

Perforin hyperreleasability and depletion in cytotoxic T cells from patients with exacerbated atopic dermatitis and asymptomatic rhinoconjunctivitis allergica

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Background: As a plasma membrane pore-forming protein, perforin is essential for T-cell cytotoxicity mediated by lytic granules. Recent studies on the immune system of perforin knockout mice demonstrated striking similarities to the immunopathology of atopic diseases.

Objective: We sought to investigate the perforin system of atopic patients.

Methods: Monoclonal antibodies were used to characterize perforin-positive PBMCs of patients with exacerbated atopic dermatitis (AD) and asymptomatic rhinoconjunctivitis allergica (RCA) by means of immunoflow cytometry. In addition, a perforin release assay was developed to quantify the velocity of ionomycin and phorbol 12-myristate 13-acetate-induced secretion of lytic granules.

Results: In atopic patients significantly fewer lymphocytes contained perforin-positive lytic granules compared with those of healthy control subjects (patients with AD: $14\% \pm 5\%$, $n = 13$, $P < .0001$; patients with RCA: $24\% \pm 5\%$, $n = 9$, $P < .01$; healthy control subjects: $33\% \pm 11\%$, $n = 13$). Of all $CD8^{hi+}$ cytotoxic T lymphocytes (CTLs), only $18\% \pm 9\%$ and $17\% \pm 12\%$ were perforin-positive in patients with AD and RCA, respectively, compared with $44\% \pm 13\%$ in control subjects ($P < .0001$). In addition, perforin-positive $CD8^{hi+}$ CTLs of atopic patients released their perforin twice as fast and more completely than control CTLs. This means that 50% of initially perforin-positive $CD8^{hi+}$ CTLs from patients with AD and RCA released their perforin completely within 32 ± 16 and 36 ± 19 minutes, respectively, and an over 85% release was reached within 113 ± 41 and 118 ± 60 minutes, respectively. In CTLs of healthy control subjects, however, it took 64 ± 40 minutes to achieve a 50% release of lytic granules, and an 85% depletion was not reached in 60% of healthy control subjects, even after 180 minutes.

Conclusion: The perforin hyperreleasability explains, at least in part, the decreased percentage of perforin-positive $CD8^{hi+}$ CTLs in atopic patients. These distortions in the system of lytic granules of atopic patients may contribute to the functional

defects observed in T-cell cytotoxicity in vivo and in vitro in patients with AD and RCA. (*J Allergy Clin Immunol* 2001;107:878-86.)

Key words: Perforin, atopic dermatitis, rhinoconjunctivitis allergica, releasability, $CD8^{+}$ lymphocytes

The 70-kd protein perforin is a key component of the cytolytic machinery of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells. After production, perforin is stored intracellularly in specialized secretory lysosomes, termed lytic granules. Target cell recognition results in polarization of the perforin-containing cell and subsequent degranulation of lytic granules toward the target cell.¹

After self-insertion into the phospholipid bilayer of the plasma membrane of the target cell, perforin polymerizes to form a pore with a diameter of about 16 nm. As a result, the poked cell dies by means of osmotic lysis. In addition, other lytic granule components, most notably proteases of the cathepsin superfamily (eg, granzyme B), use the hole fabricated by perforin to enter the target cell and to initiate apoptosis.²

Important clues about the role of perforin in vivo come from recent experiments with null mice, in which the perforin gene has been deleted (*perf*^{-/-}).³ These mice are characterized by hypergammaglobulinemia, autoantibody production, and immune deposit-related end-organ disease. They are unable to clear many viral infections.⁴⁻⁶ In addition, it was demonstrated that perforin also acts as a negative regulator of activated T cells.⁶ Such involvement of perforin in the regulation of T-cell homeostasis was recently confirmed by Kagi et al.⁷

Further evidence for perforin being engaged in down-regulation of immune responses is derived from experiments with *lpr* (Fas-deficient) and *perf*^{-/-} double knockout mice, which exhibited a striking acceleration of the spontaneous lymphoproliferative disease typically seen in *lpr* mice. By using the same experimental system, Majlessi et al⁸ recently demonstrated that perforin and Fas/Fas ligand are alternatively or simultaneously used to downregulate Ig production in vitro.

In atopic patients hyperimmunoglobulinemia and autoantibody production against IgE and other autoantigens, such as profilin, are well-known features. A disturbed T-cell homeostasis, eg, a changed T_H1/T_H2 balance, points to a defect in T cell-dependent regulation of

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Abbreviations used

AD:	Atopic dermatitis
cAMP:	Cyclic adenosine monophosphate
CLA:	Cutaneous lymphocytic antigen
CTL:	Cytotoxic T lymphocyte
ECP:	Eosinophil cationic protein
NK:	Natural killer
PFA:	Paraformaldehyde
PMA:	Phorbol 12-myristate 13-acetate
RCA:	Rhinoconjunctivitis allergica

immunologic processes. In addition, clinicians have been aware for a long time that in patients with atopic dermatitis (AD), cutaneous viral infections caused by herpes simplex (ie, eczema herpeticum), vaccinia, varicella zoster, or coxsackie A16 viruses are often prolonged and more severe compared with the population average. An increased susceptibility to infections by human papilloma and molluscum contagiosum viruses can also be observed in atopic individuals.⁹⁻¹¹

Thus we wondered whether a defect in the perforin system of these patients might contribute to the observed immunopathology. We now demonstrate that in atopic patients significantly fewer peripheral lymphocytes do contain perforin compared with those of healthy control subjects. Additionally, a perforin hyperreleasability is shown; that is, perforin-positive CD8^{hi} CTLs of atopic patients do release their perforin-containing granules twice as fast and more completely than CTLs from healthy control subjects.

METHODS

Cells and antibodies

PBMCs were obtained by means of Ficoll-Lite (Linaris) density gradient centrifugation (650g) of heparinized blood collected before initiation of systemic therapy from 13 patients with exacerbated AD (7 male and 6 female patients; age, 21 ± 8 years). All patients with AD had moderate-to-severe inflammation of the skin (SCORAD score, 49 ± 7). They were characterized by high serum levels of total IgE (8995 ± 12695 kU/L) and eosinophil cationic protein (ECP; 67 ± 56 µg/L). In addition, 9 patients (4 male and 5 female patients; age, 27 ± 4 years) with seasonal rhinoconjunctivitis allergica (RCA) without symptoms at the time of blood sampling and without any history of AD or asthmatic disease were recruited (IgE level, 668 ± 994 kU/L; ECP level, 18 ± 16 µg/L). All of the atopic patients had a positive skin prick test response to inhalant allergens and a positive screening test response to specific serum IgE against inhalant allergens (sxl; UpJohn-Pharmacia, Freiburg, Germany). PBMCs of 13 healthy volunteers (6 male and 7 female, subjects; age, 30 ± 5 years) with no history of inflammatory skin disease or RAC served as control subjects (IgE level, 32 ± 14 kU/L; ECP level, 7 ± 4 µg/L). Phycoerythrin-conjugated mAbs against surface molecules (ie, CD3 [clone: B-B11], CD4 [clone: B-F5], CD8 [clone: B-H7], CD56 [clone: B-A19], the FITC-conjugated mAb against perforin [clone: δG9] and a control FITC-conjugated mouse IgG2b immunoglobulin) were obtained from Hölzel (Cologne, Germany). The unconjugated antibodies (ie, anti-CD63 [clone: RFAC4] and anti-cutaneous lymphocytic antigen [CLA; clone: HECA-452]) were obtained from Pharmingen, and the FITC-conjugated goat anti-mouse antibody and the cy5-conjugated goat anti-rat IgM antibody were obtained from Jackson ImmunoResearch/Dianova.

Immunostaining and flow cytometry of cells

After separation, PBMCs were placed in aliquots into equivalents of 0.5 mL of blood. Cells were fixed for 7 minutes in ice-cold PBS (pH 7.4) containing 2% paraformaldehyde (PFA), spun down, and washed twice in PBS containing 10% FCS and 0.2% NaN₃. Monoclonal antibodies were diluted in PBS/FCS containing 2% saponin and anti-CD3 1:20, anti-CD4 1:20, anti-CD8 1:100, anti-CD56 1:100, anti-CLA 1:100, anti-perforin 1:750, or mouse IgG2b immunoglobulin 1:200 as an isotype control for the perforin mAb, respectively. Cell pellets were incubated with 50 µL of antibody solution overnight at 4°C, washed twice in PBS/FCS, and taken up in PBS/PFA. Double staining was performed with mAbs against perforin and CD3, CD4, or CD56, respectively. Triple staining was performed with mAbs against perforin, CD8, and CLA. The cy5-labeled secondary goat anti-rat-IgM antibody (diluted in PBS 1:100) was incubated for 2 hours at 20°C. Staining against the CD63 antigen (1:100) was performed on unfixed PBMCs in PBS (pH 7.4) for 1 hour at 4°C. Cells were washed twice in ice-cold PBS/FCS and incubated with the FITC-labeled secondary goat anti-mouse antibody (diluted in PBS 1:100) for 1 hour at 4°C. Cells were washed twice and stored in PBS/PFA at 4°C until analysis.

Measurements were performed in a flow cytometer (FACScan, Becton Dickinson) employing CellQuest software. Peripheral blood lymphocytes were identified in the forward side scatter and gated. By using Microsoft EXCEL, a *t* test assuming unequal variances was applied for statistical analysis. The level of significance was set at a *P* value of less than .05.

Perforin release assay

PBMCs (equivalents of 0.5 mL of blood) were spun down at 4°C and subsequently resuspended in 0.5 mL of warm (37°C) RPMI-1640 containing ionomycin (1 µg/mL) and phorbol 12-myristate 13-acetate (PMA; 50 ng/mL, both from Calbiochem). Stimulation was stopped after incubation at 37°C for indicated time intervals by rapid cooling of cells with 3 mL of ice-cold PBS without calcium (Dulbecco, pH 7.4). In parallel, cells were incubated in RPMI-1640 without ionophore and mitogen as unstimulated controls. Cells were spun down at 4°C, fixed immediately, washed with PBS/FCS, and triple stained overnight. By means of flow cytometry, the percentage of CD8^{hi} perforin-positive cells was quantified, and reduction of perforin-positive cells over time was used as a measure for perforin release.

RESULTS

In the first part of the study, we characterized CD3⁺ T lymphocytes and CD56⁺ NK cells of atopic patients and healthy control subjects with regard to their perforin content. In addition, CD4⁺, CD8⁺, and CLA⁺ CD8⁺ T-cell subpopulations were investigated. Results summarized in Tables I, II, and III and Figs 1 and 2 are presented in detail as follows.

Increase of the CD4/CD8 ratio and reduction of CD56⁺ cells in patients with AD

In both patients and control subjects, 70% to 80% of peripheral blood lymphocytes stained positive for the CD3 antigen (Table I). However, there was a significant reduction of cells positive for CD56 in patients with AD compared with those of healthy control subjects (patients with AD: 6% ± 2%; healthy control subjects: 15% ± 8%; *P* < .001). In patients with AD, the CD4⁺ T-cell subpopulation was increased significantly (patients with AD:

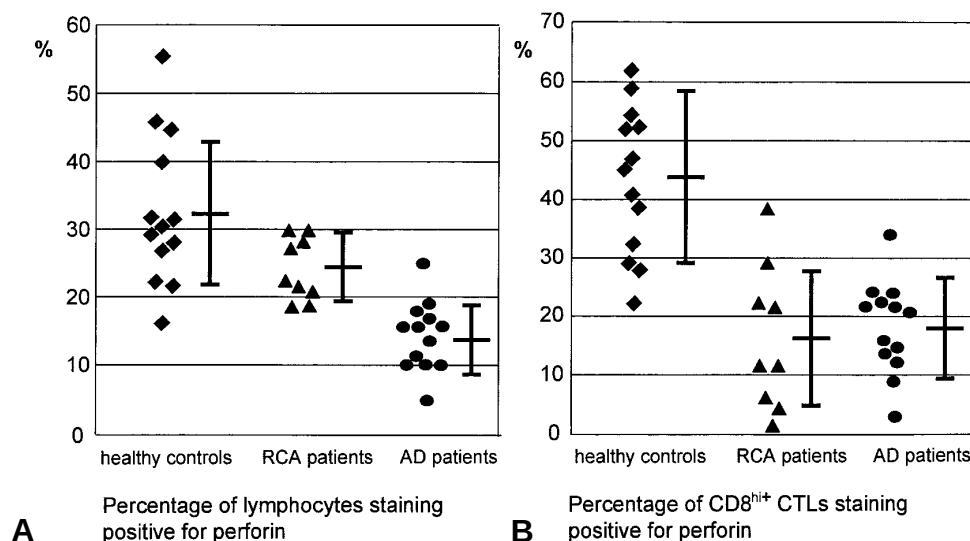


FIG 1. Reduction of perforin-positive lymphocytes in patients with atopy. **A**, Relative amount of perforin-expressing cells (mean $\pm$ SD) among the lymphocytic cell population in 13 patients with exacerbated AD, 9 patients with asymptomatic RCA, and 13 healthy control subjects. **B**, Corresponding relative portion of perforin-positive cells among CD8^{hi+} cytotoxic T cells.

TABLE I. Subpopulations of peripheral blood lymphocytes

	CD3 ⁺ cells	CD4 ⁺ cells	CD8 ⁺ cells	CD8 ^{hi+} cells	CD4/CD8	CD56 ⁺ cells
Healthy control subjects, n = 13 (%)	76 $\pm$ 7	46 $\pm$ 6	32 $\pm$ 7	26 $\pm$ 9	1.6 $\pm$ 0.5	15 $\pm$ 8
Patients with RCA, n = 9 (%)	71 $\pm$ 11	47 $\pm$ 7	28 $\pm$ 4	21 $\pm$ 4	1.7 $\pm$ 0.4	14 $\pm$ 5
Patients with AD, n = 13 (%)	79 $\pm$ 6	55 $\pm$ 11	25 $\pm$ 5	21 $\pm$ 3	2.4 $\pm$ 1.0	6 $\pm$ 2
<i>t</i> Test, healthy control subjects vs patients with RCA	<i>P</i> = .13	<i>P</i> = .31	<i>P</i> = .05	<i>P</i> = .06	<i>P</i> = .20	<i>P</i> = .47
<i>t</i> Test, healthy control subjects vs patients with AD	<i>P</i> = .13	<i>P</i> = .009	<i>P</i> = .003	<i>P</i> = .03	<i>P</i> = .007	<i>P</i> = .003

55% $\pm$ 11%; healthy control subjects: 46% $\pm$ 6%; *P* < .05), and the CD8⁺ subpopulation was decreased compared with that in healthy control subjects (patients with AD: 26% $\pm$ 4%; healthy control subjects: 32% $\pm$ 6%; *P* < .05). Thus a shift of the CD4/CD8 ratio was observed in patients with AD (patients with AD: 2.4 $\pm$ 1.0; healthy control subjects: 1.6 $\pm$ 0.5; *P* < .01; Table I). Patients with RCA did not show alterations of these lymphocytic subpopulations to the same significant extent as did patients with AD (Table I).

Reduction of perforin-positive lymphocytes in atopic patients

Staining with anti-perforin antibody revealed a significant numerical reduction of perforin-positive lymphocytes in atopic patients (patients with AD: 14% $\pm$ 5%; patients with RCA: 24% $\pm$ 5%) compared with that found in healthy control subjects 33% $\pm$ 11%; *P* = .0001 and .01, respectively; Table II and Fig 1, A). CD3⁺ lymphocytes showed reduced numbers of perforin-containing cells: 25% $\pm$ 9% of all CD3⁺ lymphocytes stained positive for perforin in healthy control subjects compared with only 8% $\pm$ 3% in patients with AD (*P* < .0001) and 10% $\pm$ 5% in patients with RCA (*P* < .0001). In CD56⁺

cells, however, no significant difference between patients with AD (85% $\pm$ 25%), patients with RCA (89% $\pm$ 6%), and healthy control subjects (89% $\pm$ 12%) was detected for the expression of perforin.

Among CD3⁺ cells, both CD4⁺ lymphocytes and CD8⁺ CTLs—defined as cells expressing high levels of CD8 (CD8^{hi+})—contributed to the reduced percentage of perforin-positive lymphocytes in atopic patients. In healthy control subjects 44% $\pm$ 13% of all CD8^{hi+} CTLs and 5% $\pm$ 3% of all CD4⁺ lymphocytes contained perforin. In atopic patients, however, only 18% $\pm$ 9% (patients with AD) and 17% $\pm$ 12% (patients with RCA), respectively, of CD8^{hi+} CTLs (*P* < .0001; Fig 1, B) and only 2% $\pm$ 1% and 1% $\pm$ 1% of CD4⁺ cells (*P* < .005) stained positive for perforin. Representative examples of the corresponding individual raw data of CD8/perforin double staining of a patient with AD and of a healthy control subject are given in Fig 2.

Percentage of perforin-positive lymphocytes correlates with serum IgE level

Patients with AD characterized by high serum IgE levels (23,500 $\pm$ 9700 kU/L; n = 6; SCORAD score, 52 $\pm$ 4) showed a significantly lower percentage of perforin-

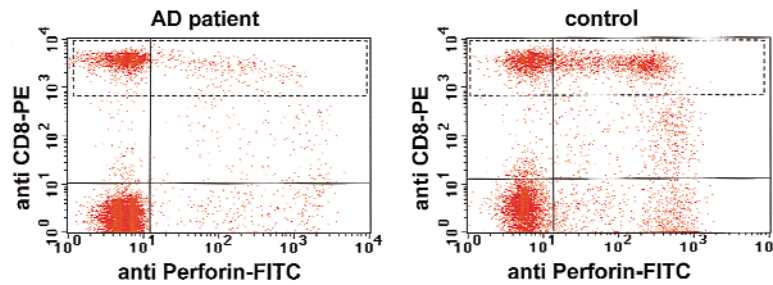


FIG 2. Representative examples of a dot plot analysis of cells stained with mAbs against CD8 and perforin in an atopic individual (patient with AD) and a healthy volunteer. The lymphocytic population was identified by its characteristics in the forward side scatter and gated. Cells expressing CD8 at high density on their surface (CD8^{hi+}) were defined as CTLs. Note the substantial difference in the CD8^{hi+} perforin-positive subpopulation between the patient with AD and the healthy control subject.

TABLE II. Relative proportions of perforin-positive cells in relation to lymphocyte subpopulations

	Perforin-positive cells of					
	All lymphocytes	CD3 ⁺ cells	CD4 ⁺ cells	CD8 ⁺ cells	CD8 ^{hi+} cells	CD56 ⁺ cells
Healthy control subjects, n = 13 (%)	33 ± 11	25 ± 9	5 ± 3	52 ± 13	44 ± 13	89 ± 12
Patients with RCA, n = 9 (%)	24 ± 5	10 ± 5	1.5 ± 1.5	40 ± 14	17 ± 12	91 ± 7
Patients with AD, n = 13 (%)	14 ± 5	8 ± 3	1.6 ± 1.2	26 ± 11	18 ± 9	89 ± 6
<i>t</i> Test, healthy control subjects vs patients with RCA	<i>P</i> = .01	<i>P</i> < .0001	<i>P</i> = .001	<i>P</i> < .0001	<i>P</i> < .0001	<i>P</i> = .33
<i>t</i> Test, healthy control subjects vs patients with AD	<i>P</i> = .0001	<i>P</i> < .0001	<i>P</i> = .001	<i>P</i> = .03	<i>P</i> = .0001	<i>P</i> = .10

TABLE III. Relative proportions of CLA⁺ cells in relation to CD8⁺ lymphocyte subpopulations

	CLA ⁺ cells of			Perforin-positive cells of	
	All lymphocytes	CD8 ⁺ cells	CD8 ^{hi+} cells	CLA ⁺ CD8 ⁺ cells	CLA ⁺ CD8 ^{hi+} cells
Healthy control subjects, n = 7 (%)	5.1 ± 3.3	5.0 ± 2.9	2.5 ± 1.0	50 ± 11	38 ± 14
Patients with RCA, n = 7 (%)	6.2 ± 2.0	3.3 ± 1.5	1.3 ± 1.3	32 ± 10	17 ± 12
Patients with AD, n = 6 (%)	9.0 ± 3.0	5.6 ± 2.9	2.2 ± 2.0	22 ± 12	18 ± 10
<i>t</i> Test, healthy control subjects vs patients with RCA	<i>P</i> = .25	<i>P</i> = .17	<i>P</i> = .06	<i>P</i> = .003	<i>P</i> = .007
<i>t</i> Test, healthy control subjects vs patients with AD	<i>P</i> = .045	<i>P</i> = .39	<i>P</i> = .42	<i>P</i> = .001	<i>P</i> = .02

positive lymphocytes (12% ± 4%) compared with patients with AD with moderately elevated serum IgE levels (701 ± 813 kU/L; n = 7; SCORAD score, 47 ± 8; 17% ± 5%, *P* < .05). In addition, significantly lower numbers of CD56⁺ lymphocytes were found in patients with AD with high IgE serum levels (4% ± 2%) compared with patients with AD with moderately elevated serum IgE levels (7% ± 1.5%, *P* < .05).

Percentage of perforin-positive cutaneous lymphocytic antigen-positive CD8^{hi+} CTLs

In patients with AD, 9% ± 2% of all peripheral blood lymphocytes expressed the cutaneous lymphocytic antigen (CLA) compared with 5% ± 3% and 6% ± 2% in healthy control subjects and patients with RCA, respectively (*P* < .05). Among CD8⁺ subpopulations, no significant difference with regard to the CLA expression was detected between atopic patients and healthy control subjects (Table III). Among perforin-positive CD8^{hi+} cells, 3.7% ± 2.2% (patients with AD), 2.4% ± 1.2% (patients

with RCA), and 3.6% ± 1.7% (healthy control subjects; *P* > .05) expressed the CLA antigen. Focusing on the percentage of perforin-positive cells, a similar reduction level of perforin staining, as described in the overall population of CD8⁺ cells and the CD8^{hi+} subpopulation (Table II), was detected among CD8⁺ CLA⁺ and CD8^{hi+} CLA⁺ cells, respectively (Table III).

Perforin release assay

To address the possibility that the observed reduction of intracellular perforin-positive lytic granules was due to an increased release of secretory lysosomes, a direct assay for measuring perforin release in CD8^{hi+} CTLs was developed. To set up this assay, we first studied CD63 as a marker for exocytosis of secretory lysosomes in Ficoll-isolated PBMCs activated with PMA and ionomycin. Data in Fig 3 demonstrate that stimulation with ionomycin/PMA indeed resulted in an increased surface expression of the CD63 antigen, indicating its translocation from lytic granules to the cell surface during the secretion process.

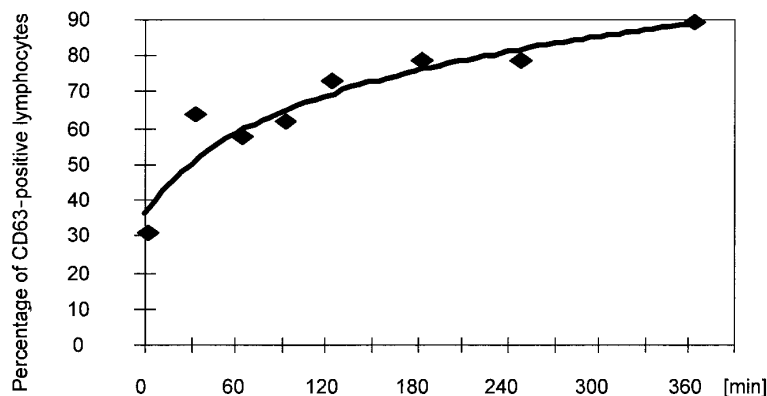


FIG 3. Time-dependent upregulation of CD63 on the surface of control lymphocytes after stimulation with ionomycin/PMA.

Subsequently, this ionomycin/PMA induction protocol was used to study the release of perforin-containing granules in lymphocytes from patients with AD, patients with RCA, and healthy control subjects (see below).

Accelerated perforin release in CD8^{hi} CTLs of atopic patients

Perforin release was quantified as the reduction of the perforin-positive portion of CD8^{hi} CTLs over time. Representative individual examples of these experiments are given for a patient with AD and a healthy control individual in Fig 4 (raw data). Cumulative relative data are summarized in Fig 5. In atopic patients, an average 50% of perforin-positive CD8^{hi} CTLs secreted their perforin completely within 32 ± 16 minutes (patients with AD) and 36 ± 19 minutes (patients with RCA), and 75% completed secretion within 74 ± 23 minutes (patients with AD) and 76 ± 44 minutes (patients with RCA), respectively. Eighty-five percent did so within 113 ± 43 minutes (patients with AD) and 118 ± 60 minutes (patients with RCA). A 90% release was reached within 240 minutes in 19 of 22 atopic patients. In contrast, perforin release in CD8^{hi} CTLs of healthy control subjects took almost twice as long. As an average, 50% of control cells released their perforin completely after 64 ± 40 minutes ($P = .01$), and 75% completed their release after 122 ± 54 minutes ($P = .02$). Eighty-five percent of release was not reached during 180 minutes in 7 of 13 healthy control subjects, and 90% release was reached only in 1 of 9 investigated control subjects. Thus perforin was released at a faster and more complete rate in atopic patients compared with healthy control subjects.

DISCUSSION

In atopic patients an allergen-induced activation of T_H2-type lymphocytes with subsequent predominance of T_H2-derived cytokines is supposed to initiate the pathophysiologic cascade. Factors discussed to contribute to T_H2 development are the cytokine milieu present at the time of antigen exposure, genetics, pharmacologic factors such as increased cyclic adenosine monophosphate

(cAMP)-phosphodiesterase enzyme activity of atopic leukocytes, and a predominant expression of costimulatory molecules favoring a T_H2 immune response, such as B7.2 in patients with AD.^{9,12} Moreover, microbial factors, such as superantigens, have been favored but are also discussed controversially as possibly decisive pathogenetic cofactors.¹³⁻¹⁶

The role of CD8⁺ lymphocytes in this process is not yet understood. Principally, they are able to regulate the T_H1/T_H2 balance.¹⁷ Recently, insights into a possible function of these cells was provided by a study reporting that CD8⁺ CLA⁺ T cells of patients with AD were stimulated to proliferate by superantigens. In addition, they were as potent as CD4⁺ CLA⁺ T cells in IgE induction and support of eosinophil survival.¹⁸ In patients with AD, a functional defect of CD8⁺ T cells was suspected early because of an increased susceptibility to viral infections of the skin, dysregulated Ig production, and autoimmune reactions found frequently in patients with AD. Indeed, several groups reported subnormal absolute and relative numbers of CD8⁺ T cells¹⁸⁻²⁰ or NK cells,²¹⁻²³ whereas the CD4⁺ subpopulation was reported to be slightly increased or unaltered. Thus a changed balance between CD8⁺ and CD4⁺ cells in the peripheral blood was found in patients with AD compared with healthy control subjects. Our results characterizing T lymphocytes and NK cells (Table I) are in line with the results of these studies.

Despite the moderate numeric reduction of CD8⁺ T cells in patients with AD, cytotoxicity evoked ex vivo by T cells was reported to be depressed rather markedly in patients with active AD.^{24,25} In addition, concanavalin A-induced suppressor cell function of CD8⁺ lymphocytes, which is now known to be mediated at least partly by T cell-mediated cytotoxicity, was shown to be decreased in patients with AD as well.²⁶⁻²⁹ However, the contribution of the perforin-granzyme system engaged by CTLs to eliminate their targets has until now not been investigated.

Here we report a significant (ie, 50%) reduction of perforin-positive lymphocytes in patients with AD ($14\% \pm 5\%$) compared with that found in healthy control subjects ($33\% \pm 11\%$, $P = .0001$; Fig 1, A). This was main-

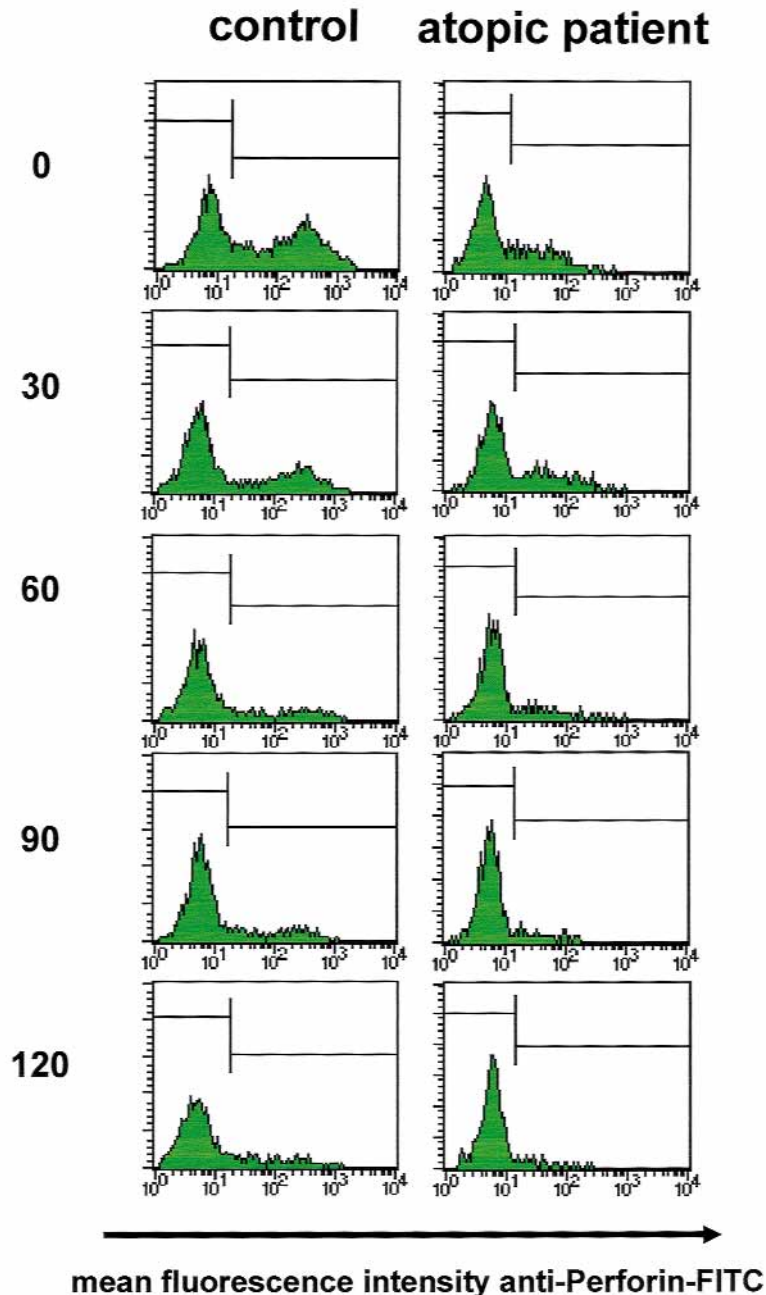


FIG 4. Accelerated perforin release from CD8^{hi+} CTLs of patients with atopy as detected in vitro during ionomycin/PMA stimulation (time course in minutes after onset of stimulation). Representative histogram analyses of CD8^{hi+} perforin-positive lymphocytes stimulated with ionomycin/PMA are presented for an individual patient with AD and compared with that of a healthy control subject. The y axis indicates counts of CD8^{hi+} lymphocytes.

ly caused by perforin depletion of CD3⁺ T cells, which dominate the lymphocytic population. In patients with AD, only $8\% \pm 3\%$ of CD3⁺ T cells stained positive for perforin, which was just about one third of control levels ($25\% \pm 9\%$). The percentage of perforin-positive CD56⁺ NK cells, however, was not altered significantly ($89\% \pm 6\%$ in patients with AD vs $89\% \pm 12\%$ in healthy control subjects, Table II). Thus perforin expression seems not to be generally depressed in patients with AD. NK cells did

contribute to the observed reduction of perforin expression in lymphocytes of patients with AD versus that of healthy control subjects only indirectly through the reduction of their relative amount within the lymphocytic population (ie, $6\% \pm 2\%$ vs $15\% \pm 8\%$, respectively).

Relating the percentage of perforin-positive cells to all lymphocytes, the overall population of CD8⁺ lymphocytes, and the subpopulation of CD8^{hi+} CTLs, an interesting difference between patients with AD and patients

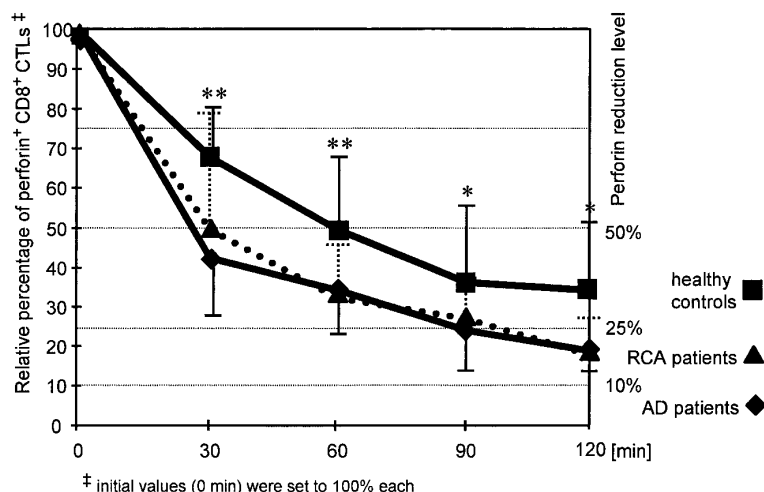


FIG 5. Perforin hyperreleasability in CD8^{hi} CTLs of patients with atopy. CD8^{hi} lymphocytes were analyzed for perforin content over 120 minutes after ionomycin/PMA stimulation. The initial value of the perforin-positive portion of CD8^{hi} lymphocytes was set to 100% each. Note that atopic patients released their perforin-containing cytolytic granules twice as fast and in a more complete manner compared with the healthy control subjects (* $P < .05$, ** $P < .005$).

with RCA was observed. Comparing patients with AD and RCA, there was a similar reduction of perforin-positive cells among the CD8^{hi} CTL population, whereas the reduction of perforin-positive cells was less pronounced in patients with RCA when relating to all lymphocytes, as well as to overall CD8⁺ cells (Table II). This may be explained by a relatively large amount of CD8^{lo} cells expressing perforin in patients with RCA.

We recently reported increased numbers of perforin-positive peripheral blood lymphocytes in other inflammatory skin diseases, such as exacerbated psoriasis and generalized drug eruptions.³⁰ Similar observations were made in patients with multiple sclerosis.³¹ The significant numeric reduction of perforin-positive T cells, however, seems to be a unique feature of atopic patients only.

Our perforin values for subpopulations of healthy control subjects are in line with data previously published by others.³¹⁻³³

Interestingly, patients with AD with serum IgE levels greater than 2000 kU/L showed significantly lower numbers of perforin-positive lymphocytes compared with patients with AD with moderate serum IgE levels (ie, <2000 kU/L). This correlation serves as indirect evidence that the observed perforin defect indeed could play an important role in dysregulating IgE production *in vivo*. CLA⁺ CD8⁺ AD lymphocytes were shown to be capable of inducing IgE production *ex vivo*.¹⁸ This may not be contradictory to our data because of a well-known functional heterogeneity among the CD8⁺ population.

To relate our findings to skin immunopathology, the skin homing receptor CLA was investigated. CLA⁺ percentages of CD8⁺ cells were in line with levels reported previously.¹⁹ Among CD8^{hi} CTLs, only 1.5% to 2.5% expressed the CLA antigen. There was no significant difference detectable between atopic patients and healthy control subjects. However, with regard to their perforin

content, CLA⁺ CD8^{hi} CTLs of atopic patients showed a similar degree of perforin depletion (as related to healthy control subjects) as that found in the total CD8^{hi} CTL population (Table II vs Table III). Thus in addition to a possible role of perforin-deficient CD8^{hi} CTLs in systemic immunopathology (ie, peripheral blood circulation), skin homing perforin-deficient CD8^{hi} CTLs may contribute most decisively to the immunologic imbalance observed in atopic lesions.

As to possible reasons for the striking feature of perforin depletion in atopic lymphocytes, either a diminished production of perforin or an increased rate of perforin secretion may come into consideration. To address the latter question, we developed a secretion assay for perforin-positive cytolytic granules focusing on CD8^{hi} lymphocytes.

To establish this assay, surface expression of the CD63 antigen was studied as a transmembranous protein with a steady state distribution on the plasma membrane and in secretory lysosomes. CD63 is brought to the plasma membrane during the secretion process.³⁴ By demonstrating that stimulation with ionomycin and PMA resulted in CD63 upregulation on the cell surface (Fig 3), experimental conditions were defined to study releasability of perforin-containing cytolytic granules further.

Therefore focusing on CD8^{hi} CTLs, the velocity of perforin release was determined as the relative reduction of perforin-positive CTLs over time by setting the initial value (percentage of perforin-positive CD8^{hi} CTLs before starting the stimulation) to 100%.

By using this experimental approach, a perforin hyperreleasability in CTLs of atopic patients was found as a striking and until now unknown feature (Fig 5). Whereas in healthy control subjects half of all initially perforin-positive CD8^{hi} CTLs released their perforin completely within 64 ± 40 minutes of stimulation and 75% did so

within 122 ± 54 minutes, perforin release was almost twice as fast in atopic patients (ie, 50% release within 32 ± 15 minutes in patients with AD and 35 ± 18 minutes in patients with RCA and 75% release within 74 ± 23 minutes in patients with AD and 76 ± 44 minutes in patients with RCA).

As an additional feature of this perforin hyperreleasability, CD8^{hi} CTLs of atopic patients did release their perforin more completely, as was the case in healthy control subjects. After 113 ± 43 minutes, 85% of initially perforin-positive CD8^{hi} CTLs of patients with AD had secreted their perforin-containing granules (patients with RCA, 118 ± 60 minutes). The majority of lymphocytes from healthy control subjects did not reach a similar extent of perforin reduction, even after 240 minutes (data not shown).

This hyperreleasability in CTLs of atopic patients might explain the reduced percentage of perforin-positive lymphocytes. However, whether perforin production might also be altered remains to be investigated in the future. An additional prospective study with newborns of atopic individuals and ex vivo experiments trying to induce hyperreleasability in CTLs of healthy control subjects may help to elucidate whether the distortions of the perforin system in atopic patients represents a primary or a secondary defect.

Recently, perforin-mediated killing of keratinocytes was demonstrated by using sulfamethoxazole-specific CD4⁺ and CD8⁺ T-cell clones. This effect was dependent on the presence of IFN- γ .³⁵ Therefore perforin hyperreleasable CD8^{hi} CTLs could contribute to local skin pathology by degranulation after an unspecific activation signal in the inflammatory microenvironment. In addition, CLA⁺ T cells were shown to be capable of Fas/Fas ligand-mediated killing of keratinocytes.³⁶ Because the perforin system is involved in downregulation of Fas-mediated killing,⁶ one may speculate that perforin-deficient lymphocytes might enhance Fas-based killing of keratinocytes by AD lymphocytes.

A faster release kinetics for histamine from basophilic granulocytes is a well-known feature in patients with atopy.^{28,37-40} Thus our data demonstrating hyperreleasability of cytotoxic T-cell granules can be integrated in a pathogenetic concept of altered releasability, which may be a factor relatively independent of the cell type in the pathomechanism of atopy.

In basophilic granulocytes of atopic patients, a decreased intracellular cAMP level caused by an increased activity of cAMP-phosphodiesterase is supposed to be involved in the accelerated histamine release kinetics.²⁸ Because the intracellular cAMP response is known to be decreased in atopic lymphocytes as well,^{41,42} and an increase of cAMP by, for example, forskolin and 3-isobutyl-1-methyl-xanthine can inhibit perforin secretion,⁴³ it may be speculated that the disturbed cAMP metabolism might be involved in the pathomechanism of perforin hyperreleasability observed in patients with AD. Interestingly, inhibitors of phosphodiesterase activity demonstrated a beneficial therapeutic effect in recent clinical studies with atopic patients. The influence of these substances on perforin hyperreleasability in vivo and ex vivo will have to be determined.

Taken together, our data demonstrate a significant reduction of CD8^{hi} CTLs containing perforin in atopic patients. In addition, perforin-containing CTLs of atopic patients do release their perforin faster and more completely in comparison with those of healthy control subjects. These results may contribute to a better future understanding of the molecular defects involved in the pathogenesis of dysregulated immune reactions in atopic patients.

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