

Enhanced expression of Fas ligand (CD95L) on T cells after segmental allergen provocation in asthma

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Background: Little is known about the termination of the T-cell driven inflammation found in patients with allergic asthma.

Objective: Because signals delivered through Fas/Fas ligand can lead to T-cell apoptosis, we investigated the expression of Fas and Fas ligand on peripheral blood- and bronchoalveolar lavage fluid (BALF)-derived T cells and the percentage of apoptotic BALF cells in asthma.

Methods: Nine atopic subjects with mild asthma and 9 control subjects underwent segmental sham and allergen challenge. Flow cytometry was used to determine the T-cell expression of Fas and Fas ligand, and the terminal dUTP nick end labeled technique was applied to detect apoptotic BALF cells.

Results: In asthmatic and control subjects almost all T cells in the BALF expressed Fas antigen without changes after saline or allergen challenge. A small percentage of T cells in BALF expressed the Fas ligand. In asthmatic subjects, but not in control subjects, there was a significant increase in Fas ligand after allergen challenge (CD3: $0.8\% \pm 0.6\%$ [baseline] vs $3.2\% \pm 1.2\%$ [allergen challenge]; CD4: $1.8\% \pm 0.0\%$ vs $4.3\% \pm 1.8\%$; CD8: $2.8\% \pm 2.4\%$ vs $9.1\% \pm 4.8\%$) but not after saline challenge, with a significant correlation to the percentage of BALF eosinophils. Apoptotic BALF cells were localized exclusively in macrophages at a very low frequency (0.03% to 0.15%) and without changes after saline or allergen challenge in both groups.

Conclusion: In asthma there is an upregulation of Fas ligand on T cells in BALF after allergen challenge. Because there is no evidence for increased apoptosis, this phenomenon may reflect antigen-induced T-cell activation rather than apoptosis. (*J Allergy Clin Immunol* 1999;103:649-55.)

Key words: Apoptosis, Fas, Fas ligand, T cell, asthma

Asthma is characterized as a chronic inflammatory disease of the bronchial mucosa, and T cells play an important role in orchestrating the disease process.¹ Lung T cells in patients with asthma show signs of activation and an enhanced expression of T_{H2}-type cytokines.^{2,3} After allergen challenge, an accumulation

Abbreviations used

BAL:	Bronchoalveolar lavage
BALF:	Bronchoalveolar lavage fluid
FL-1:	Fluorescence 1
PE:	Phycoerythrin
TdT:	Terminal deoxynucleotidyl transferase
TUNEL:	Terminal dUTP nick end labeling

of CD4⁺ T cells in the lung has been demonstrated,⁴ but little is known about the mechanisms that terminate the T cell-driven process. Because lymphocyte numbers in the lung are the result of entry, exit, proliferation, and cell death, data on the ultimate fate of T cells in asthma are important to understand the dynamic changes after allergen challenge.⁴ T cells triggered through the T-cell receptor can lead to activation-induced cell death (apoptosis),^{5,6} and apoptosis after allergen-specific T-cell activation might be involved in the elimination of T cells in allergic inflammation. A possible mechanism of this programmed cell death is mediated through the Fas antigen, a cell surface molecule that is a member of the TNF receptor family.⁷ It has been shown that the interaction of Fas antigen (CD95) with its ligand (CD95L), which is also expressed on activated T cells,⁸ is involved in activation-induced T-cell apoptosis.⁹ Recently, in vivo experiments in a murine model have shown for the first time that lung lymphocyte elimination after intratracheal antigen challenge is mediated by apoptosis, which is dependent on the expression of Fas and its ligand.¹⁰ It is not known, however, whether these mechanisms are involved in allergic inflammation (eg, in asthma). Therefore the hypothesis was proposed that in asthma there is enhanced expression of Fas antigen and/or Fas ligand, leading to increased T-cell apoptosis, especially after allergen exposure. The aim of this study was to determine the expression of Fas and Fas ligand on peripheral blood- and bronchoalveolar lavage fluid (BALF)-derived T cells in asthmatic subjects undergoing segmental allergen challenge and to examine the appearance of apoptosis in BALF cells.

METHODS

Study subjects

Nine patients with mild asthma (mean age, 25 ± 3 years; 2 women and 7 men; mean FEV₁, $99\% \pm 12\%$ of predicted value; geometric mean PC₂₀, 5.1 mg/mL histamine; geometric mean IgE,

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155 kU/L) and 9 normal control subjects (mean age, 28 ± 4 years; 2 women and 7 men; mean FEV₁, $102\% \pm 4\%$ of predicted value; geometric mean PC₂₀, 14.5 mg/mL histamine; geometric mean IgE, 10 kU/L) participated in the study. All patients had mild allergic asthma as defined in the consensus report.¹¹ They had a history of intermittent wheeze with reversible airflow obstruction, and asthma had previously been diagnosed by an independent physician. Most of them had seasonal asthma ($n = 8$) and were tested out of their season. Each patient had a positive skin prick test response, defined as a greater than 4 mm diameter skin wheal response to 1 or more of 8 common allergens (*Dermatophagoides pteronyssinus*, *D farinae*, mixed grass pollen, mixed tree pollen, dog hair, feather, cat fur, and *Alternaria* spp; allergen extraction from Abelló, Bornheim, Germany). The allergen extract used for segmental allergen challenge (Mixed grass pollen or *D pteronyssinus*; Abelló) was that which produced the largest wheal response on skin prick testing. On the day of bronchoscopy, a skin wheal dose-response series was undertaken with this allergen using 10-fold dilutions. The concentration chosen for the endobronchial challenge was one tenth the dilution in saline that elicited a 3 mm diameter skin wheal response. The instilled amount of antigen was 0.01 to 0.1 µg for mixed grass pollen (mixture of the major allergens Dac g 5, Fes p 5, Lol p 5, Phl p 5, and Poa p 5) and 0.6 µg for *D pteronyssinus* (mixture of the major allergens Der p 1 and Der p 2). Bronchial hyperresponsiveness was determined at a screening visit by a modified bronchoprovocation test with histamine as described elsewhere.¹² Histamine was nebulized in a 10-L reservoir by a pressure nebulizer (Pari Provocation test II, Starnberg, Germany) with increasing doubling concentrations, and aerosol was inhaled through a 1-way valve. The PC₂₀ was calculated as the cumulative concentration of histamine that reduced FEV₁ by 20% of postsaline baseline as derived by linear interpolation between the last 2 points of the log concentration-response curve. Patients were only using salbutamol, administered by metered-dose inhaler, when required for relief of symptoms; none were treated with corticosteroids, sodium cromoglycate, or theophylline. The normal control subjects had no history of allergic or other diseases, negative skin prick test responses, normal IgE (≤ 100 IU/mL), normal lung function test results, and no bronchial hyperresponsiveness (PC₂₀ > 8 mg/mL). All study subjects were nonsmokers, and no subject had an episode of acute bronchitis 4 weeks before the investigations. All subjects were volunteers and gave their written consent after being fully informed about the purpose and nature of the studies which were approved by the Ethical Committee of Hannover Medical School.

Segmental allergen challenge

Segmental allergen challenge was performed as previously described.¹³ A commercially available endotoxin detection assay (E-toxate, Limulus ameobocyte lysate; Sigma, St Louis, Mo) was used to demonstrate that the instilled saline and allergen solutions were free of endotoxin. Lung function tests and collection of 5 mL of venous blood in tubes containing EDTA were performed, and all subjects received nebulized salbutamol (1.25 mg), atropine (0.5 mg subcutaneously), and midazolam (mean, 4.7 mg; range, 2 to 8 mg) before the bronchoscopy. Oxygen (100%) was delivered by means of the nasal cannulae throughout the procedure (2 to 4 L/min), and oxygen saturation was monitored with a digital oximeter. Lidocaine (4%) was sprayed into the upper airways to achieve local anesthesia. The bronchoscope (P30; Olympus Optical, Tokyo, Japan) was then passed through the mouth or nares, and lidocaine (2%) was introduced through the bronchoscope to anesthetize the larynx and lower airways. The instrument was wedged into the inferior lingular bronchus, and the bronchus underwent lavage with 100 mL of sterile saline solution prewarmed to 37°C. Bronchoalveolar lavage (BAL) was performed by instilling 20-mL aliquots, which were

aspirated by using gentle suction. The instrument was passed into the superior lingular bronchus, and 10 mL of prewarmed saline solution was instilled as a control challenge. Finally, the bronchoscope was passed to the medial segment of the middle lobe, and 10 mL of prewarmed allergen solution was instilled. After control and allergen instillation, the appearance of the airways was observed for 5 minutes. Bronchoconstriction was observed in each case after allergen provocation but in no case after saline instillation. After 24 hours, subjects (all asthmatic subjects and 5 control subjects) were rebronchoscoped with the same premedication, and the superior lingular bronchus and the medial middle bronchus underwent lavage with 100 mL of prewarmed saline. After each bronchoscopy, all subjects were observed closely for 4 hours and given a contact telephone number when they left the department.

Processing of cells and flow cytometric analysis

BALF and peripheral blood samples were processed as described.¹⁴ Briefly, BALF samples were filtered through a 100-µm filter (Becton Dickinson, Heidelberg, Germany), centrifuged at 250g for 10 minutes, and washed in PBS. The total count of nucleated cells was performed by using a Neubauer hemocytometer, and an aliquot was separated for cytospin preparations. Cytospin slides were made with a Shandon cytospin device (Life Sciences International, Frankfurt, Germany), and differential cell counts were performed after staining with May-Grünwald-Giemsa stain, with 300 cells per slide being counted.

Flow cytometry was used to identify T-cell subsets and to determine the T-cell expression of Fas and Fas ligand. BAL cells resuspended in PBS (100 µL, 1×10^6 cells/mL) or whole blood (100 µL) were incubated for 15 minutes in the presence of anti-CD95-phycoerythrin (PE) (final concentration, 0.5 µg/100 µL mouse IgG1, Pharmingen), biotinylated anti-CD95L (0.5 µg/100 µL mouse IgG1, Pharmingen), and one of the following antibodies: anti-CD3-FITC (0.5 µg/100 µL mouse IgG1, Becton Dickinson), anti-CD4-FITC (0.5 µg/100 µL mouse IgG1; Medac, Hamburg, Germany), or anti-CD8-FITC (1.5 µg/100 µL mouse IgG1; Coulter, Krefeld, Germany). Cells were washed and incubated with 5 µL of streptavidin-Tricolor (Medac) for 15 minutes. Erythrocytes were lysed (lysing solution, Becton Dickinson), leukocytes were washed with PBS, and cells were kept at 4°C for no more than 24 hours in the dark until analyzed. Negative controls consisted of isotype-matched, PE- or FITC-labeled, nonspecific antibodies (Becton Dickinson), and in the case of the biotinylated antibody, the primary antibody was omitted. Flow cytometric analysis was performed with scatter gates on the lymphocyte fraction by using a flow cytometer (Calibur, Becton Dickinson) equipped with an argon ion laser and appropriate filters for FITC (fluorescence-1 [FL-1]), PE (FL-2), and Tricolor (FL-3). A total number of 40,000 to 50,000 events was recorded to ensure that more than 4000 events were detectable in the lymphocyte gate. The percentages of CD3⁺, CD4⁺, and CD8⁺ lymphocytes and the percentage of CD3⁺, CD4⁺, and CD8⁺ lymphocytes bearing the surface markers CD95 or CD95L were calculated from the counted events in the quadrants of the FL-1/FL-3 (CD95L) and FL-1/FL-2 (CD95) dot plots.

Detection of apoptotic BALF cells

To detect cells with DNA fragmentation undergoing apoptosis, a modified terminal dUTP nick end labeled (TUNEL) method was used as previously described.¹⁵ Briefly, BALF-cytospin slides were fixed with 4% paraformaldehyde (pH 7.4 in PBS) for 20 minutes and incubated with 70% ethanol for 30 minutes at room temperature. After washing (0.05% Tween in Tris-buffered saline), the slides were incubated with FITC-labeled dUTP (1.7 nmol/L) and

TABLE I. Leukocytes and lymphocyte subpopulations in BALF at baseline and after diluent and allergen challenge

	Total cell count ($\times 10^6$)	Macrophages (%)	Eosinophils (%)	Neutrophils (%)	Lymphocytes (%)	CD3 ⁺ (%)	CD4 ⁺ (%)	CD8 ⁺ (%)
Asthma								
BAL baseline (n = 9)	10.2 $\pm$ 3.4	87.9 $\pm$ 10.9	0.9 $\pm$ 1.2	0.9 $\pm$ 1.6	10.9 $\pm$ 10.6	93.4 $\pm$ 2.0	57.9 $\pm$ 15.2	35.8 $\pm$ 15.0
BAL diluent (n = 9)	19.2 $\pm$ 11.8	66.5 $\pm$ 21.7*	0.4 $\pm$ 0.6	27.0 $\pm$ 23.2*	6.0 $\pm$ 4.7	91.0 $\pm$ 4.1	60.7 $\pm$ 11.9	29.9 $\pm$ 12.8
BAL allergen (n = 9)	67.1 $\pm$ 113.7	43.6 $\pm$ 21.9*	21.7 $\pm$ 21.3*	21.4 $\pm$ 22.6*	3.9 $\pm$ 1.7*	85.2 $\pm$ 3.6	65.8 $\pm$ 12.3	26.6 $\pm$ 10.3
Control								
BAL baseline (n = 9)	6.5 $\pm$ 2.2	92.8 $\pm$ 2.4	0.1 $\pm$ 0.2	1.1 $\pm$ 1.2	5.9 $\pm$ 2.7	92.2 $\pm$ 2.2	55.8 $\pm$ 13.2	34.8 $\pm$ 10.0
BAL diluent (n = 5)	22.8 $\pm$ 26.3	72.6 $\pm$ 18.7*	0.0 $\pm$ 0.0	23.6 $\pm$ 19.7*	3.8 $\pm$ 1.5	93.7 $\pm$ 2.1	57.4 $\pm$ 14.9	31.9 $\pm$ 13.0
BAL allergen (n = 5)	40.2 $\pm$ 44.1	64.2 $\pm$ 15.5*	0.0 $\pm$ 0.0	31.6 $\pm$ 16.7*	4.4 $\pm$ 1.6	91.9 $\pm$ 2.4	59.7 $\pm$ 11.1	25.1 $\pm$ 8.4

Values are expressed as means $\pm$ SD.

Statistical analysis of diluent BAL and allergen BAL challenges versus baseline BAL: * $P < .05$ as determined by paired t test.

CD3⁺, CD4⁺, and CD8⁺ cells are expressed as a percentage of positive cells within the lymphocyte population.

terminal deoxynucleotidyl transferase (TdT) (12.5 U) in the TdT buffer (200 mmol/L potassium cacodylate, 25 mmol/L Tris-HCl, 1.25 mg/mL BSA [pH 6.6], and 5 mmol/L cobalt chloride solution) for 60 minutes at 37°C. After washing, the incorporated dUTP was revealed by a biotin-conjugated anti-FITC antibody and subsequent immunocytochemistry as described.¹⁶ The number of apoptotic bodies was counted in at least 50,000 BALF cells and calculated for 100,000 BALF cells. Positive controls consisted of pretreatment with DNase before incubation with dUTP and TdT (inducing artificial DNA fragmentation), paraformaldehyde-fixed cryostat sections of rat thymus (containing a substantial percentage of apoptotic Tc cells), and cytospin preparations of irradiated (0 to 100 Gy) PBMCs undergoing apoptosis.

Statistical analysis

Results are expressed as means $\pm$ SD. Differences between groups were analyzed by using the paired t test. Differences with P values less than .05 were considered statistically significant.

RESULTS

Clinical course

The segmental allergen instillation induced an immediate bronchoconstriction with airway edema in all asthmatic subjects but not in control subjects. Saline instillation did not induce visible alterations. At the time of BAL sampling, 24 hours after the provocation, the endobronchial alterations were no longer visible. The allergen provocation was well tolerated by all asthmatic patients. FEV₁ percent predicted dropped from 103.7% $\pm$ 11.2% before the allergen challenge to 99.9% $\pm$ 14.1% 1 hour after the procedure without statistically significant differences. Peak flow measurements performed hourly on the day of the allergen challenge did not show a late response in any asthmatic patient.

BAL recovery, differential cell count, and T-cell subsets

BAL recovery did not differ between baseline BAL, sham-challenged BAL, and allergen-challenged BAL,

either in asthmatic subjects (baseline: 75 $\pm$ 10 mL; sham-challenged: 75 $\pm$ 3 mL; allergen-challenged: 68 $\pm$ 10 mL) or in control subjects (baseline: 77 $\pm$ 10 mL; sham-challenged: 77 $\pm$ 5 mL; allergen-challenged: 78 $\pm$ 12 mL). There was an increased total cell count after saline and allergen challenge in asthmatic and control subjects, which did not achieve statistical significance (Table I). The percentage of macrophages was decreased and the percentage of neutrophils was increased in both asthmatic and control subjects, whereas the percentage of eosinophils was elevated after allergen challenge in asthmatic subjects. The percentage of lymphocytes in asthmatic subjects was decreased after allergen challenge, but the total number did not change. The proportions of lymphocytes expressing CD3, CD4, or CD8 were not influenced by the challenge.

T-cell expression of Fas and Fas ligand

In peripheral blood from either asthmatic or control subjects, a mean of 26% to 30% of T cells (asthmatic subjects: CD3⁺, 29% $\pm$ 11%; CD4⁺, 30% $\pm$ 12%; CD8⁺, 29% $\pm$ 8%; control subjects: CD3⁺, 26% $\pm$ 6%; CD4⁺, 28% $\pm$ 8%; CD8⁺, 26% $\pm$ 4%) expressed the Fas antigen, whereas in BALF the mean of Fas-positive T cells ranged from 92% to 99% in both groups without differences between the groups (asthmatic subjects: CD3⁺, 97% $\pm$ 5%; CD4⁺, 99% $\pm$ 1%; CD8⁺, 93% $\pm$ 1%; control subjects: CD3⁺, 98% $\pm$ 1%; CD4⁺, 99% $\pm$ 1%; CD8⁺, 95% $\pm$ 2%). After saline (asthmatic subjects: CD3⁺, 97% $\pm$ 3%; CD4⁺, 99% $\pm$ 1%; CD8⁺, 92% $\pm$ 6%; control subjects: CD3⁺, 96% $\pm$ 7%; CD4⁺, 99% $\pm$ 1%; CD8⁺, 95% $\pm$ 3%) or allergen challenge (asthmatic subjects: CD3⁺, 98% $\pm$ 3%; CD4⁺, 99% $\pm$ 1%; CD8⁺, 93% $\pm$ 5%; control subjects: CD3⁺, 96% $\pm$ 7%; CD4⁺, 99% $\pm$ 1%; CD8⁺, 95% $\pm$ 2%) there was no further upregulation of CD95 in the BALF of asthmatic and control subjects. In peripheral blood essentially no differences were found compared with prechallenge data (data not shown).

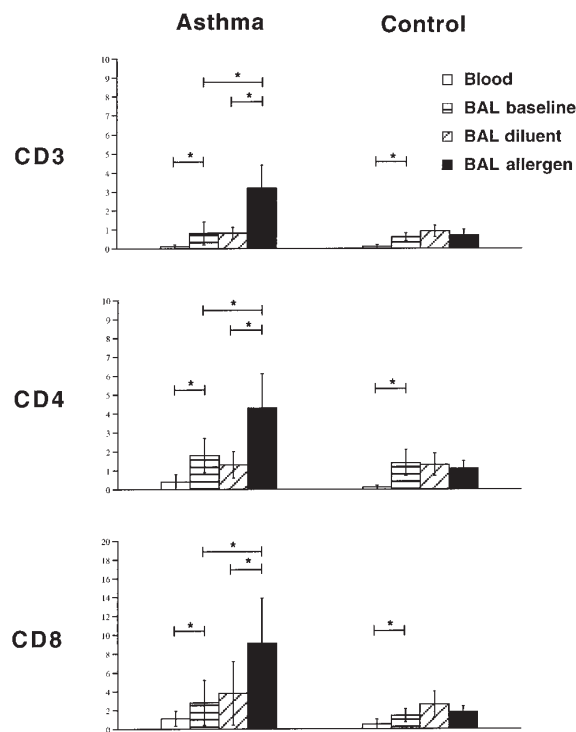


FIG 1. Percentage of CD3⁺, CD4⁺, and CD8⁺ cells expressing Fas ligand (CD95L). Cells were obtained from peripheral blood and different BALF samples of allergic asthmatic subjects ($n = 9$, for CD3⁺ $n = 8$) and nonallergic control subjects ($n = 5$). BAL was performed at baseline and 24 hours after segmental, endobronchial saline (BAL diluent), and allergen challenge (BAL allergen) (mean $\pm$ SD; * $P < .05$, paired t test).

The percentages of CD3⁺, CD4⁺, and CD8⁺ T cells expressing CD95L are shown in Fig 1. In contrast to the Fas antigen there was hardly any expression of Fas ligand on peripheral blood T cells in asthmatic and control subjects (CD3: $0.1\% \pm 0.1\%$ [asthma] and $0.1\% \pm 0.1\%$ [control]; CD4: $0.4\% \pm 0.4\%$ and $0.1\% \pm 0.1\%$; CD8: $1.1\% \pm 0.8\%$ and $0.5\% \pm 0.5\%$) without changes after challenge (data not shown). In BALF there was a slightly higher expression of CD95L ($P < .05$) on T cells compared with peripheral blood in asthmatic and control subjects (CD3: $0.8\% \pm 0.6\%$ and $0.6\% \pm 0.2\%$; CD4: $1.8\% \pm 0.9\%$ and $1.4\% \pm 0.7\%$; CD8: $2.8\% \pm 2.4\%$ and $1.4\% \pm 0.7\%$). Although in control subjects there was no further upregulation of Fas ligand on BALF T cells after saline or allergen challenge, in asthmatic subjects the percentage of Fas ligand-positive T cells increased significantly after allergen challenge (CD3: $3.2\% \pm 1.2\%$, $P = .002$; CD4: $4.3\% \pm 1.8\%$, $P = .0006$; CD8: $9.1\% \pm 4.8\%$, $P = .006$) but not after saline challenge (CD3: $0.8\% \pm 0.3\%$; CD4: $1.3\% \pm 0.7\%$; CD8: $3.8\% \pm 3.4\%$). All Fas ligand-positive T cells coexpressed the Fas antigen. Fig 2 shows a typical dot plot of T cells expressing CD95L in the different BAL samples from an asthmatic subject, demonstrating the upregulation of CD95L after allergen provocation. In asthmatic subjects a significant correlation was found between the percentage of

CD95L-positive T cells after allergen challenge and the percentage of eosinophils in BALF (Fig 3).

Apoptotic BALF cells

When the percentage of BALF cells undergoing apoptosis was determined, a very low percentage of TUNEL-positive nuclei was found, and they were almost exclusively located intracellularly in macrophages (Fig 4). Because they were partly digested, it was impossible to further determine their phenotype. Therefore the total number of intracellular apoptotic cells was determined from 100,000 BALF cells. Neither in asthmatic subjects (baseline: 55 ± 27 ; saline challenge: 106 ± 80 ; allergen challenge: 67 ± 47) nor in control subjects (baseline: 34 ± 26 ; saline challenge: 88 ± 60 ; allergen challenge: 150 ± 133) was there a statistically significant difference after saline or allergen challenge.

DISCUSSION

Allergen-driven T-cell recruitment and activation is a well-recognized feature of bronchial inflammation in asthma,⁴ but mechanisms to terminate this process are poorly understood. T cells triggered through the T-cell receptor can lead to activation-induced cell death (apoptosis),^{5,6} and it is reasonable to speculate that increased apoptosis is a possible mechanism to eliminate T cells in allergic inflammation. It has been shown that signals transmitted by means of the Fas/Fas ligand pathway are involved in activation-induced T-cell apoptosis in vitro,⁹ but it is not known whether this mechanism plays a role in allergic asthma. In this study we evaluated the T-cell expression of Fas and Fas ligand in asthmatic and control subjects undergoing segmental allergen challenge. In contrast to peripheral blood, essentially all BALF T cells expressed Fas antigen before and after allergen challenge in both asthmatic and control subjects. Although at baseline in both groups Fas ligand was virtually not expressed on peripheral T cells and at low frequencies on BALF T cells, there was a significant upregulation of Fas ligand on BALF T cells in asthmatic subjects after allergen challenge, although not in control subjects. This was seen on both CD4⁺ and CD8⁺ T cells. Despite high levels of Fas expression and upregulated Fas ligand expression on BALF T cells, it was not possible to detect an increased percentage of apoptotic BALF cells after allergen challenge.

In accordance with our data, Herry et al¹⁷ have shown that most BALF T cells (CD3⁺, CD4⁺, and CD8⁺ cells) of normal subjects express the Fas antigen, whereas only about 50% of peripheral blood T cells are CD95⁺. We show here that in asthma there are no differences compared with normal subjects, even after allergen challenge. Because essentially all BALF cells express the CD45RO⁺ memory phenotype characteristic of previously activated cells¹⁸ and Fas expression was found exclusively on CD45RO⁺ cells,¹⁹ this finding was not unexpected. Thus Fas expression might be a marker for previously activated T cells, which is a general phenomenon

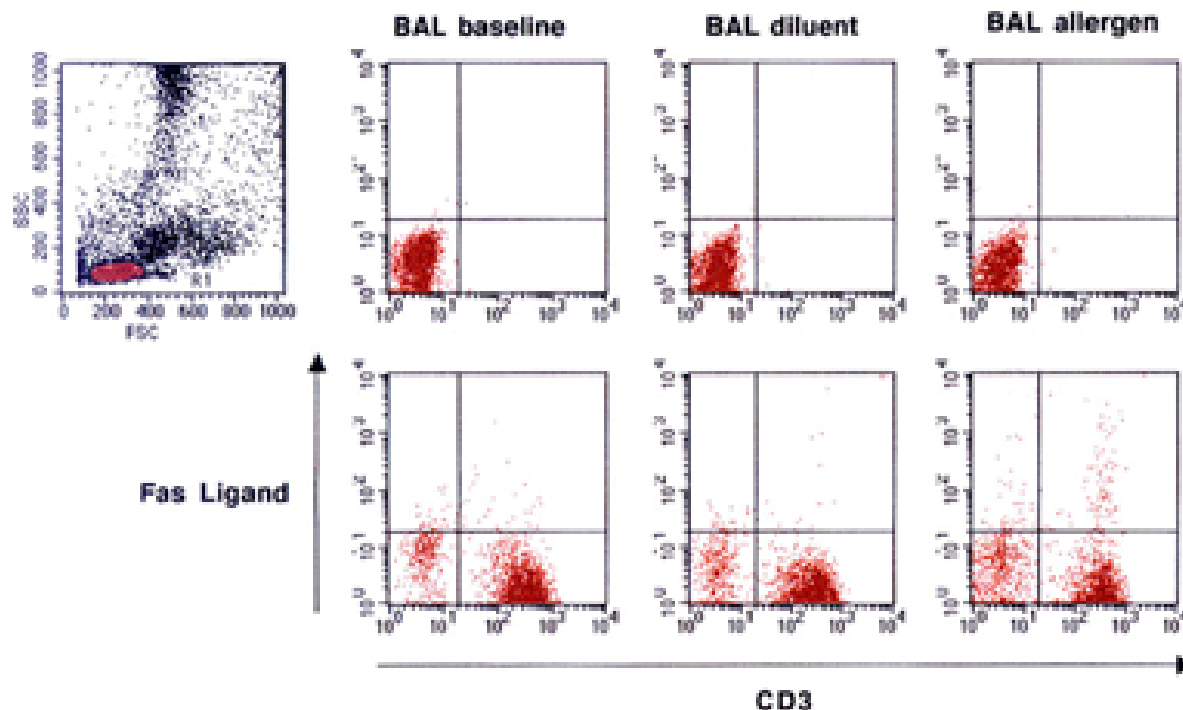


FIG 2. Flow cytometric expression of Fas ligand (CD95L) on CD3⁺ lymphocytes from different BAL samples in an allergic asthmatic subject. BAL was performed at baseline and 24 hours after segmental, endobronchial saline (*BAL diluent*), and allergen challenge (*BAL allergen*). Negative controls with irrelevant control antibodies are shown in the top row. Forward/side scatter plot for lymphocyte cluster gate (shown in red color) is illustrated on the left. The allergen BAL sample is illustrated.

of BALF cells in contrast to peripheral blood T cells. In contrast to our findings, a recent report has found very low percentages of CD95⁺ T cells in peripheral blood (8.5%) and BALF (15%) of normal subjects and no Fas expression on BALF T cells from asthmatic subjects (0.9%).²⁰ Together with a lack of Fas mRNA expression in BALF, the authors conclude that in asthma there is a defective expression of Fas resulting in prolonged inflammation. The reason for these discrepant results is not known, but it is difficult to understand why in asthmatic subjects Fas expression should be completely absent. Surprisingly, in this study the percentage of CD45RO⁺ BALF T cells was also very low in control subjects (18.7%) and asthmatic subjects (36.5%), whereas others have clearly shown that in both groups almost all T cells express the memory phenotype.²¹

In contrast to Fas antigen, its ligand was only expressed on a minority of BALF T cells in control subjects and subjects with mild asthma. However, after allergen challenge, a small but substantial proportion of CD4⁺ and an even greater proportion of CD8⁺ cells became Fas ligand positive. This upregulation of Fas ligand did not occur in control subjects or after saline challenge, indicating that it is allergen specific. This is further supported by the fact that the increase in Fas ligand expression correlates positively with the allergic response, determined as the percentage of BALF eosinophils after allergen challenge. Fas ligand expression has been shown in

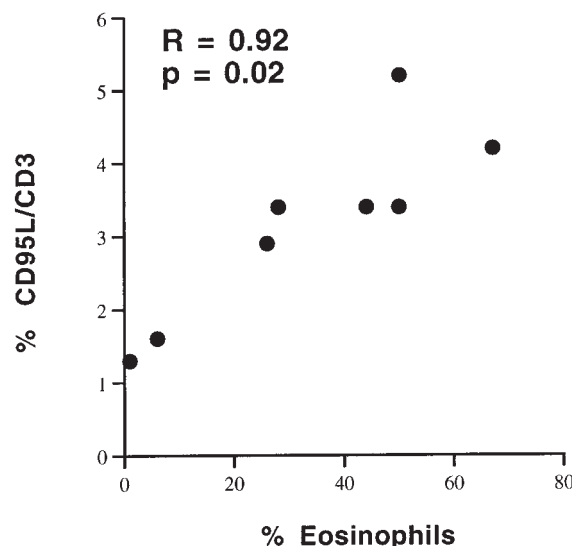


FIG 3. Correlation between the expression of Fas ligand (CD95L) on CD3⁺ lymphocytes and the percentage of eosinophils in BALF from allergic asthmatic patients (n = 8) 24 hours after segmental endobronchial allergen challenge.

vitro on T cells and several T-cell lines after polyclonal and T cell-specific activation inducing Fas-dependent cytotoxicity.⁸ One could speculate that allergen-induced T-cell expression of Fas ligand leads to enhanced apop-

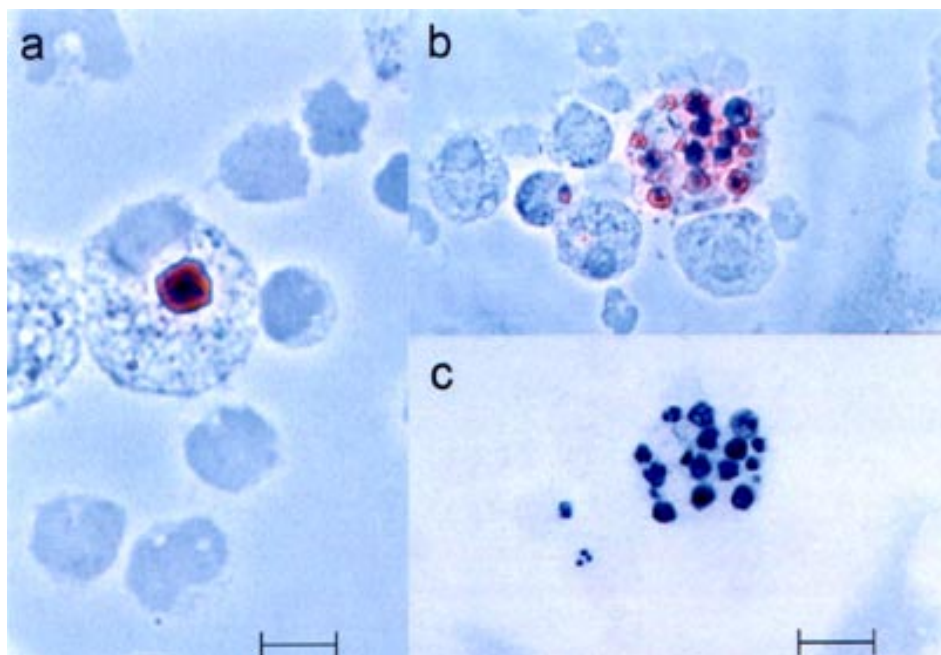


FIG 4. Cytospin preparations of BALF cells stained with the TUNEL technique. A macrophage with a recently phagocytosed apoptotic lymphocyte (red) is shown in phase-contrast microscopy (**a**). Phase-contrast (**b**) and bright field microscopy (**c**) show the same region of BALF cells demonstrating macrophages filled with a number of residual bodies of apoptotic cells (**a**, bar represents 15 μ m; **b** and **c**, bar represents 20 μ m).

tosis either by cell-cell contact to other Fas-positive T cells or by autocrine cell suicide of the Fas ligand expressing T cells coexpressing Fas.²² Furthermore, soluble Fas ligand, as a cytokine, could induce apoptosis in other Fas-positive T cells. However, we were unable to detect soluble Fas ligand by ELISA technique in all BALF samples (data not shown). Other investigators have presented preliminary data showing an increased proportion of Fas ligand-positive cells in bronchial epithelium from asthmatic subjects compared with control subjects without further identifying the cells.²³ On the other hand, in a murine asthma model Clara cells have been identified as a primary source of Fas ligand, and cell-associated staining for Fas ligand mRNA and protein was reduced in the airway epithelium during allergic airway inflammation.²⁴ When we determined the number of BALF cells undergoing apoptosis by using the TUNEL method, we found only a very low frequency of TUNEL-positive cells without differences before and after allergen challenge. Therefore our study provides no evidence that increased apoptosis is present in BALF cells from asthmatic subjects after allergen challenge. This finding is in accordance with preliminary data from other authors who found no differences in the number of apoptotic cells in the bronchial mucosa of asthmatic subjects compared with control subjects despite upregulation of Fas ligand.²³ Because they detected increased coexpression of Fas and Fas ligand together with markers for proliferation, they suggested that Fas/Fas ligand interaction is associated with a proliferative rather than

an apoptotic signal. Interestingly, in vitro experiments have shown that Fas-mediated signals may indeed induce CD4⁺ and CD8⁺ T-cell activation and proliferation.²⁵ In our study we did not look for T-cell activation or proliferation markers.

A limitation of this study is the fact that we were not able to identify free apoptotic T cells in the BALF. Although we believe that the TUNEL method is an appropriate method to detect cells with DNA fragmentation, which is by definition a hallmark of apoptosis, phagocytosis of apoptotic BALF cells by macrophages made it impossible to identify the phenotype of the digested cells. Thus we were only able to determine the total number of apoptotic, phagocytosed cells in BALF, and we might have missed an increased proportion of T cells in the constant number of total apoptotic cells. Other methods to identify cells undergoing apoptosis (detecting cell surface expression of phosphatidylserine²⁶ or hypodiploid DNA content by using propidium iodide and flow cytometry²⁷) might be more appropriate to detect early apoptotic cells before phagocytosis has taken place.

In conclusion, an upregulation of Fas ligand is demonstrated on BALF-derived T cells after allergen challenge in mild asthma, which we believe is a sign of allergen-specific T-cell activation. Further studies are necessary to determine whether Fas ligand expression is linked to T-cell apoptosis or T-cell activation in asthma and whether interaction with these mechanisms might open therapeutic options.

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