

T_H2 lymphocytes from atopic patients treated with immunotherapy undergo rapid apoptosis after culture with specific allergens

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Background: In atopic patients treatment with specific immunotherapy (SIT) induced a shift in the balance of T-cell immune response away from a T_H2-type (producing mostly IL-4) in favor of a T_H1-type T-lymphocyte response (with the preferential production of IFN- γ). However, the mechanisms through which SIT acts are less clear. We have recently shown that allergens may induce an activation-induced cell death process in lymphocytes from SIT-treated atopic patients. **Objective:** This study aimed to determine whether allergen-induced apoptosis can occur in a specific subset of cells.

Methods: The study was performed in lymphocytes from normal subjects and atopic patients, some of whom were treated with SIT. Cells were cultured in the presence of gramineous pollen (*Lolium perenne*) allergenic extracts. Cell phenotype and intracellular cytokine expression were measured by means of fluorescent mAbs. Apoptosis was measured by using terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling. Fluorescence was analyzed in a FAC-Scan flow cytometer, and the data were evaluated with Consort 30 software.

Results: Our results showed that allergens induce apoptosis of lymphocytes in SIT-treated atopic patients. Apoptosis occurs mainly in T_H2 lymphocytes with the IL-4⁺/CD4⁺ phenotype and subsequently increases the percentage of IFN- γ ⁺ cells in the culture.

Conclusion: These results suggest that the shift from T_H2 to T_H1 induced by SIT in atopic patients may be mediated, at least in part, by the induction of an activation-induced cell death process in allergen-responder T_H2 cells. (J Allergy Clin Immunol 2001;107:647-53.)

Key words: Allergy, apoptosis, immunotherapy, lymphocytes, T_H1, T_H2

Abbreviations used

AICD: Activation-induced cell death
PE: Phycoerythrin
PMA: Phorbol-12-myristate-13-acetate
SIT: Specific immunotherapy
TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling

Allergy is characterized by the increased ability of some immunocompetent cells to respond to a group of ubiquitous antigens that activate the immune system after their inhalation, ingestion, or penetration through the skin.¹⁻³ In the course of the past few years, evidence has accumulated that suggests that allergen-reactive T cells play an important role in triggering the activation, recruitment, or both of B cells, mast cells, and eosinophils, the cell triad involved in allergic disease.^{4,5} Recent reports have demonstrated that T_H2 T lymphocytes (CD4⁺CD45RO⁺) that secrete cytokines, such as IL-4, IL-5, IL-10, and IL-13, are the principal type of T lymphocyte involved in the allergic process^{3,6,7} and that PBMCs from allergic patients respond with a T_H2 cytokine profile when activated in vitro with allergens.⁸⁻¹⁰

Lymphocytes become activated when antigen receptors on the cell surface are cross-linked or when they are exposed to agents that mimic these signals. Lymphocyte activation leads to the production of immune mediators (eg, antibodies and cytokines) and entry into the cell cycle. However, alternatively and under abnormal conditions, lymphocyte activation may lead to death caused by apoptosis in a process termed *activation-induced cell death* (AICD).¹¹ The AICD process was first observed in developing thymocytes and has been suggested as a mechanism for negative selection, by which immature cells with the potential for autoreactivity are removed. In addition, AICD has also been observed in normal mature lymphocytes under certain conditions,¹²⁻¹⁵ including those that may account for the phenomenon of peripheral depletion, in which mature T cells are eliminated on exposure to high doses of antigens. In addition, it has been suggested that the AICD process in lymphocytes may explain certain manifestations (eg, shortened lym-

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phocyte survival times, lymphopenia, inhibition of lymphocyte transformation, and increased presence of suppressor T cells) that have been reported in diseases characterized by cyclic activation of immune cells.^{13,15,16} In this respect we have recently shown that in PBMCs from atopic patients treated with specific immunotherapy (SIT), allergen stimulation induces an AICD process, and we suggest that this process may be involved in the mechanism of SIT.¹⁶

SIT with allergen-standardized extracts is probably the most effective treatment of allergy caused by a large number of allergens, including pollens, animal hair, and dander.^{3,17-24} Although it is clear that SIT reduces the symptoms of the allergic disease, the basic immunologic mechanisms involved in the amelioration of the allergic symptoms are not fully understood. Several changes in the T cell-mediated immune response, such as increased production of IFN- γ and a reduction in the production of IL-4, have been demonstrated in atopic patients undergoing SIT, suggesting that a shift in the balance between the lymphocyte response from T_H2 to T_H1 is involved in the SIT mechanism.^{3,19,25,26}

In this study we postulate that the T_H2 subset of lymphocytes from atopic patients treated with SIT undergo death by apoptosis when they are stimulated by allergens and that this mechanism may explain the change in the T_H response observed in atopic patients treated with SIT. To test this hypothesis, lymphocytes from atopic patients either untreated or treated with SIT were stimulated in vitro with allergens; after culture, we simultaneously determined, by using flow cytometry, the CD4/CD8 and CD45RO/CD45RA phenotypes, IL-4/IFN- γ production, and the percentage of cells undergoing apoptosis.

METHODS

Subjects

Patients were selected in accordance with the routine study protocols used in the allergy service at this hospital. According to the protocols, patients were allocated to one of 3 groups.

Group 1 consisted of atopic patients without immunotherapy ($n = 25$). These were patients with clinical rhinoconjunctivitis and seasonal asthma and with sensibility demonstrated by using the prick test, an IgE-specific test in the upper superior range at 2 for the CAP System (Pharmacia, Uppsala, Sweden), and test of ocular provocation to gramineous pollen (*Lolium perenne*). These patients had not previously been subjected to immunotherapy with allergenic extracts.

Group 2 consisted of atopic patients with immunotherapy ($n = 25$). These were patients with clinical rhinoconjunctivitis and seasonal asthma, who showed sensibility to *Lolium perenne* by means of the prick test, specific IgE test, and ocular provocation test. They also had been subjected to immunotherapy with an allergenic extract standardized in biologic units (E.H. RETARD C.B.F. LETI), the effectiveness of which was demonstrated for our group in a previous study,²⁷ according to a weekly standard rule until they reached the maximum monthly dose. By the time of the study, this group had been treated for more than 2 years with the maximum tolerated dose.

Group 3 consisted of nonatopic subjects ($n = 10$). These were subjects without clinical allergology, whose sensitivity to *Lolium perenne* after application of the protocol described above had not been demonstrated.

Preparation of human mononuclear cells

Circulating human PBLs were obtained from whole blood donated by 10 healthy volunteers and 50 atopic patients. Buffy coat cells were separated by using a differential centrifugation gradient (Ficoll/Hypaque, Pharmacia). The mononuclear cells were then washed and used for culture.

Cell culture

Cells were seeded for 2 days on 96-well culture plates (Falcon; Becton Dickinson and Co, Paramus, NJ) in complete culture medium containing RPMI-1640 culture medium supplemented with L-glutamine (2 mmol/L), HEPES (20 mmol/L), sodium pyruvate (1 mmol/L), streptomycin (50 ng/mL), penicillin (100 UI/mL), and 10% human autologous serum (Bio-Whittaker, Walkersville, Md). Serum was heated to 56°C for 60 minutes to inactivate the complement. Under these conditions, cells were cultured for 48 hours at 2×10^5 cells per well in the presence of 30 μ g/mL allergenic extract (*Lolium perenne*). The quantity of extract to be used had previously been determined by means of a dose-response assay. In addition, phorbol-12-myristate-13-acetate (PMA) at 10 μ g/mL plus ionomycin 0.5 μ mol/L (Sigma-Aldrich, St Louis, Mo) was used as cell stimulation, and incubation with medium alone was used as a negative control. In some of the experiments, bee venom (250 ng/mL) was used as an unrelated control in PBL cultures from atopic subjects with and without SIT. Because cytokines produced as a consequence of cell activation are released outside the cells, Brefeldin A (Sigma-Aldrich) inhibits intracellular transport, and cytokines produced during the last hours of cell stimulation will be retained inside the cell. Brefeldin A, 2 μ g/mL, was added to in vitro cultures 4 hours before the end of culture.

Cell fluorescence

Three hundred thousand cells were washed with PBS buffer (PBS/0.1% Na₃ and 5% human autologous serum) and incubated for 30 minutes at 4°C with 20 μ L of the appropriate mAb. Cells were then washed in cold PBS buffer and analyzed in the flow cytometer. Background fluorescence was determined by using mAb-conjugated mouse IgG. Cytofluorometric analysis was performed with a FACScan cytometer (Becton Dickinson, San Jose, Calif). Data were evaluated with Consort 30 software (Becton Dickinson). In these experiments the forward- and side-light scatter analysis in the flow cytometer identified a gate in which PBLs were grouped, and other cells, mainly monocytes and leukocytes, were gated out according to their forward-light and side-light scatter characteristics. Necrotic cells were eliminated on the basis of their forward-light scatter characteristics.²⁸

In all cases mAbs (and their respective isotype-matched controls) were fluorescence-conjugated antibodies. Antibodies used to detect surface antigens were labeled with TRI-COLOR (R-PE-Cy5 Tanden) and were anti-CD3, anti-CD4, anti-CD8, anti-CD45RA, and anti-CD45RO (Caltag Laboratories, Burlingame, Calif). Cells (10^5 /mL) were incubated for 30 minutes at 4°C with the mAb.

Intracellular cytokine staining

After surface phenotyping (see above), cells (10^6 /mL) were washed, fixed, and permeabilized by using the FIX & PERM cell permeabilization kit (Caltag Laboratories). Briefly, for each sample to be analyzed, 100 μ L of cells was incubated with the fixation medium. After incubation for 5 minutes at room temperature, 4 mL of precooled absolute methanol was added and incubated for 10 minutes at 4°C. Cells were then washed (for 5 minutes at 1500 rpm) in wash buffer (PBS, 0.1% Na₃, and 10% autologous serum). To permeabilize the cells, the supernatant was removed, and 100 μ L of permeabilization reagent and the appropriate conjugated antibody or corresponding isotype control were added. After incubation, cells were washed and resuspended in 0.5 mL of 1% formaldehyde and stored at 4°C until flow cytometer

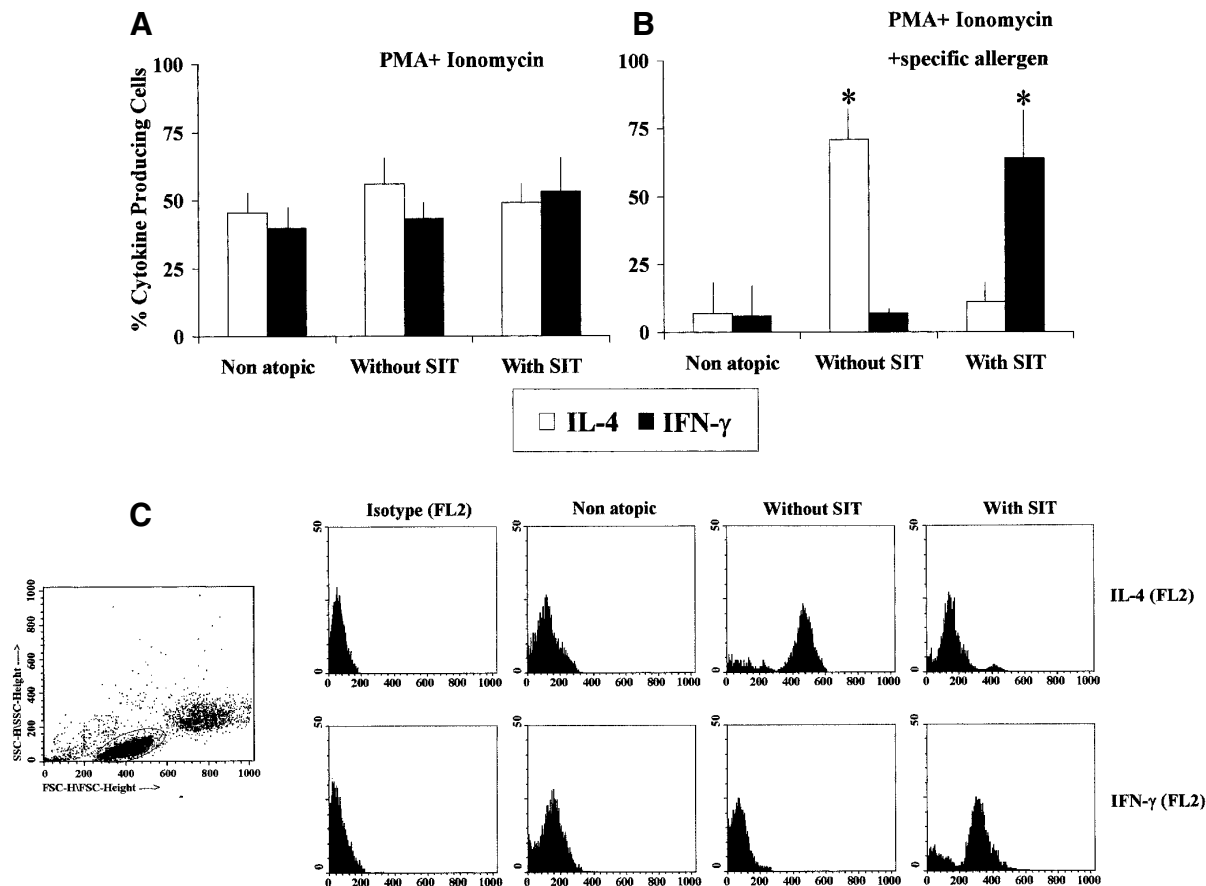


FIG 1. Intracytoplasmic cytokine expression in peripheral blood cells from control subjects and atopic patients after stimulation with allergens. **A**, Cells (10^6 /mL) were cultured for 4 hours with $10 \mu\text{g/mL}$ PMA plus $0.5 \mu\text{mol/L}$ ionomycin and $2 \mu\text{g/mL}$ Brefeldin A. Intracellular IFN- γ and IL-4 production was analyzed with specific mAbs. **B**, Cells (10^6 /mL) were cultured for 48 hours with $30 \mu\text{g/mL}$ allergenic extract (*Lolium perenne*). After culture, cells were incubated with $10 \mu\text{g/mL}$ PMA, $0.5 \mu\text{mol/L}$ ionomycin, and $2 \mu\text{g/mL}$ Brefeldin A for 4 hours, washed, fixed, and permeabilized, and intracellular cytokine production was determined by using specific mAbs. The data represent the mean $\pm$ SE from 50 atopic patients (25 treated with SIT and 25 SIT-untreated patients) and 10 nonatopic subjects (* $P < .01$ vs nonatopic subjects). **C**, The selected cell population (forward-light scatter and side-light scatter) and intracellular cytokine marking is shown, including the anti-isotype control in cells of atopic and nonatopic patients with and without SIT-treated specific allergens.

analysis. For intracellular cytokines, mAbs were conjugated with phycoerythrin (PE) and were anti-IL-4 and anti-IFN- γ (Becton Dickinson). In all cases a fluorescent isotype control antibody was used.

Cell apoptosis

Apoptosis-associated DNA strand breaks were studied by using a kit based on the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) method (Boehringer Mannheim). In accordance with the manufacturer's instructions, 10^6 cells were fixed with 4% paraformaldehyde for 30 minutes at room temperature, washed, and permeabilized for 2 minutes on ice with 0.1% Triton X-100. After washing, cells were decanted and resuspended in $50 \mu\text{L}$ of TUNEL reaction mixture ($5 \mu\text{L}$ of TUNEL enzyme containing terminal deoxynucleotidyl transferase mixed with $45 \mu\text{L}$ of TUNEL label containing fluorescein-dUTP and deoxyribonucleoside triphosphate nucleotides) or in $50 \mu\text{L}$ of TUNEL label as a negative control. After 60 minutes of incubation at 37°C in a humidified atmosphere,

cells were washed 3 times in wash buffer (PBS, 0.1% NaN_3 , and 10% autologous serum) and analyzed by means of flow cytometry.

Simultaneous detection of apoptosis, cytokine expression, and surface phenotype

Cells were previously labeled with TRI-COLOR-conjugated antibodies, as described above. After washing, cells were fixed, permeabilized, and labeled with PE-conjugated anticytokine antibodies and TUNEL. In all cases the respective PE-, TRI-COLOR-, and TUNEL-labeled controls were used. After staining, cells were analyzed in a FACScan flow cytometer acquiring 10,000 events.

Statistical analysis

Differences between means were analyzed with ANOVA, followed by the Duncan test. An unpaired t test was used to compare 2 means. Results are presented as the mean $\pm$ SE.

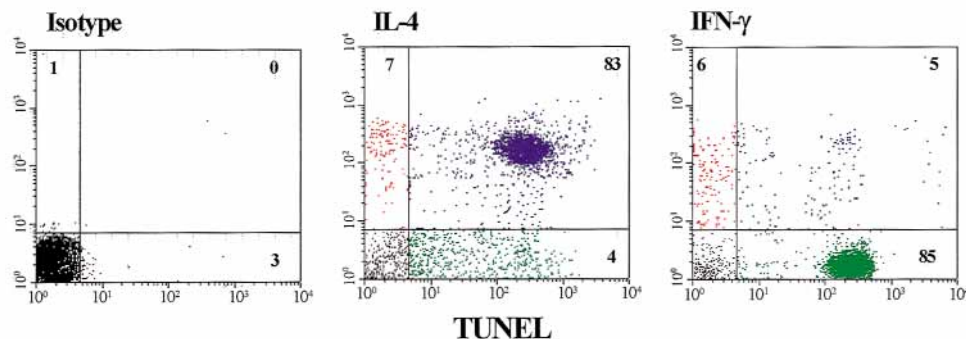


FIG 2. Representative flow cytometric simultaneous study of intracytoplasmic cytokine production and apoptosis in cells from SIT-treated atopic patients cultured for 48 hours with 30 μ g/mL allergenic extract. IL-4/IFN- γ is presented on the y-axis, and TUNEL labeling is presented on the x-axis. Dot plots show events gated in the lymphocyte region of the forward/side-light scatter dot plot corresponding to apoptotic cells. Percentages of events in quadrants are indicated on each dot plot.

TABLE I. Expression of IL-4 and IFN- γ by CD4 or CD8 lymphocytes

Patients	IL-4		IFN- γ	
	CD4	CD8	CD4	CD8
No SIT	89 $\pm$ 12	11 $\pm$ 4	22 $\pm$ 13	27 $\pm$ 7
SIT	91 $\pm$ 18	6 $\pm$ 3	32 $\pm$ 9	34 $\pm$ 8

The percentage of CD4⁺ and CD8⁺ cells was studied in PBLs expressing intracytoplasmic IL-4 or IFN- γ (histogram region in IL-4⁺ or IFN- γ ⁺ PBLs). Results are the mean $\pm$ SE of all patients studied. For each patient, data were calculated as the mean of 3 different experiments. No significant differences were observed between patients receiving and not receiving SIT.

TABLE II. Expression of IL-4 and IFN- γ by CD45RA/RO lymphocytes

Patients	IL-4		IFN- γ	
	CD45RO	CD45RA	CD45RO	CD45RA
No SIT	76 $\pm$ 17	13 $\pm$ 4	81 $\pm$ 16	17 $\pm$ 8
SIT	83 $\pm$ 19	9 $\pm$ 6	82 $\pm$ 9	12 $\pm$ 7

The percentage of CD45RA/RO cells was studied in PBLs expressing intracytoplasmic IL-4 or IFN- γ (histogram region in IL-4⁺ or IFN- γ ⁺ PBLs). Results are the mean $\pm$ SE of all patients studied. For each patient, data were calculated as the mean of 3 different experiments. No significant differences were observed between patients receiving and not receiving SIT.

RESULTS

Allergens induce cytokine production in PBMCs from atopic patients

The IL-4/IFN- γ cytokine profile was studied in lymphocytes that had been isolated from normal and atopic subjects treated or untreated with SIT and cultured with PMA plus ionomycin and specific allergens. No significant differences were found in the expression of IFN- γ or IL-4 in cells from allergic or control subjects with or without SIT stimulated with PMA plus ionomycin (Fig 1, A). After culture with allergens, only cells from atopic patients secreted cytokines (Fig 1, B and C).

The cytokine profile in cells from SIT-untreated or SIT-treated patients shows that in atopic patients without SIT, a low percentage of cells produce IFN- γ (7% $\pm$ 3%), whereas a high percentage produce IL-4 (71% $\pm$ 12%). In contrast, in cells from atopic patients treated with SIT, the percentage of cells that produces IFN- γ was 64% $\pm$ 18%, whereas the percentage that produces IL-4 was 11% $\pm$ 7% (Fig 1, B). In all cases the controls from cytokine production in cells stimulated with PMA plus ionomycin show a similar percentage of IFN- γ - and IL-4-producing cells.

Simultaneous study of cytokine expression and CD4/CD8 and CD45RO/RA phenotype

To study whether cytokine production induced by allergens occurs preferentially in particular subsets of lymphocytes, a simultaneous analysis of cytokine production and cell phenotype was performed by using a dual-color analysis with FACScan.

IL-4 is expressed mainly by CD4⁺ cells in both SIT-untreated patients (84% $\pm$ 12%) and SIT-treated patients (91% $\pm$ 18%). In contrast, IFN- γ was similarly produced by CD4⁺ and CD8⁺ cells, and no differences between SIT treated or untreated patients could be seen (Table I). The study of the CD45RO/RA phenotype shows that in both patient groups, cytokines were produced mainly in CD45RO cells (Table II), and these cells produce either IL-4 or IFN- γ .

Allergens induce apoptosis of cells from SIT-treated atopic patients in whom IL-4 cytokine is expressed

To study whether PBMC apoptosis induced by allergens in patients undergoing SIT occurs preferentially in a subset of cells with a specific cytokine profile, we simultaneously studied, in the same permeabilized cell, the expression of cytokines and apoptosis.

Lymphocytes from atopic patients not undergoing SIT cultured with allergens did not increase apoptosis. In

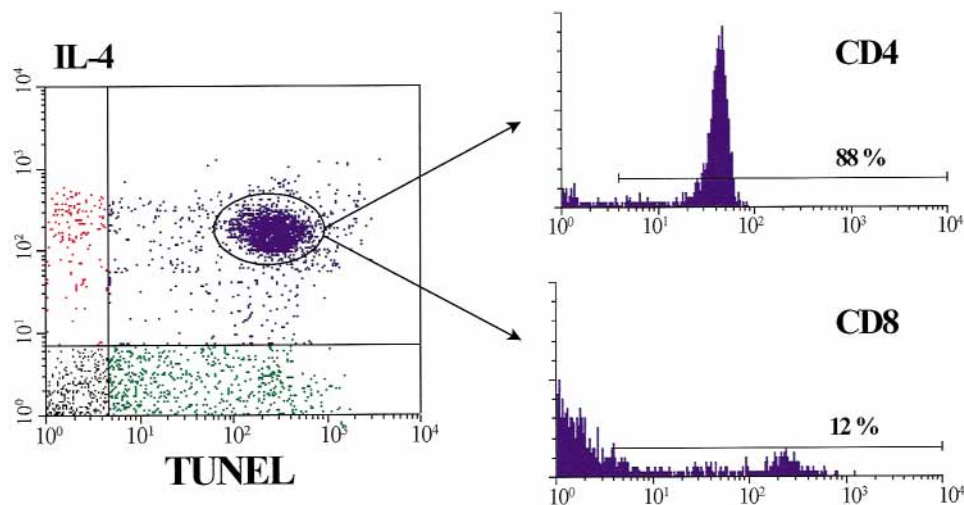


FIG 3. Study of intracytoplasmic cytokine production and CD4/CD8 phenotype in apoptotic cells. Triple labeling was performed on cells from SIT-treated atopic patients cultured for 48 hours with 30 μ g/mL allergenic extract. After culture, cells were permeabilized and labeled simultaneously with PE-conjugated anti-cytokine antibodies, TRI-COLOR-conjugated antibodies, and TUNEL by using the respective PE-, TRI-COLOR-, and TUNEL-labeled controls. After staining, cells were studied by using flow cytometry. Apoptotic cells were gated, and their IL-4/IFN- γ expression was determined. Cytokine-producing cells were then also studied for CD4/CD8 expression. The figure shows a representative example of the triple cell staining.

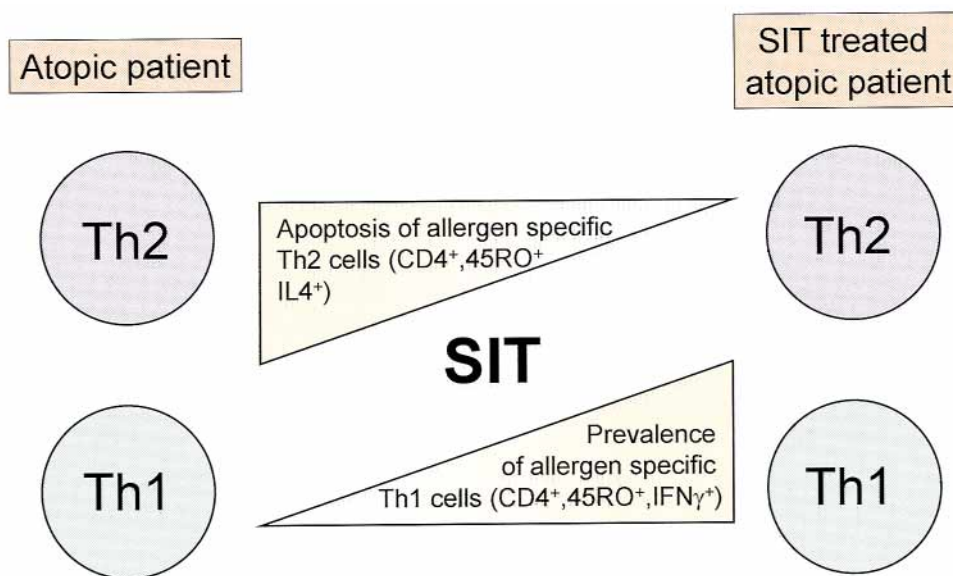


FIG 4. Schematic model of the possible involvement of allergen-induced apoptosis in the T_H2 -to- T_H1 shift after SIT. Repeated allergen inoculation induces apoptosis of T_H2 cells in atopic patients, which will cause a decrease of T_H2 cells and a prevalence of T_H1 cells after SIT.

contrast, a significant percentage of cells from atopic patients receiving SIT ($39\% \pm 14\%$) undergo apoptosis after culture with allergens. In both groups of patients, no apoptosis was observed when control cells were stimulated with PMA.

In apoptotic cells from patients receiving SIT, the cytokine profile shows that apoptotic cells mainly expressed IL-4 ($84\% \pm 11\%$, Fig 2), whereas a smaller percentage of cells ($4\% \pm 2\%$) expressed IFN- γ . The phe-

notype shows that $88\% \pm 17\%$ of apoptotic cells that were IL-4 $^+$ exhibit the CD4 $^+$ phenotype (Fig 3).

DISCUSSION

It is becoming clear that cytokines play an important role in allergy. In this respect the shift from T_H1 to T_H2 , induced after immunotherapy treatment in atopic patients, has been suggested as one of the possible mech-

anisms through which specific immunotherapy operates effectively. In an earlier report we demonstrated that allergens may induce apoptosis in allergen-responder lymphocytes from atopic patients treated with specific immunotherapy.¹⁶ In this case we studied in vitro cells from atopic patients either untreated or treated with SIT and the cytokine profile of allergen-responder cells and attempted to determine whether the apoptosis induced by allergens occurs specifically in a T_H subset of cells. For this purpose, we cultured peripheral blood cells for 48 hours with specific allergens and measured the profile of T_H1 or T_H2 cytokine production and apoptosis in those cells. Our results show that PBMCs from atopic patients produce cytokines after culture with allergens. In atopic patients without SIT, allergen-responder cells were mainly T_H2 cells that produced IL-4. In contrast, in atopic patients with SIT, allergen-responder cells exhibit a predominantly T_H1 profile and produce IFN- γ . In both patient groups CD4⁺ cells were the principal cells that produced IL-4. In contrast, IFN- γ was expressed similarly by both CD4⁺ and CD8⁺. The CD45RO/RA phenotype showed that either IL-4 or IFN- γ was produced mainly by CD45RO⁺ cells. Allergens induce apoptosis only in cells from SIT-treated patients, and apoptotic cells were mainly in T_H2 cells that expressed the CD4⁺ phenotype. These results suggest that induction by allergens of apoptosis in the IL-4-producing cells may be involved at least in part in the T_H1 -to- T_H2 shift induced in atopic patients by using SIT.

Freshly isolated lymphocytes did not normally produce cytokines unless stimulated. Therefore to study the ability of PBMCs from atopic patients to produce cytokines, cells were cultured with specific allergens. In accordance with the classical cytokine model of allergy,²⁹ we have shown that the highest proportion of cytokine-produced PBMCs from atopic patients without SIT was T_H2 cells and produced IL-4. In contrast, the largest percentage of cytokines produced by cells from atopic patients receiving SIT consisted of T_H1 cells and produced IFN- γ . These results support that the allergen-specific T-cell responses belong to the T_H2 subset. Furthermore, the fact that patients receiving SIT cells modified their cytokine profile from IL-4 to IFN- γ may indicate that some of the effect of immunotherapy may be associated with changes in cytokine-producing cells.

This study shows that CD4⁺ and CD45RO⁺ are the principal types of T cells involved in the production of IL-4 in the allergic response. Earlier reports demonstrated an increase in CD4⁺/CD45RO⁺ cells as a consequence of allergic reactions.^{30,31}

Our results indicate that the IL-4-producing cells were primarily CD4⁺ in spite of the fact that the percentage of CD4 cells that produce IL-4 is much lower than in patients not receiving SIT.

The fact that only the cells cultured with allergens induce apoptosis in CD4⁺ cells from patients receiving SIT suggests that apoptosis is triggered by specific mechanisms. Furthermore, apoptotic cells were also IL-4⁺ cells, which supports the idea that the subsets of cells that

express IL-4 may be involved in the atopic response.

Our results indicate that allergens induce apoptosis in IL-4⁺/CD4⁺ cells from atopic patients receiving SIT. It is likely that, as has been observed in vitro, in vivo immunotherapy with allergens will induce apoptosis in IL-4⁺/CD4⁺ cells, and this mechanism may be involved in immunotherapy treatment. On the other hand, these results suggest (Fig 4) a new perspective that may explain the shift from T_H2 to T_H1 phenotype observed in SIT-treated patients, suggesting that immunotherapy did not change the profile of cytokine production by T cells, while inducing the elimination of T_H2 cells and encouraging the preponderance of T_H1 cells.

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