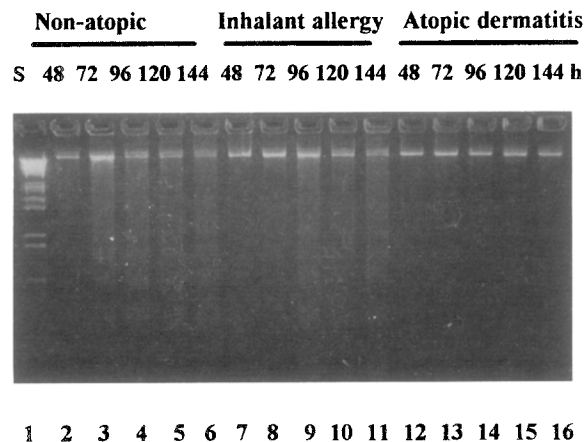


FIG. 2. DNA was extracted from eosinophils of a NA subject (lanes 2 to 6), a patient with IA (lanes 7 to 11), and a patient with AD (lanes 12 to 16) as described in Material and Methods. Lane 1 contains DNA standard made by *Hind*III-digested lambda DNA. After electrophoresis of the DNA in 1.8% agarose and staining with ethidium bromide, the DNA was viewed by UV fluorescence. After 48 to 72 hours, DNA extracted from the NA subject but not DNA from the patient with IA or the patient with AD exhibited a typical ladder consisting of characteristic 200 base pair DNA fragments. In IA, PCD occurred after 96 hours, whereas in AD, PCD was not evident up to 144 hours. One representative example is shown. Hours in culture are indicated above the photograph.

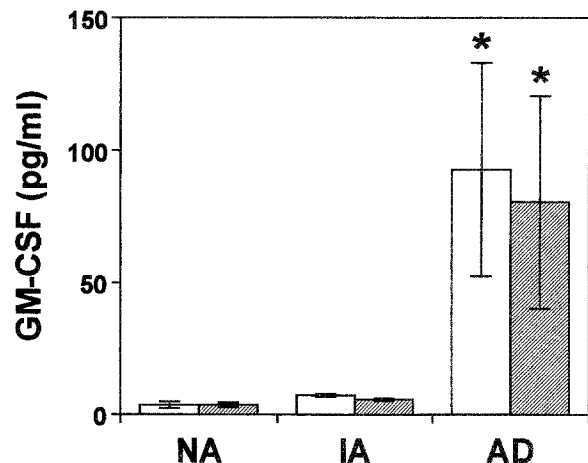
statistically significant at each time point from 72 to 144 hours ($p < 0.05$ up to $p < 0.0001$). In IA, IL-3, IL-5, and GM-CSF were equally able to prolong eosinophil survival ($p < 0.05$), whereas in AD only IL-5 and GM-CSF resulted in increased viability at later time points (120 hours and 144 hours).

Detection of nucleosomal DNA fragments by ELISA

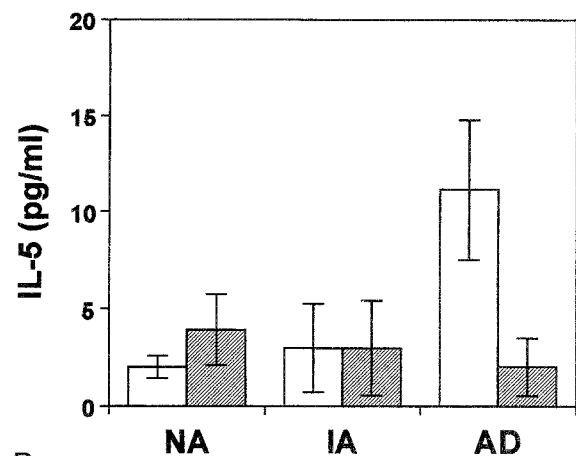
Nucleosomal DNA fragments were detected in supernatants of cultured eosinophils undergoing PCD. There was an inverse correlation between eosinophil survival and the measured absorbance representing the amount of nucleosomal DNA fragments in the supernatants, thus indicating programmed cell death ($r = -0.67$; $p = 0.0058$). With this quantitative assay it was confirmed that IA and AD eosinophils showed delayed PCD in culture, which could be further delayed by addition of GM-CSF, IL-3, and IL-5 (data not shown).

Detection of nucleosomal DNA fragments by DNA ladder assay

After 48 to 72 hours, unstimulated eosinophils from NA subjects exhibited the classical "ladder" pattern of internucleosomal DNA cleavage indicative of endogenous endonuclease activation, the hallmark of apoptosis (Fig. 2). In contrast, PCD of eosinophils from subjects with IA showed a marked delay, with DNA fragmentation occurring at 96 hours. In AD, PCD mostly occurred after 120 hours or later (see Fig. 2). GM-CSF, IL-3, and IL-5 were able to delay PCD in all subjects studied. However, when AD eosinophils were cultured with these



A



B

FIG. 3. Detection of proeosinophilic cytokines released into the eosinophil supernatants of subjects with inhalant allergy (IA) or atopic dermatitis (AD) and nonatopic subjects (NA), as assessed by specific enzyme immunoassays. Data are given as mean $\pm$ SEM values of 4 to 10 different donors in each group. Unfilled bars represent supernatants obtained after 72 hours of culture; filled bars represent supernatants obtained after 144 hours in culture. The minimum detectable concentration for GM-CSF and IL-5 was 7.8 pg/ml. **A**, Detection of GM-CSF. **B**, Detection of IL-5. * $p = 0.006$ versus NA and $p < 0.05$ versus IA.

cytokines, survival was prolonged for more than 240 hours (not shown).

Detection of cytokines in eosinophil supernatants

The amounts of transforming growth factor (TGF)- β -1, IL-3, IL-5, and GM-CSF were measured in the 72 and 144 hour supernatants of unstimulated cultured eosinophils of 95% or higher purity by the double sandwich ELISA procedure. Whereas IL-3 and TGF- β -1 levels were low in all supernatants studied (data not shown), significant amounts of GM-CSF were found to be released from subjects with AD irrespective of

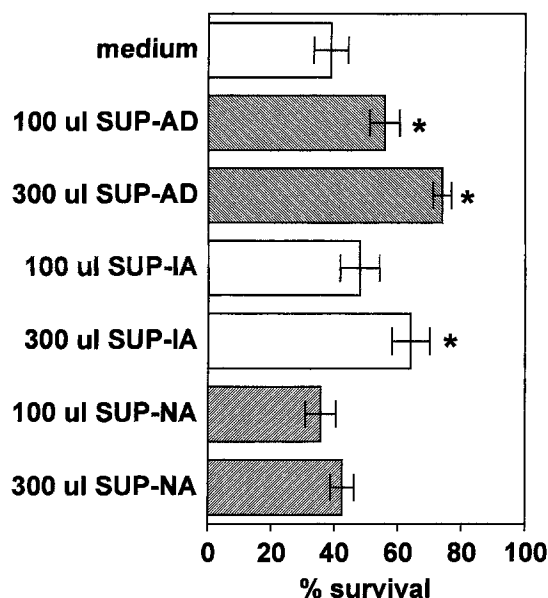


FIG. 4. Eosinophil supernatants derived from subjects with IA or AD, but not from NA subjects, significantly prolong the survival of eosinophils from NA subjects in culture. Eosinophils from NA subjects were incubated for 72 hours with 300 μ l medium or with 100 μ l or 300 μ l of eosinophil supernatants derived from subjects with AD (SUP-AD), IA (SUP-IA), or NA subjects (SUP-NA) after 72 hours in culture.

whether they had ADe or ADi (Fig. 3, A). Additionally, 72-hour supernatants from AD eosinophils contained elevated levels of IL-5 (Fig. 3, B). In contrast, eosinophil supernatants from IA patients and NA subjects did not contain detectable levels of GM-CSF or IL-5. Interestingly, some AD patients produced either IL-5 or GM-CSF, whereas others produced both cytokines (not shown).

Investigation of the inhibition of PCD in eosinophils from NA subjects by supernatants from subjects with AD or IA, clearly showed that supernatants of AD eosinophils produced greater inhibition of PCD in NA eosinophils than did supernatants of IA eosinophils. Supernatants of NA eosinophils were ineffective (Fig. 4).

To verify that the observed effects in AD were, in fact, mediated by an autocrine production of proeosinophilic cytokines, AD eosinophils were incubated with or without neutralizing anti-GM-CSF mAbs. It was shown that the addition of these mAbs significantly reduced eosinophil survival (Fig. 5).

Constitutive and stimulated eosinophil CD95 (Fas antigen) surface expression

CD95 is a functional molecule on human peripheral blood eosinophils that can regulate eosinophil apoptosis *in vitro*.^{37, 38} To assess potential differences of CD95 expression on eosinophils in NA subjects, patients with IA, and patients with AD, a flow cytometric analysis of unstimulated eosinophils in culture was first performed.

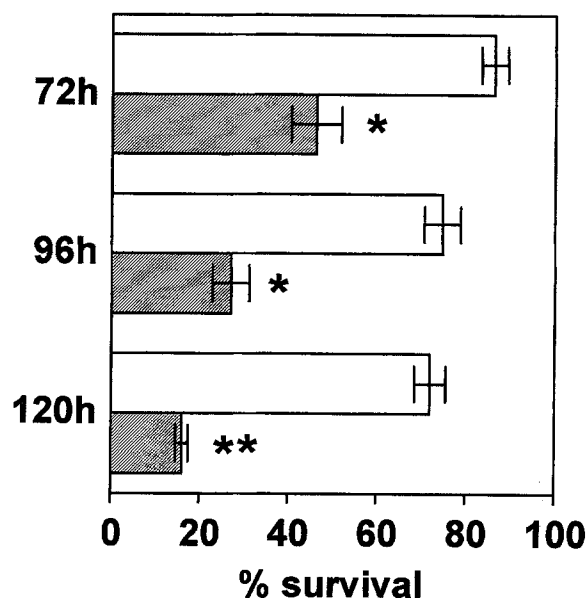


FIG. 5. Neutralizing effect of anti-GM-CSF mAbs. Eosinophils from AD patients were incubated for 72 hours without (unfilled bars) or with (filled bars) anti-GM-CSF mAbs neutralizing 1000 U of GM-CSF bioactivity.

There were no obvious differences in constitutive CD95 expression on eosinophils between the groups studied, and expression did not increase over time in culture. Moreover, no significant modulation of CD95 could be observed after stimulation with IL-3, IL-5, and GM-CSF (data not shown).

Expression of the eosinophil activation marker CD69

Constitutively, unstimulated eosinophils expressed low levels of CD69, as described previously.³⁹ However, IA eosinophils and particularly AD eosinophils were found to have a markedly enhanced surface expression of CD69 over time in culture without the need for exogenous growth factors, whereas on NA eosinophils CD69 expression remained unchanged (Table I).

In confirmation of preliminary reports³⁹ proeosinophilic cytokines upregulated CD69 expression. However, this upregulation was seen in IA and AD but not in NA subjects (Table II). Notably, in AD, GM-CSF (100 U/ml) and IL-5 (100 U/ml) were not able to further increase CD69 surface expression when compared to eosinophils stimulated with medium alone. In contrast, IL-3 (100 U/ml) significantly enhanced expression of CD69 (see Table II).

DISCUSSION

The data presented clearly demonstrate by qualitative and quantitative assays that PCD is significantly delayed in eosinophils of subjects with IA and particularly in those with AD, as compared to NA controls. Furthermore, the results suggest that delayed PCD may be

TABLE I. Enhanced CD69 expression on eosinophils of subjects with IA or AD after 48 to 96 hours in culture

	NA subjects	Subjects with IA	Subjects with AD
0 hr	3.0 ± 1.2	4.5 ± 1.0	6.9 ± 1.3
48 hr	5.4 ± 2.4	10.4 ± 1.0	36.2 ± 4.5*
96 hr	7.6 ± 0.7	27.2 ± 4.3*	33.3 ± 5.9*

Expression was determined on fresh eosinophils (0 hr) and after culture for 48 hr and 96 hr. Values are mean fluorescence intensity (MFI) of eosinophils ± SEM after subtraction of nonspecific MFI obtained with a control isotype mAb, calculated from six separate experiments.

* $p < 0.05$, indicated time point versus 0 hr. After 48 hr, the p value was less than 0.05 for AD versus NA or IA; after 96 hours, the p value was less than 0.05 for IA or AD versus NA.

mediated at least in part by an autocrine production of GM-CSF and probably of IL-5.

The demonstrated survival rates of eosinophils from NA subjects in culture are consistent with published work showing low viability in healthy subjects without atopy after 96 hours in culture.^{25, 40, 41} However, regarding the present findings some caution is required when comparing eosinophil survival rates among different studies because some previous data did not recognize an atopic constitution among blood donors. It is worthwhile to mention that we found comparable survival rates between the extrinsic and intrinsic type of AD, suggesting that IgE-mediated specific sensitization may not be directly involved in prolonged eosinophil survival. It is tempting to speculate that atopic disorders may be better characterized by inhibited eosinophil PCD than by the sensitization to specific allergens.

Inhibited PCD may be responsible for the well-known accumulation of eosinophils in the circulation and tissues of atopic subjects. In this study, however, circulating eosinophil numbers did not differ between NA subjects and subjects with IA, ADe, or ADi. However, eosinophil survival rates differed, being the highest and the same in patients with ADe and ADi, decreasing in patients with IA, and being the lowest in NA subjects. NA subjects with blood eosinophilia up to 31% (e.g., NA subjects with scabies) in this study did not show prolonged eosinophil survival in vitro. This observation implies that inhibited PCD did not result in peripheral blood eosinophilia, and this is confirmed by data demonstrating no prolonged survival of eosinophils from patients with hypereosinophilic syndrome in vitro (viability less than 20% at 96 hours).²⁷ In contrast, Owen et al.⁴² reported that the hypodense eosinophil population in three patients with corticosteroid-unresponsive idiopathic hypereosinophilic syndrome was uniquely long lived ex vivo in the absence of exogenous cytokines (about 45% survival after 96 hours). Both studies were carried out with eosinophils of three subjects with HES. Thus, further data will have to clarify whether peripheral blood eosinophils in HES demonstrate prolonged survival.

Previous work in patients with hypereosinophilic syn-

TABLE II. CD69 expression on peripheral blood eosinophils of NA subjects, subjects with IA, and subjects with AD after stimulation with proeosinophilic cytokines

	NA subjects	Subjects with IA	Subjects with AD
48 hr			
Medium	5.4 ± 2.4	10.4 ± 1.0	36.2 ± 4.5
GM-CSF	2.3 ± 0.5	19.5 ± 1.5*	31.6 ± 3.9
IL-5	4.2 ± 1.7	19.3 ± 1.4*	41.8 ± 6.7
IL-3	6.0 ± 2.2	56.3 ± 2.9*	58.6 ± 8.1*
96 hr			
Medium	7.6 ± 0.7	27.2 ± 4.3	33.3 ± 5.9
GM-CSF	3.8 ± 0.8	31.3 ± 2.4	29.9 ± 5.7
IL-5	6.3 ± 3.2	30.0 ± 0.9	37.9 ± 4.7
IL-3	7.3 ± 0.9	73.0 ± 19.3*	70.8 ± 14.1*

Expression was determined after stimulation with medium control, GM-CSF, IL-3, or IL-5 (100 U/ml each) for 48 hr and 96 hr. Values are mean fluorescence intensity (MFI) of eosinophils ± SEM after subtraction of nonspecific MFI obtained with a control isotype mAb, calculated from six separate experiments.

* $p < 0.05$, indicated stimulus versus medium control at respective time point.

drome has shown that IL-5 is more potent than IL-3 and GM-CSF in prolonging the survival of eosinophils.²⁷ In contrast, our data reveal that IL-3 was most potent in prolonging the survival of eosinophils in NA subjects, followed by GM-CSF and IL-5, whereas in IA and AD, significant differences between cytokines were not found as a result of increased eosinophil survival per se (see Fig. 1). However, we examined only a single dose of cytokine (100 U/ml), which may provide insufficient data to base any conclusions on relative potencies.

Although triggering the CD95 (Fas antigen) cell-surface receptor has been shown to induce eosinophil apoptosis, the present work suggests that modulated expression of Fas antigen seems not to be involved in delayed eosinophil PCD. However, the possibility that Fas antigen may not function in IA or AD has to be addressed in further studies. Because atopy can be transferred by bone marrow transplantation, the genetically determined trait might be expressed in blood leukocytes that circulate and infiltrate skin and respiratory tissues. It may be postulated that genes regulating PCD could be slightly dysregulated in IA and more significantly dysregulated in AD. This thesis may also be in line with the recently observed inhibited PCD in monocytes derived from patients with chronic AD⁴³ and implicates a common genetic dysregulation in several proinflammatory target cells in atopic disorders. Alternatively, in IA and particularly in AD, eosinophils may be primed in vivo to undergo delayed PCD. Additional support for this thesis is provided by recent work indicating that eosinophils from nonallergic donors needed in vitro priming by GM-CSF, IL-3, and IL-5 to migrate through endothelial layers, whereas AD eosinophils penetrated spontaneously.⁴⁴ In addition, refractoriness of eosinophils from eosinophilic subjects to migratory

and priming effects of IL-5 was shown and has been explained by prior *in vivo* priming with IL-5.⁴⁵ Because studies over the past few years have demonstrated that eosinophils synthesize and secrete several important inflammatory and regulatory cytokines including TGF- α , TGF- β , GM-CSF, IL-1 α , IL-3, IL-5, IL-6, IL-8, TNF- α , and MIP1,⁴⁶ priming mediators may not only be provided by T-lymphocytes⁴⁷ but may also be produced in an autocrine manner by eosinophils themselves.⁴⁸⁻⁵¹ Indeed, our data provide strong evidence for an eosinophil autocrine production of GM-CSF and probably of IL-5, at least in AD. Supernatants of AD eosinophils, more than those of IA eosinophils, showed survival-prolonging effects on NA eosinophils. Moreover, in AD eosinophils, culture with neutralizing anti-GM-CSF mAbs abrogated the survival-prolonging effect. The eosinophil preparations were highly purified (95%) and the contaminating cells consisted of neutrophils only. Moreover, recent data demonstrated only minimal amounts of GM-CSF in supernatants of neutrophils.⁵² Thus, the possibility that these increased amounts of cytokines were contributed by other cell types seems unlikely.

As shown previously, unstimulated peripheral blood eosinophils of donors with mild eosinophilia do not express CD69. In contrast, elevated CD69 expression has been demonstrated on bronchoalveolar lavage eosinophils in asthma and in eosinophilic pneumonia, indicating eosinophil preactivation.^{39, 53} The observation that we found an upregulation of CD69 expression over time in culture in eosinophils of subjects with IA and particularly in eosinophils of subjects with AD, further supports the autocrine production of cytokines. Only IL-3 was able to further upregulate the *per se* enhanced CD69 surface expression in IA and AD eosinophils over time in culture, perhaps because eosinophils were not shown to produce significant amounts of IL-3 on their own. It was recently demonstrated that ligation of CD69 by a specific monoclonal antibody induced apoptosis and cell death in human eosinophils cultured with GM-CSF.⁵⁴

In conclusion, our data strongly suggest that delayed eosinophil PCD may be a common feature of IA and AD irrespective of the contribution of specific sensitization. Because no relationship between inhibited eosinophil PCD and the peripheral blood eosinophil count was observed, inhibition of programmed eosinophil death may not be a pathogenic event for eosinophilia in general, as proposed previously.⁵⁵ Future studies will have to address the question of whether inhibited PCD may result in local accumulation of eosinophils. Solving the puzzle of inhibited eosinophil PCD will not only improve our understanding of atopic disorders but may also have major therapeutic implications.

REFERENCES

1. Rothe MJ, Grant-Kels JM. Atopic dermatitis: an update. *J Am Acad Dermatol* 1996;35:1-13.
2. Halbert AR, Westoon WL, Morelli JG. Atopic dermatitis: is it an allergic disease? *J Am Acad Dermatol* 1995;33:1008-18.
3. Kapp A. Atopic dermatitis: the skin manifestation of atopy. *Clin Exp Allergy* 1995;25:210-19.
4. Wierenga EA, Snoek M, Jansen HM, Bos JD, Van Lier RA, Kapsenberg ML. Human atopy-specific types 1 and 2 T helper cell clones. *J Immunol* 1991;147:2942-9.
5. Sager N, Feldmann A, Schilling G, Kreitsch P, Neumann C. House dust mite-specific T cells in the skin of subjects with atopic dermatitis: frequency and lymphokine profile in the allergen patch test. *J Allergy Clin Immunol* 1992;89:801-10.
6. Leiferman KM. Eosinophils in atopic dermatitis. *J Allergy Clin Immunol* 1994;94:1310-17.
7. Bousquet J, Chané P, Lacoste JY, Enander J, Venge P, Peterson C, et al. Eosinophilic inflammation in asthma. *N Engl J Med* 1990;323:1033-9.
8. Venge P, Hakansson L. Current understanding of the role of the eosinophil granulocyte in asthma. *Clin Exp Allergy* 1991;21(Suppl 3):31-7.
9. Martin LB, Kita H, Leiferman KM, Gleich GJ. Eosinophils in allergy: role in disease, degranulation, and cytokines. *Int Arch Allergy Immunol* 1996;109:207-15.
10. Leiferman KM, Ackerman SJ, Sampson HA, Haugen HS, Venencie PY, Gleich GJ. Dermal deposition of eosinophil-granule major basic protein in atopic dermatitis. *N Engl J Med* 1985;313:282-7.
11. Charlesworth EN, Hood AF, Soter NA, Kagey-Sobotka A, Norman PS, Lichtenstein LM, et al. Cutaneous late-phase response to allergen: mediator release and inflammatory cell infiltration. *J Clin Invest* 1989;83:1519-26.
12. Terada N, Konno A, Togawa K. Biochemical properties of eosinophils and their preferential accumulation mechanism in nasal allergy. *J Allergy Clin Immunol* 1994;94:629-42.
13. Bruijnzeel-Koomen C, Storz E, Menz G, Bruijnzeel P. Skin eosinophilia in patients with allergic and nonallergic asthma and atopic dermatitis. *J Allergy Clin Immunol* 1992;89:52-9.
14. Kägi MK, Joller-Jemelka H, Wüthrich B. Correlation of eosinophils, eosinophil cationic protein and soluble interleukin-2 receptor with the clinical activity of atopic dermatitis. *Dermatology* 1992;185:88-92.
15. Kapp A, Czech W, Krutmann J, Schöpf E. Eosinophil cationic protein (ECP) in sera of patients with atopic dermatitis. *J Am Acad Dermatol* 1991;24:555-8.
16. Paganelli R, Fanale-Belasio E, Carmini D, Scala E, Meglio P, Businco L, et al. Serum eosinophil cationic protein in patients with atopic dermatitis. *Int Arch Allergy Appl Immunol* 1991;96:175-8.
17. Carlson M, Hakansson L, Peterson C, Stalenheim G, Venge P. Secretion of granule proteins from eosinophils and neutrophils is increased in asthma. *J Allergy Clin Immunol* 1991;87:27-33.
18. Czech W, Krutmann J, Schöpf E, Kapp A. Serum eosinophil cationic protein (ECP) is a sensitive measure for disease activity in atopic dermatitis. *Br J Dermatol* 1992;126:351-5.
19. Kapp A. The role of eosinophils in the pathogenesis of atopic dermatitis: eosinophil granule proteins as markers of disease activity. *Allergy* 1993;48:1-5.
20. Sugai T, Sakiyama Y, Matumoto S. Eosinophil cationic protein in peripheral blood of pediatric patients with allergic diseases. *Clin Exp Allergy* 1992;22:275-81.
21. Krutmann J, Czech W, Diepgen T, Niedner R, Kapp A, Schöpf E. High dose UVA1 therapy in the treatment of patients with atopic dermatitis. *J Am Acad Dermatol* 1992;26:225-30.
22. Borres MP, Odelram H, Irander K, Kjellman NI, Björkstén B. Peripheral blood eosinophilia in infants at 3 months of age is associated with subsequent development of atopic disease in early childhood. *J Allergy Clin Immunol* 1995;95:694-8.
23. Calbi M, Scarpellino B, Giacchetti L. Usefulness of neonatal eosinophil counts as a marker of atopy? *Pediatr Allergy Immunol* 1993;4:86-8.
24. Her E, Frazer J, Austen KF, Owen WF Jr. Eosinophil hematopoietins antagonize the programmed cell death of eosinophils. Cytokine and glucocorticoid effects on eosinophils maintained by endothelial cell-conditioned medium. *J Clin Invest* 1991;88:1982-7.
25. Rothenberg ME, Owen WF Jr., Silberstein DS, Woods JJ, Soberman RJ, Austen KF, et al. Human eosinophils have prolonged survival,

- enhanced functional properties, and become hypodense when exposed to human interleukin 3. *J Clin Invest* 1988;81:1986-92.
26. Yamaguchi Y, Suda T, Ohta S, Tominaga K, Mioura Y, Kasahara T. Analysis of the survival of mature human eosinophils: interleukin-5 prevents apoptosis in mature human eosinophils. *Blood* 1991;78:2542-7.
27. 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.
28. Wallen N, Kita H, Weiler D, Gleich GJ. Glucocorticoids inhibit cytokine-mediated eosinophil survival. *J Immunol* 1991;147:3490-5.
29. Savill JS, Wyllie AH, Henson JE, Walport MJ, Henson PM, Haslett C. Macrophage phagocytosis of aging neutrophils in inflammation. *J Clin Invest* 1989;83:865-9.
30. Hanifin JM, Rajka G. Diagnostic features of atopic dermatitis. *Acta Dermato Venereol (Stockh)* 1980;92(Suppl):44-7.
31. Walker C, Kagi MK, Ingold P, Braun P, Blaser K, Bruijnzeel-Koomen CA, et al. Atopic dermatitis: correlation of peripheral blood T cell activation, eosinophilia and serum factors with clinical severity. *Clin Exp Allergy* 1993;23:145-53.
32. Elsner J, Oppermann M, Czech W, Dobos G, Schöpf E, Norgauer J, et al. C3a activated reactive oxygen radical species production and intracellular calcium transients in human eosinophils. *Eur J Immunol* 1994;24:518-22.
33. Zeck-Kapp G, Kroegel C, Riede UN, Kapp A. Mechanisms of human eosinophil activation by complement protein C5a and platelet activating factor: similar functional responses are accompanied by different morphologic alterations. *Allergy* 1995;50:34-47.
34. Kapp A, Zeck-Kapp G, Czech W, Schöpf E. The chemokine RANTES is more than a chemoattractant: characterization of its effect on human eosinophil oxidative metabolism and morphology in comparison with IL-5 and GM-CSF. *J Invest Dermatol* 1994;102:906-14.
35. Leist M, Gantner F, Bohlinger I, Germann PG, Tiegs G, Wendel A. Murine hepatocyte apoptosis induced in vitro and in vivo by TNF-alpha requires transcriptional arrest. *J Immunol* 1994;153:1778-88.
36. Wedi B, Elsner J, Czech W, Butterfield JH, Kapp A. Modulation of intercellular adhesion molecule 1 (ICAM-1) expression on the human mast cell line HMC-1 by inflammatory mediators. *Allergy* 1996;51:676-84.
37. Matsumoto K, Schleimer RP, Seito H, Iikura Y, Bochner BS. Induction of apoptosis in human eosinophils by anti-Fas antibody treatment in vitro. *Blood* 1995;86:1437-43.
38. Druilhe A, Cai Z, Hailè S, Chouaib S, Pretolani M. Fas-mediated apoptosis in cultured human eosinophils. *Blood* 1996;87:2822-30.
39. Hartnell A, Robinson DS, Kay AB, Wardlaw AJ. CD69 is expressed by human eosinophils activated in vivo in asthma and in vitro by cytokines. *Immunology* 1993;80:281-6.
40. Takanashi S, Nonaka R, Xing Z, O'Byrne P, Dolovich J, Jordana M. Interleukin-10 inhibits lipopolysaccharide-induced survival and cytokine production by human peripheral blood eosinophils. *J Exp Med* 1994;180:711-5.
41. Alam R, Forsythe P, Stafford S, Fukuda Y. Transforming growth factor β abrogates the effects of hematopoietins on eosinophils and induces their apoptosis. *J Exp Med* 1994;179:1041-5.
42. Owen WF, Rothenberg ME, Petersen J, Weller PF, Silberstein D, Sheffer A, et al. Interleukin 5 and phenotypically altered eosinophils in the blood of patients with the idiopathic hypereosinophilic syndrome. *J Exp Med* 1989;170:343-8.
43. Bratton DL, Hamid Q, Boguniewicz M, Doherty DE, Kailey JM, Leung DYM. Granulocyte macrophage colony-stimulating factor contributes to enhanced monocyte survival in chronic atopic dermatitis. *J Clin Invest* 1995;95:211-8.
44. Moser R, Fehr J, Bruijnzeel PL. IL-4 controls the selective endothelium-driven transmigration of eosinophils from allergic individuals. *J Immunol* 1992;149:1432-8.
45. Sehmi R, Cromwell O, Wardlaw AJ, Moqbel R, Kay AB. Interleukin-5 is a chemoattractant for eosinophils purified from subjects with a blood eosinophilia but not from normal healthy subjects. *Clin Exp Allergy* 1993;23:1027-36.
46. Spry CFJ, Kay AB, Gleich GJ. Eosinophils 1992. *Immunol Today* 1992;13:384-7.
47. Humbert M. Pro-eosinophilic cytokines in asthma. *Clin Exp Allergy* 1996;26:123-7.
48. Kita H, Ohnishi T, Okubo Y, Weiler D, Abrams JS, Gleich GJ. Granulocyte/macrophage colony-stimulating factor and interleukin-3 release from human peripheral blood eosinophils and neutrophils. *J Exp Med* 1991;174:745-8.
49. Tanaka Y, Delaporte E, Dubucquoi S, Gounni AS, Porchet E, Capron A, et al. Interleukin-5 messenger RNA and immunoreactive protein expression by activated eosinophils in lesional atopic dermatitis skin. *J Invest Dermatol* 1994;103:589-92.
50. Moqbel R, Levi-Schaffer F, Kay AB. Cytokine generation by eosinophils. *J Allergy Clin Immunol* 1994;94:1183-8.
51. Broide H, Paine M, Firestein G. Eosinophils express interleukin 5 and granulocyte macrophage-colony-stimulating factor mRNA at sites of allergic inflammation. *J Clin Invest* 1992;90:1414-24.
52. Cassatella MA. The production of cytokines by polymorphonuclear neutrophils. *Immunol Today* 1995;16:21-7.
53. Nishikawa K, Morii T, Hirofumi A, Hamada K, Saito S, Narita N. In vivo expression of CD69 on lung eosinophils in eosinophilic pneumonia: CD69 a possible activation marker for eosinophils. *J Allergy Clin Immunol* 1992;90:169-74.
54. Walsh GM, Williamson ML, Symon FA, Willars GB, Wardlaw AJ. Ligation of CD69 induces apoptosis and cell death in human eosinophils. *Blood* 1996;87:2815-21.
55. Simon H-U, Blaser K. Inhibition of programmed cell eosinophil death: a key pathogenic event for eosinophilia? *Immunol Today* 1995;16:53-5.