

Evaluation of apoptosis of eosinophils, macrophages, and T lymphocytes in mucosal biopsy specimens of patients with asthma and chronic bronchitis

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Background: Apoptosis regulates inflammatory cell survival, and its reduction contributes to the chronicity of an inflammatory process. Apoptosis is controlled by suppressing or inducing genes, such as *bcl-2* and *p53*, respectively.

Objective: We sought to assess apoptosis of eosinophils, macrophages, and T lymphocytes in bronchial biopsy specimens from asthmatic subjects and to examine its regulation by evaluating the expression of B-cell lymphoma leukemia-2 (*Bcl-2*) and *P53* proteins. We also sought to explore the relationships between cell apoptosis and GM-CSF, a cytokine able to increase eosinophil and macrophage survival.

Methods: Apoptosis in eosinophils, macrophages, and T lymphocytes was evaluated in bronchial biopsy specimens obtained from 30 asthmatic subjects, 26 subjects with chronic bronchitis, and 15 control subjects by combining the terminal deoxynucleotidyl transferase-mediated dNTP nick end-labeling technique and immunohistochemistry. The expression of *P53*, *Bcl-2*, and GM-CSF was studied through immunohistochemistry by using specific mAbs.

Results: The number of apoptotic eosinophils and macrophages was lower in subjects with asthma than in those with chronic bronchitis ($P < .007$ and $P < .001$, respectively) and inversely correlated with the clinical severity of asthma ($P < .001$ and $P < .002$, respectively). Few T lymphocytes were apoptotic in all groups studied. In asthma GM-CSF+ cells correlated with the number of nonapoptotic eosinophils and macrophages ($P = .0001$) and with the severity of the disease ($P < .003$). In asthma *Bcl-2*+ cells were higher than in control subjects and subjects with chronic bronchitis ($P < .002$ and $P < .015$, respectively), they outnumbered *P53*+ cells, and they correlated with the number of T lymphocytes ($P < .001$) and with the severity of the disease ($P < .003$).

Conclusion: Airway inflammation in asthma is associated with

an enhanced survival of different cell types caused by reduced apoptosis. (*J Allergy Clin Immunol* 1999;103:563-73)

Key words: Apoptosis, asthma, chronic bronchitis, eosinophils

Airways inflammation has been widely seen in all forms of asthma, and an association between the extent of inflammation and the clinical severity of the disease has been demonstrated.^{1,2} However, the mechanisms underlying the persistence of inflammation in the airways of asthmatic patients still remain poorly understood.

Apoptosis, a dynamic process involved in the control of the "tissue load" of immune effector cells at inflamed sites, tends to limit inflammatory tissue injury and to promote resolution rather than progression of inflammation.^{3,4} Apoptosis is morphologically characterized by chromatin condensation, genomic DNA fragmentation, and cell membrane dissociation. The genetic regulation of apoptosis involves a large number of genes classified into 3 categories: genes suppressing apoptosis, such as those belonging to the *bcl-2* family⁵; genes acting as effectors of apoptosis, such as caspases⁶; and intermediate genes upstream, such as Fas/Fas ligand, *p53*, *myc*, and *WAF 1*.^{7,8} Among apoptosis-inducing genes, an important role is played by *p53*, which is considered a guardian of the genome and is necessary for some forms of programmed cell death.^{9,10} Among apoptosis-suppressing genes, a prominent role is played by *bcl-2*, which is able to prevent cell death either through altering cell cycle rates or by activating antioxidant-associated mechanisms.^{11,12}

Compelling evidence has been accumulated showing that a decreased or suppressed apoptosis of immune effector cells in inflamed tissues is crucial to the chronic evolution of an inflammatory process in different organs.^{11,13-15} In rheumatoid arthritis the active suppression of T-cell death by the synovial microenvironment has been shown to be an important mechanism for the persistence of T-cell infiltrates in chronically inflamed rheumatoid arthritis joints.¹¹ More recently, the delayed eosinophil apoptosis in nasal polyps has been indicated as a novel mechanism by which eosinophils specifically accumulate in human tissues.¹³

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Abbreviations used

APAAP:	Alkaline phosphatase–antialkaline phosphatase
Bcl-2:	B-cell lymphoma leukemia-2
TUNEL:	Terminal deoxynucleotidyl transferase–mediated dNTP nick end-labeling

The reduced apoptosis of inflammatory cells, and in particular of eosinophils, may play a crucial role in the chronic persistence of airway inflammation in asthma. In this disease several proinflammatory cytokines released in the bronchial microenvironment can directly regulate the survival and death of inflammatory cells, such as eosinophils.^{14,16,17} Among the cytokines regulating eosinophil survival, an important role is played by GM-CSF, which is overexpressed in asthma^{18,19} and is able to enhance cell survival by blocking apoptotic pathways.^{16,20,21}

In this study we evaluated whether a reduction of inflammatory cell apoptosis was associated with airway inflammation in asthma. We also studied cell apoptosis in chronic bronchitis to verify whether a reduction of cell apoptosis was specific for asthma or was a common feature in the chronic inflammatory process occurring in the airways. We used the terminal deoxynucleotidyl transferase–mediated dNTP nick end-labeling (TUNEL) technique to study apoptosis in bronchial biopsy specimens obtained from control subjects and subjects with asthma and chronic bronchitis. To investigate the possible mechanism involved in cell apoptosis, we examined the expression of P53 and B-cell lymphoma leukemia-2 (Bcl-2) proteins, which are able to induce or to inhibit cell apoptosis, respectively. In addition, by combining the TUNEL and immunohistochemistry techniques, we studied the number of apoptotic eosinophils, macrophages, and lymphocytes infiltrating the bronchial mucosa. Moreover, we evaluated the relationship between the expression of GM-CSF and cell apoptosis in the 3 groups of subjects studied. Finally, to evaluate whether the number of apoptotic cells may depend on an increased rate of apoptotic cell removal by macrophages, we studied the expression of CD36, a marker commonly used to examine the phagocytosis of apoptotic cells by macrophages.²²

METHODS**Subjects**

Thirty asthmatic subjects (age range, 22 to 61 years; mean, 36 ± 11 years) were selected according to the criteria of the American Thoracic Society, as previously described.¹ The clinical severity of asthma was assessed according to the validated score of Aas,²³ which grades the different forms of chronic asthma from very mild (score 1) to very severe (score 5). Some patients with a severe form of the disease were studied and should have received corticosteroids according to guidelines. However, they were seen for the first time in our clinic and had a bronchoscopy the following day. None of the subjects participating in this study was then a current smoker. Allergy was tested by skin prick tests²⁴ with a battery of extracts based on the common aeroallergens found in the Mediterranean area. Serum-specific IgE was titrated by the CAP System (Pharmacia-Upjohn) in patients with positive skin prick test responses. Subjects

who had any bronchial or respiratory tract infection during the month preceding the test were excluded from the study. Patients were excluded from the study if they had had a severe exacerbation of asthma resulting in hospitalization during the month preceding the study, or if they had taken any drug that might modify the results of the study, particularly systemic corticosteroids during the previous 2 months, inhaled corticosteroids during the previous month, and nedocromil sodium or cromoglycate during the previous 2 weeks. No patient was taking long-acting β_2 -agonists or oral theophylline. Short-acting β_2 -agonists were stopped for 12 hours.

Twenty-six patients with chronic bronchitis (age range, 45 to 70 years; mean ± SEM 56.2 ± 6.6 years) were selected according to the criteria of the American Thoracic Society, as previously reported.²⁵ Because an overlap between chronic bronchitis and asthma may occur in subjects with so-called “asthmatic bronchitis,” patients showing 1 of the following features were excluded: a history of perennial allergic rhinitis and/or a positive diagnosis of immediate-type hypersensitivity,¹ wheezing, an improvement of FEV₁ capacity of over 12% from baseline, or an absolute value of 200 mL after inhalation of 200 μ g of salbutamol.²⁶ Smoking habits were carefully checked, and a minimum of 30 pack-years was required (range, 30 to 150 pack-years) for inclusion in the chronic bronchitis group. To distinguish chronic bronchitis from emphysema, all patients with chronic bronchitis underwent routine chest x-ray and computed tomography scans. Patients with obvious emphysema, as assessed by routine chest x-ray and computed tomography scans, were excluded. Subjects who had any bronchial or respiratory tract infection during the month preceding the test were excluded from the study. Patients were excluded from the study if they had taken any of the drugs listed above.

Fifteen healthy subjects (age range, 17 to 67 years; mean ± SEM, 45.3 ± 18.0 years) were used as a control group. Their pulmonary function was within normal range. Subjects who had any bronchial or respiratory tract infection during the month preceding the test were excluded from the study.

The study was performed after informed consent was obtained from the participants and with the approval of the Ethics Committee of the Hospital.

Pathologic examination of bronchial mucosa

Biopsy specimens were recovered by using fiberoptic bronchoscopy performed as previously described in the subsegmental bronchi.¹ Specimens were fixed in 3.7% wt/vol formaldehyde and embedded in paraffin blocks. Four-micrometer sections were deparaffinized and rehydrated before hematoxylin and eosin staining or immunohistochemistry. The total number of leukocytes infiltrating the submucosa was evaluated by an anti-CD45 mAb (CD45 Clones 2B11+PD7/26; Dako, Glostrup, Denmark) used as a pan-leukocyte marker at the dilution of 1:50.

Detection of apoptosis in bronchial biopsy specimens

Apoptosis was detected by using the TUNEL technique (Oncor, Inc, Gaithersburg, Md), which detects DNA strand breaks in cells undergoing apoptosis.²⁷ Briefly, terminal deoxynucleotidyl transferase, an enzyme that catalyzes a template-independent addition of deoxyribonucleotide triphosphate to the 3'-OH ends of double or single stranded DNA, was used to incorporate biotinylated deoxyuridine at sites of DNA cleavage. Avidin peroxidase was used as the detection agent. As a positive control we used slides prepared with paraffin-embedded mammary glands obtained at the fourth day after weaning (kindly provided by Oncor, Inc, Gaithersburg, Md).²⁷ Negative control slides were created by substituting 32 μ L of distilled water for terminal deoxynucleotidyl transferase enzyme.

Apoptosis was also evaluated on the basis of the morphologic appearance of apoptosis. In particular, marked loss of cytoplasm and nuclear condensation were considered as the 2 major criteria for the determination of cells having undergone apoptosis by light microscopy. Apoptotic cells were therefore identified as those cells stained by using the TUNEL technique and showing the morphologic appearance of apoptosis.

For the overall length of each biopsy specimen, the number of apoptotic cells in the submucosa, defined as the zone 100 μ m deep to the limit of the reticular basement membrane, was quantified by using an eyepiece graticule per unit area (square millimeters). The results were expressed as the medians and 25-75 percentiles of the total measurements (at least 4 measurements were performed to calculate averages). All the slides were coded and evaluated blindly by 2 independent investigators (AMV and GC). The interinvestigator coefficient of agreement was excellent ($\kappa = 0.93$).

Detection of apoptosis in eosinophils, macrophages, and activated T cells

To evaluate whether eosinophils, macrophages, and activated T cells were in an apoptotic state, the slides were first stained by the alkaline phosphatase–antialkaline phosphatase (APAAP) method (Dako LSAB, Dako) to identify the cell phenotype and then subsequently stained by the TUNEL technique to detect apoptotic cells. The APAAP technique was performed by using mAbs specific for eosinophil cationic protein (EG2) (Kabi Pharmacia, Uppsala, Sweden)²⁸ at a dilution of 1:5, for a subset of antigen-primed T cells (anti-UCHL1, Dako)²⁹ at the dilution of 1:50, and for macrophages (anti-CD68, Dako) at the dilution of 1:50. Control slides were prepared by using an irrelevant mouse antibody of the same isotype and at the same concentration as the specific primary mAb (Dako). The identification of apoptotic eosinophils, activated T cells, and/or macrophages was given by the colocalization of the dark brown nuclear staining caused by the TUNEL technique and of the red cytoplasmic staining caused by the APAAP immunoreactivity with EG2, UCHL1, or CD68 mAbs. All the slides were coded, and the immunoreactivity of each of the markers studied was evaluated blindly by 2 independent investigators (AMV and GC). The interinvestigator coefficient of agreement was excellent ($\kappa = 0.95$).

Evaluation of the expression of P53, Bcl-2, and GM-CSF and identification of eosinophils and macrophages

Slides were incubated with the mouse mAbs against the wild-type proteins P53 at a dilution of 1:3 (Dako), against Bcl-2 (Dako) at a dilution of 1:7, and against GM-CSF (Genzyme, Cambridge, Mass) at the dilution of 1:180. The reaction was revealed by using the APAAP method (Dako LSAB), according to the manufacturer's instructions. Control slides were prepared by using an irrelevant mouse antibody of the same isotype and at the same concentration as the specific primary mAb (Dako). All the slides were coded, and the immunoreactivity of each of the markers studied was evaluated blindly by 2 independent investigators (AMV and GC).

For the overall length of each biopsy specimen, the number of positively stained cells beneath the basement membrane was quantified per unit area (square millimeters) of submucosa. The results were expressed as the medians and 25-75 percentiles of the total measurements (at least 4 measurements were performed to calculate averages).

Detection of bcl-2 mRNA by in situ hybridization

In situ hybridization was performed on specimens fixed in 10% neutral-buffered formalin and embedded in paraffin blocks. Four-

TABLE I. Demographic characteristics of the subjects

	Control subjects	Asthmatic subjects	Subjects with chronic bronchitis
No. of subjects	15	30	26
Age (y)	45 (37-50)	36 (34-40)	56 (54-62)
Sex (% male)	53	55	70
Duration of disease (y)	NA	15 (11-20)	15 (13-17)
Allergy (%)	0	64	0
FEV ₁ (% predicted)	100 (100-101)	72.5 (57-87)	82.5 (79-86)
FVC (L)	—	120 (110-126)	78 (68-95)
RV (L)	—	120 (111-140)	172 (151-216)

Results are expressed as medians and 25-75 percentiles.

NA, Not applicable; FVC, forced vital capacity; RV, residual volume.

micrometer sections were deparaffinized in xylene and rehydrated in decreasing alcohol concentrations (100%, 70%, 50%) and in diethyl pyrocarbonate-water. Then sections were digested in proteinase-K (Boehringer Mannheim, Mannheim, Germany) solution in PBS at 10 μ g/mL at 37°C for 30 minutes. After digestion, the sections were fixed in precooled 0.4% paraformaldehyde for 15 minutes at 4°C. Sections were then incubated (without probes) for 1 hour at 37°C in hybridization solution (hybridization buffer [Amersham], deionized formamide, salmon sperm DNA 100 mg/mL [Sigma], RNase-inhibitor [Boehringer], and diethyl pyrocarbonate-water). The in situ hybridization technique was performed by using human bcl-2 oligo cocktail at 100 pmol/L consisting of an equimolar mixture of 4 individual single stranded DNA antisense oligonucleotides (each of 30 basis) (by Primm s.r.l., Milan, Italy). The sequences of the oligonucleotides were selected within the "Open Reading Frame bcl-2 sequences" as described by Tsujimoto and Croce.³⁰ The oligonucleotide sequences used were as follows: antisense sequence-1, 5'-GTGCCGGTTCAGGTACTCAGTCATCCACAG-3'; antisense sequence-2, 5'-CAGCTTATAATGGATGTACTTCATCACTAT-3'; antisense sequence-3, 5'-CCAGGAGAAATCAAACAGAGGCCCGCATGCT-3'; and antisense sequence-4, 5'-AGTTCACAAAGGCATCCAGCCTCCGTTA-3'.

bcl-2 oligonucleotides used as a cocktail (100 pmol/L final concentration) were enzymatically labelled at their 3'-end with terminal transferase by incorporation of a DNA tail of 40 to 50 nucleotides in length with several digoxigenin molecules incorporated by using a Dig Oligonucleotide Tailing kit (Boehringer).³¹ Sections were incubated with 200 ng/mL of the oligonucleotide cocktail overnight at 37°C. Hybridized oligonucleotides were detected by using antidigoxigenin horseradish peroxidase-conjugated Fab fragments (Boehringer) at the dilution of 1:80 at room temperature for 1 hour. Color development was achieved by adding a freshly prepared substrate solution consisting of 1 mg/mL DAB chromogen, TRIS-HCl, and H₂O₂ 0.02% (Dako) for 5 minutes at room temperature. To ensure that results were not caused by methodological artefacts, control sections were prepared in a similar fashion during the same experimental series either without the bcl-2-labeled oligonucleotide cocktail, by using the sense oligonucleotides labeled, by omitting the antidigoxigenin horseradish peroxidase-conjugated Fab fragments, or after digestion with RNase (Sigma Chemical Company) 1 mg/mL for 1 hour at 37°C.

Combination of in situ hybridization and immunohistochemistry

To identify cells bearing transcript for bcl-2, we combined the immunohistochemistry and in situ hybridization. The sections were

TABLE II. Results of bronchial biopsies

	Control subjects	Asthmatic subjects	Subjects with chronic bronchitis	P value		
				C/A	A/CB	C/CB
Apoptotic cells*	0 (0-5)	7.5 (0-15)	55 (45-60)	NS	.001	.001
EG2+ cells						
Apoptotic cells	0	0 (0-1)	3 (0-8)	NS	.007	.0006
Total cells	0	76 (0-540)	5 (2-15)	.002	.02	.01
CD68+ cells						
Apoptotic cells*	0 (0-6.5)	1 (0-5)	28 (20-40)	NS	.001	.0001
Total cells*	125 (100-152)	325 (65-548)	205 (150-320)	.04	NS	.01
UCHL1+ cells						
Apoptotic cells*	0	0	0	NS	NS	NS
Total cells*	4 (0-17)	80 (25-180)	37.5 (15-50)	.0001	.01	.0001
bcl-2+ cells*	0 (0-0)	15 (3-150)	0 (0-29)	.002	.015	.004
p53+ cells*	0 (0-19)	0 (0-0)	11 (0-37)	NS	.03	.01
GM-CSF+ cells*	5 (3-10)	80 (38-198)	7.5 (0-19)	.001	.001	NS

Results are expressed in medians and 25-75 percentiles. Statistical analysis was done with the Mann-Whitney *U* test and Bonferroni's correction.

C, Control subjects; A, asthmatic subjects; CB, subjects with chronic bronchitis.

*Cells/mm².

first stained by the APAAP method to identify the cell phenotype and then subsequently stained by the in situ hybridization technique to detect bcl-2 mRNA. The APAAP method was performed with an mAb specific for a subset of antigen-primed T cells (CD 45RO clone UCHL1 DAKO) at the dilution of 1:50 at room temperature. Control slides were prepared by using an irrelevant mouse antibody of the same isotype and at the same concentration as the specific primary mAb (Dako). The identification of activated T cells was given by the colocalization of the red cytoplasmic staining caused by the APAAP immunoreactivity and dark brown cytoplasmic bcl-2 mRNA staining caused by the in situ hybridization technique.

Detection of CD36+ macrophages

The expression of CD36 was studied in frozen biopsy specimens obtained from 8 control subjects, 8 asthmatic subjects, and 8 subjects with chronic bronchitis. The biopsy specimens were embedded in Tissue Tek II OCT (Miles Scientific, Naperville, Ill), frozen within 30 minutes in isopentane precooled in liquid nitrogen, and stored at -80°C. Cryostat sections (5 µm thick) were cut, brought to room temperature, and fixed in acetone for 2 minutes. The sections were stained with the mAb anti-CD36 (Pharmigen, San Diego, Calif) at the concentration of 1:10 for 1 hour at room temperature.

Statistical analysis

Results are given as medians and 25-75 percentiles, except for some demographic characteristics, which are normally distributed. Normally distributed data were analyzed by using parametric statistics. Nonparametric tests were used for the other data. The Kruskal-Wallis test was used to compare the 3 groups. The Mann-Whitney *U* test was used for unpaired comparisons, and the Spearman rank test was used for correlations. For multiple correlations, Bonferroni's correction was used. The correlation between the Aas score and the percentage of stained cells was done with the Kendall *t* test.

RESULTS

Patient characteristics

The characteristics of the patients are given in Table I. Fourteen patients had mild asthma (Aas scores 1 and 2), 6 had moderate asthma (Aas score 3), and 10 had severe

asthma (Aas scores 4 and 5). FEV₁ values ranged from 40% to 100% of predicted values.

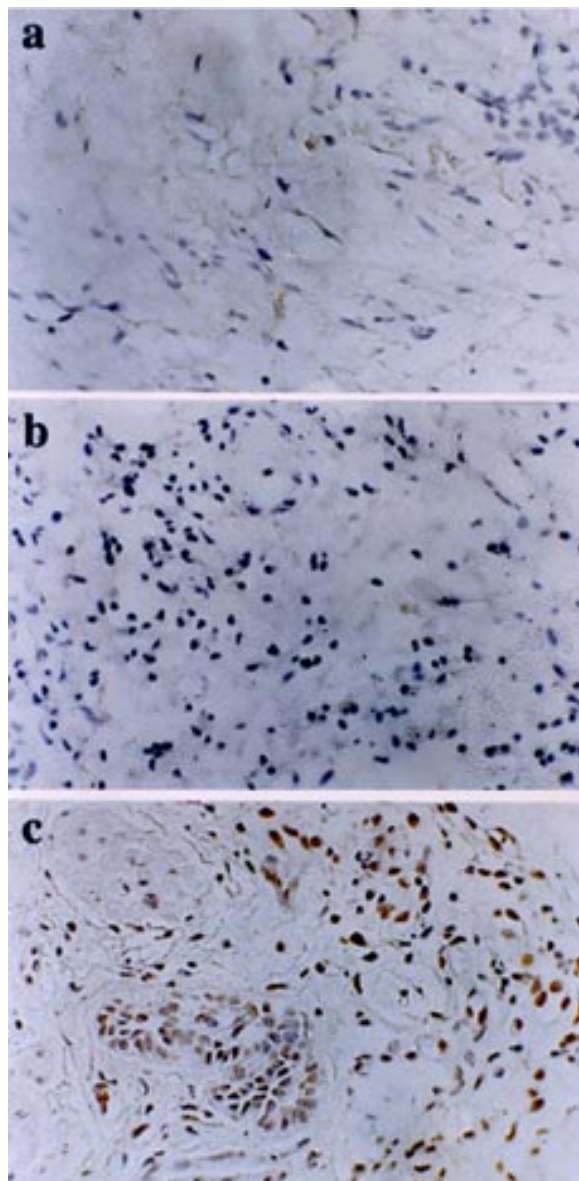
Patients with chronic bronchitis were significantly older than asthmatic patients ($P < .02$, Mann-Whitney *U* test) and were nonsignificantly older than control subjects. In patients with chronic bronchitis, FEV₁ values ranged from 72% to 89% of predicted values.

Pathologic examination of the biopsy specimens

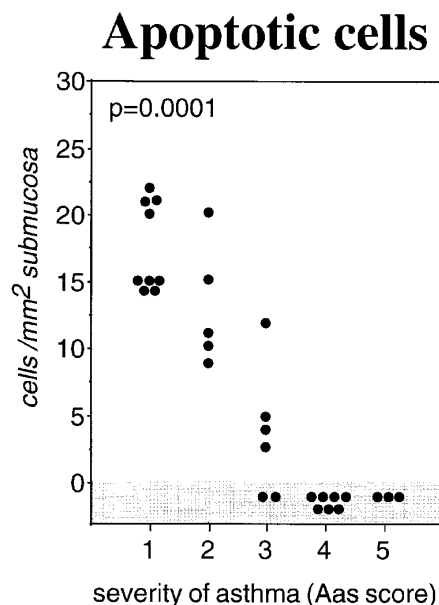
Immunohistochemistry with the anti-EG2 mAb showed that in the submucosa of asthmatic patients the number of eosinophils was significantly higher than in patients with chronic bronchitis or normal subjects ($P = .01$ and $P < .002$, respectively; Mann-Whitney *U* test). Immunohistochemistry with the anti-UCHL1 mAb showed that in the submucosa from patients with asthma and chronic bronchitis the number of activated T cells was significantly higher than that found in normal subjects ($P < .0001$, Mann-Whitney *U* test). The number of macrophages identified by using the anti-CD68 mAb was significantly higher in patients with chronic bronchitis and asthma than in normal subjects ($P < .01$ and $P < .04$, respectively; Mann-Whitney *U* test) (Table II). The number of CD45+ cells was higher in patients with asthma and chronic bronchitis than in control subjects ($P < .001$, Mann-Whitney *U* test).

Evaluation of cell apoptosis

The total number of apoptotic cells was similar in asthmatic and control subjects but significantly lower in patients with chronic bronchitis ($P < .001$, Mann-Whitney *U* test) (Fig 1 and Table II). The ratio of total apoptotic cells/CD45+ cells ranged from 0.5% (0% to 3%) in control subjects to 1% (0% to 4%) in asthmatic subjects and 16% (8% to 22%) in subjects with chronic bronchitis ($P < .001$, subjects with asthma vs subjects with



The absolute number of apoptotic eosinophils was significantly lower in subjects with asthma than in those with chronic bronchitis ($P < .007$, Mann-Whitney U test) (Figs 3 and 4 and Table II) and correlated with the severity of asthma ($P < .0001$, Kendall's τ test). In addition, the median ratio between apoptotic eosinophils and total



Bcl-2 expression was significantly increased in asthmatic subjects compared with subjects with chronic bronchitis and control subjects ($P < .02$ and $P < .002$, respectively; Mann-Whitney U test) (Figs 6 and 7 and Table II). Bcl-2 immunoreactivity was also localized in

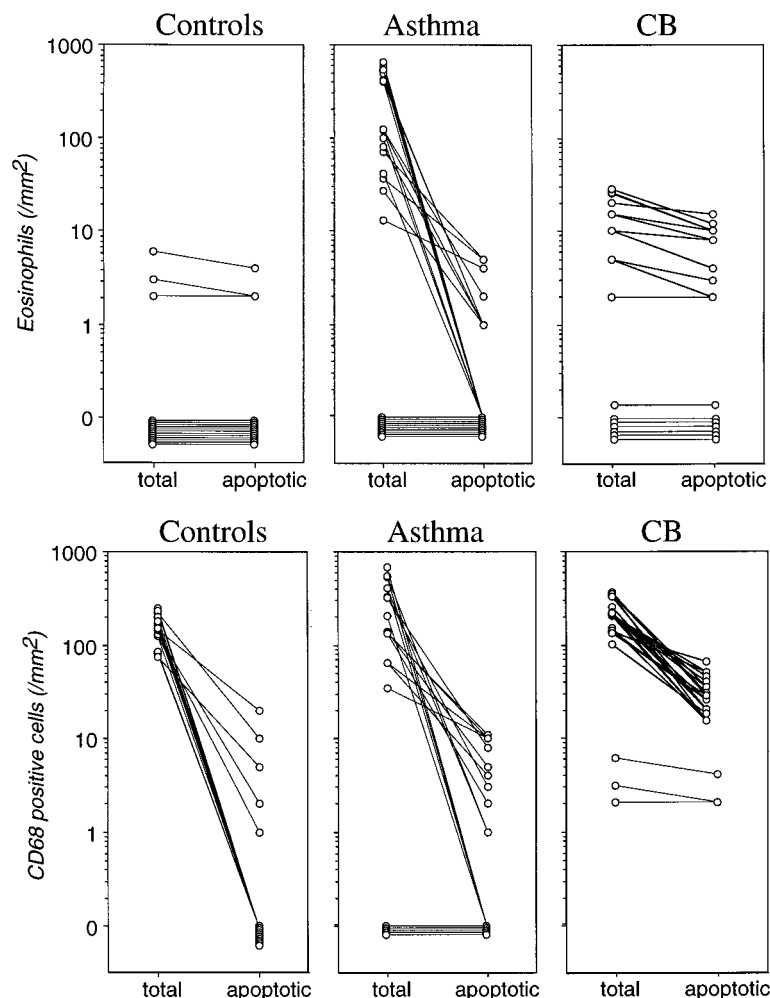


FIG 3. Number of apoptotic eosinophils and macrophages in control subjects, subjects with asthma, and subjects with chronic bronchitis (CB). *Total* indicates the number of eosinophils or macrophages identified by using anti-EG2 or anti-CD68 in bronchial biopsy specimens. *Apoptotic* indicates the number of EG2- or CD68+ cells stained with the TUNEL technique. Statistical analysis was done with the Mann-Whitney *U* test.

mucous glands and smooth muscle cells. In asthma the number of Bcl-2+ cells was directly correlated with the number of lymphocytes infiltrating the bronchial submucosa ($P < .001$, Spearman test). Furthermore, Bcl-2+ cells were significantly correlated with the severity of asthma ($P < .003$, Spearman test) (Fig 8).

Finally, in asthma the number of Bcl-2+ cells was significantly higher than the number of P53+ cells ($P < .001$, Wilcoxon test). By contrast, in patients with chronic bronchitis there was nonsignificant difference between the number of submucosal cells expressing Bcl-2 or P53.

Evaluation of Bcl-2 mRNA in lymphocytes

Transcripts for Bcl-2 were found on the same cells expressing this protein in biopsy specimens from control subjects, subjects with asthma, and subjects with chronic

bronchitis. By combining the in situ hybridization for Bcl-2 and immunohistochemistry, we detected Bcl-2 mRNA only in lymphocytes (Fig 9). The number of lymphocytes bearing Bcl-2 transcripts was 37/mm² (range, 12 to 90/mm²) in subjects with asthma, 19/mm² (range, 7 to 25/mm²) in subjects with chronic bronchitis, and 2/mm² (range, 0 to 7/mm²) in control subjects.

Expression of GM-CSF

The number of GM-CSF+ cells in the submucosa was found to be significantly higher in asthmatic subjects than in control subjects or subjects with chronic bronchitis ($P < .001$, Mann-Whitney *U* test) (Fig 9 and Table II). In subjects with asthma there was a significant correlation between the number of cells expressing GM-CSF and the number of nonapoptotic eosinophils and macrophages ($P = .0001$, Spearman rank correlation).

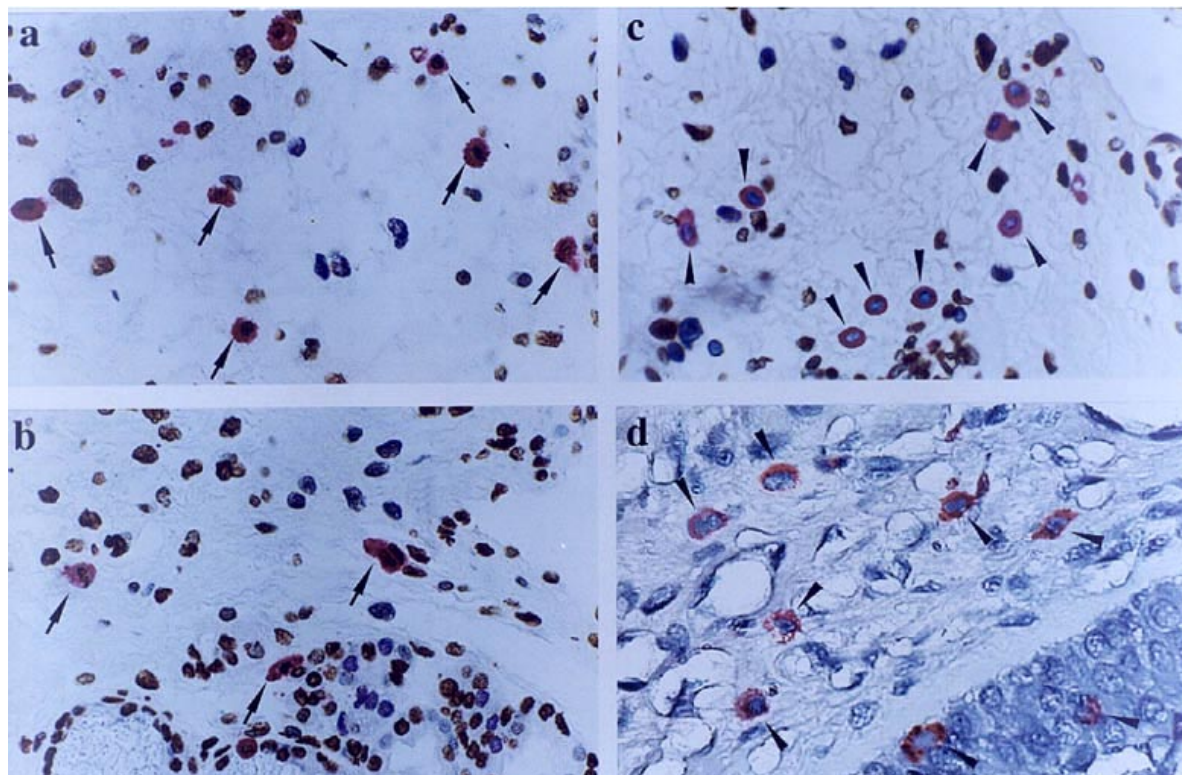


FIG 4. Identification of apoptosis in eosinophils (**b, d**) and macrophages (**a, c**) in bronchial biopsy specimens obtained from a subject with asthma (**c, d**) and a subject with chronic bronchitis (**a, b**). Apoptotic cells are identified by dark brown nuclear staining with the TUNEL technique. Eosinophils and macrophages are identified by red cytoplasmic staining caused by APAAP immunoreactivity with EG2 or CD68 mAbs (final magnification, 200 $\times$). Arrows indicate apoptotic macrophages in **a** and apoptotic eosinophils in **b**. Arrowheads indicate nonapoptotic macrophages in **c** and nonapoptotic eosinophils in **d**.

Furthermore, GM-CSF+ cells were significantly correlated with the severity of asthma ($P < .003$, Spearman test) (Fig 7).

Detection of CD36+ macrophages

The number of cells expressing CD36 was very low and not statistically different in bronchial biopsy specimens obtained from control subjects and subjects with asthma and chronic bronchitis.

DISCUSSION

The survival of inflammatory cells depends on the fate they undergo during the evolution of airway inflammation. With its potential for modulation by external mediators, apoptosis plays a key dynamic role in the control of the "tissue load" of granulocytes at inflamed sites and tends to limit inflammatory tissue injury and to promote resolution rather than progression of inflammation.^{3,4} Thus although the activation of apoptosis may serve to terminate the inflammatory processes by eliminating the increased number of inflammatory cells, a delayed apoptosis may contribute to the chronic persistence of an inflammatory cell infiltration in the inflamed tissues.^{11,13,14,16,32}

The chronic infiltration of the airways by several inflammatory cells is a prominent feature of bronchial asthma.³³ After an initial phase characterized by the generation of proinflammatory cytokines and the increased expression of adhesion molecules by the endothelium and the epithelium, airway inflammation evolves toward a chronic phase characterized by the infiltration of the airways by inflammatory cells.^{34,35} Among them, eosinophils and macrophages have been shown to play an important role in the pathogenesis of airway inflammation associated with asthma.^{1,28} The number of eosinophils is increased in the airways of asthmatic subjects,^{1,29} show morphologic and histochemical features of cellular activation,³⁶⁻³⁸ and appear to be more degranulated than in subjects with chronic bronchitis.²⁵ In addition, the number of eosinophils significantly correlates with the clinical severity of the disease.¹ In asthma airway inflammation is also characterized by a significantly increased number of macrophages.²⁹ Many of these cells are activated³⁹ and show phenotypic characteristics of monocytes, suggesting that there is an active recruitment of these cells from the bloodstream into the airways.²⁹ Although the active recruitment of eosinophils and macrophages has been widely demonstrated, it is still unclear whether these cells can persist in the airways of

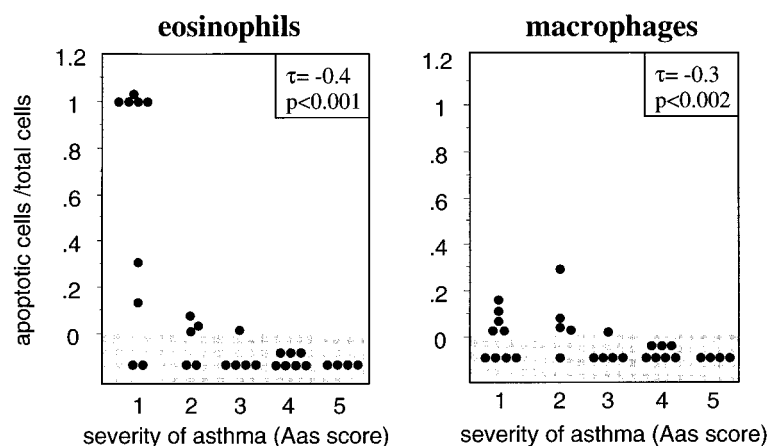


FIG 5. Correlation between the apoptotic eosinophils:total eosinophils ratio and the apoptotic macrophages:total macrophages ratio and the Aas score. Results are expressed as median ratios.

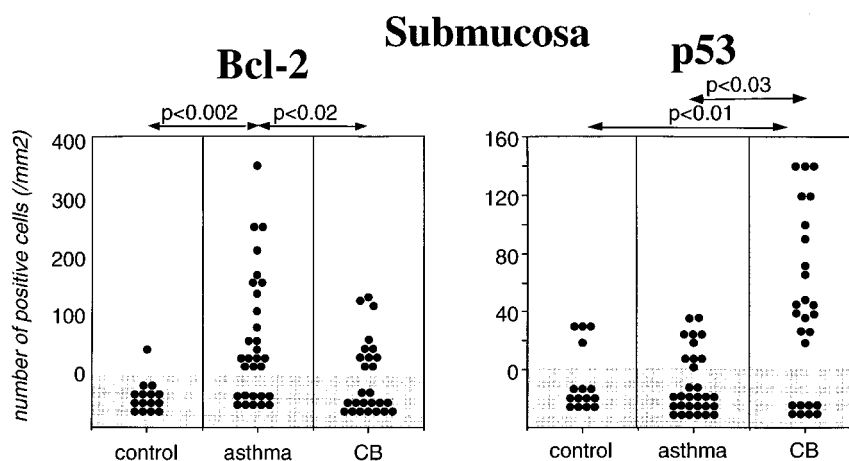


FIG 6. Expression of Bcl-2 and P53 in the submucosa of bronchial biopsy specimens obtained from control subjects, asthmatic subjects, and subjects with chronic bronchitis. Results are expressed as number of positive cells per square millimeter of submucosa.

asthmatic subjects and whether this persistence is due to a reduced or delayed apoptosis.

There are different methods for the detection of apoptosis, and each of them has advantages and drawbacks. The choice of method depends on the characteristics of the biologic sample, as well as the need to identify the phenotype of the cells undergoing apoptosis. In this study we used the TUNEL technique because it is the most useful technique to detect cell apoptosis in paraffin-embedded tissues. In addition, this technique has been used and validated in many studies and offers the major advantages of revealing early DNA breaks during apoptosis, quantitating the percentage of apoptotic cells, simultaneously determining cell surface antigens by immunohistochemistry, and phenotypically characterizing the cells undergoing apoptosis within heterogeneous populations.⁴⁰ To complement the TUNEL technique and to investigate possible mechanisms involved in cell apoptosis, we also evaluated the expression of P53 and Bcl-2,

2 molecules that play an important role in the regulation of this phenomenon.⁴¹⁻⁴³

This study shows for the first time that the number of nonapoptotic eosinophils and macrophages is significantly higher in subjects with asthma than in those with chronic bronchitis and suggests that these cells can survive longer in the airways of asthmatic subjects. In addition, we show that the persistence of these cells may have important clinical consequences as shown by the significant correlation between the number of nonapoptotic eosinophils and macrophages and the severity of asthma. Thus these results suggest that the chronic accumulation of both eosinophils and macrophages in asthma, but not in chronic bronchitis, may be due to an increased recruitment of these cells in the airways, as well as an inhibition of their programmed cell death. This hypothesis is also supported by the evidence that, although the number of CD45+ cells was similar in subjects with asthma and subjects with chronic bronchitis, the ratios between

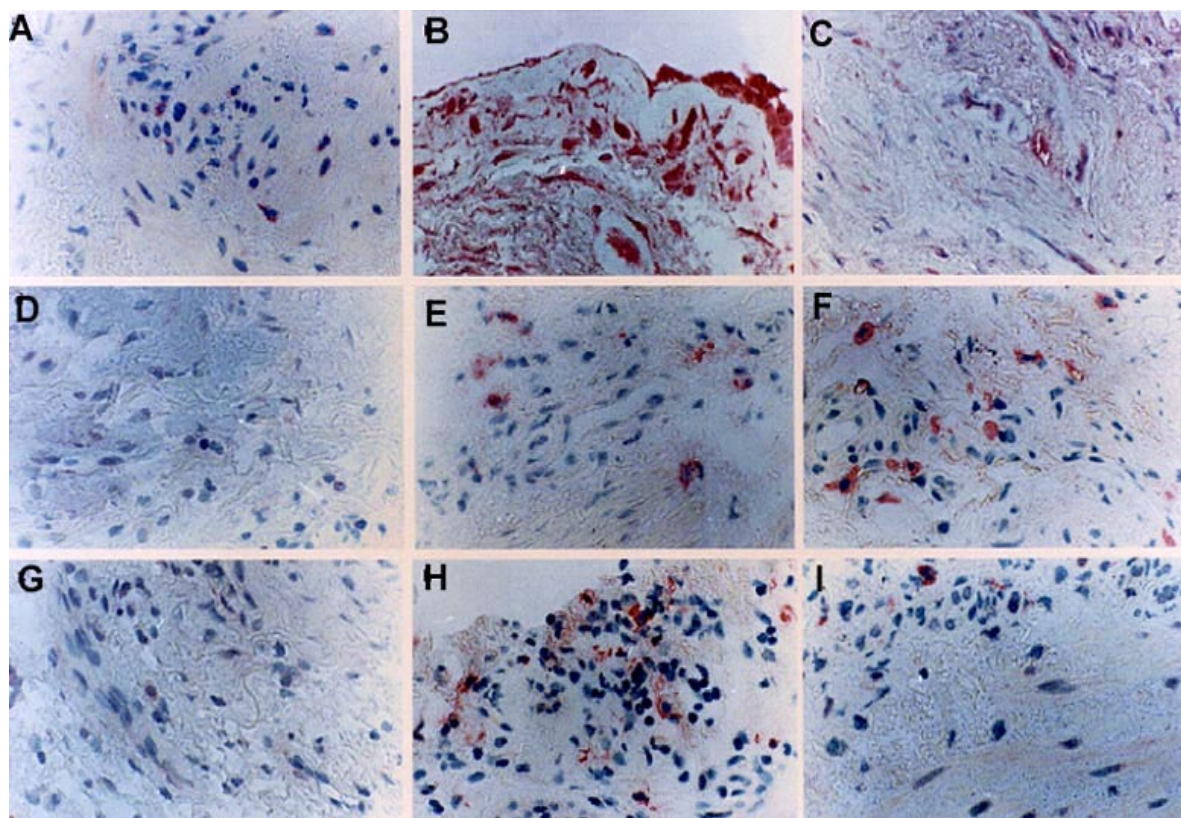


FIG 7. Immunoreactivity for GM-CSF (**a**, **b**, and **c**), P53 (**d**, **e**, and **f**), and Bcl-2 (**g**, **h**, and **i**) in the submucosa of a bronchial biopsy specimen obtained from a control subject (**a**, **d**, and **g**), an asthmatic subject (**b**, **e**, and **h**), and a subject with chronic bronchitis (**c**, **f**, and **i**) (final magnification, 100 $\times$).

apoptotic cells/CD45+ cells were significantly lower in subjects with asthma than in those with chronic bronchitis. It is conceivable that the reduced cell apoptosis is due to the effects of proinflammatory cytokines overexpressed in the airways of asthmatic subjects. Among the possible eosinophil and macrophage survival factors, an important role is played by GM-CSF.^{16,44} GM-CSF is overexpressed in asthma^{18,19} and can modulate the recruitment, activation, and survival of these cells.^{15,16,21,44} Recently, the use of GM-CSF analogues has been found to induce apoptosis of normal and activated eosinophils and to regulate their numbers and activities.⁴⁵ This study confirms the increased GM-CSF expression in asthma and suggests that this growth factor can directly affect the survival of both eosinophils and macrophages in the inflamed airways of asthmatic subjects. This hypothesis is supported by the significant correlation between the expression of GM-CSF and the number of nonapoptotic eosinophils and macrophages identified in the submucosa of bronchial biopsy specimens taken from asthmatic patients.

In this study we also show that both asthma and chronic bronchitis are characterized by a very low number of activated apoptotic T lymphocytes, suggesting that these cells are less susceptible to undergoing apoptosis than eosinophils and macrophages. This evidence confirms

and extends previous results obtained by using allergen-induced cutaneous late-phase response⁴⁶ and suggests that the increased numbers of T lymphocytes observed in asthma and chronic bronchitis are due to an active recruitment associated with a delayed apoptosis of these cells.

With regard to the reduction of the number of apoptotic eosinophils, macrophages, and lymphocytes, it may be argued that this may result from an increased rate of apoptotic cell removal by macrophages. In this study we addressed this important point by evaluating the expression of CD36 in bronchial biopsy specimens but failed to find a significant difference among the 3 study groups, suggesting a similar clearance rate of apoptotic cells in normal and inflamed airways. We acknowledge, however, that the clearance rate is difficult to assess in tissues and that this event may be studied more adequately in other biologic samples, such as sputum or bronchoalveolar lavage fluids.

Among the mechanisms regulating cell apoptosis, a crucial role is played by the balance between proto-oncogenes, such as *bcl-2*, and anti-oncogenes, such as p53. The oncogene *bcl-2* is capable of inhibiting cell apoptosis,^{41,42} whereas p53 is involved in the induction of this phenomenon.⁴³ *bcl-2* plays a prominent role in tissue development or differentiation, in the maintenance of

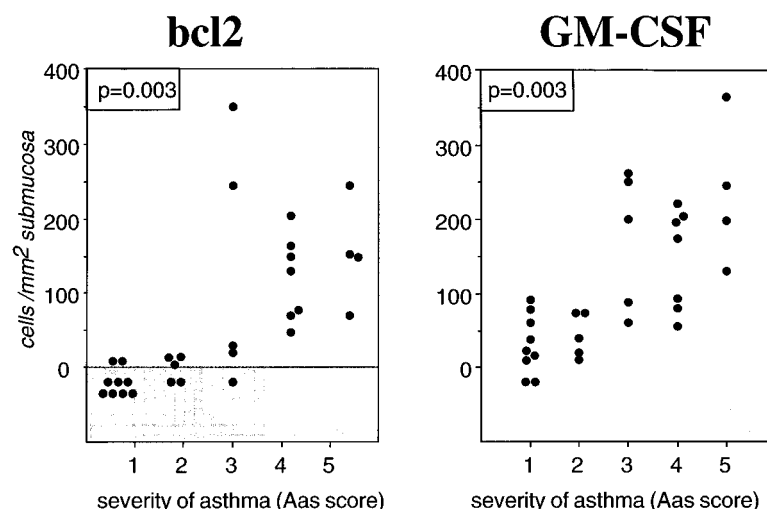
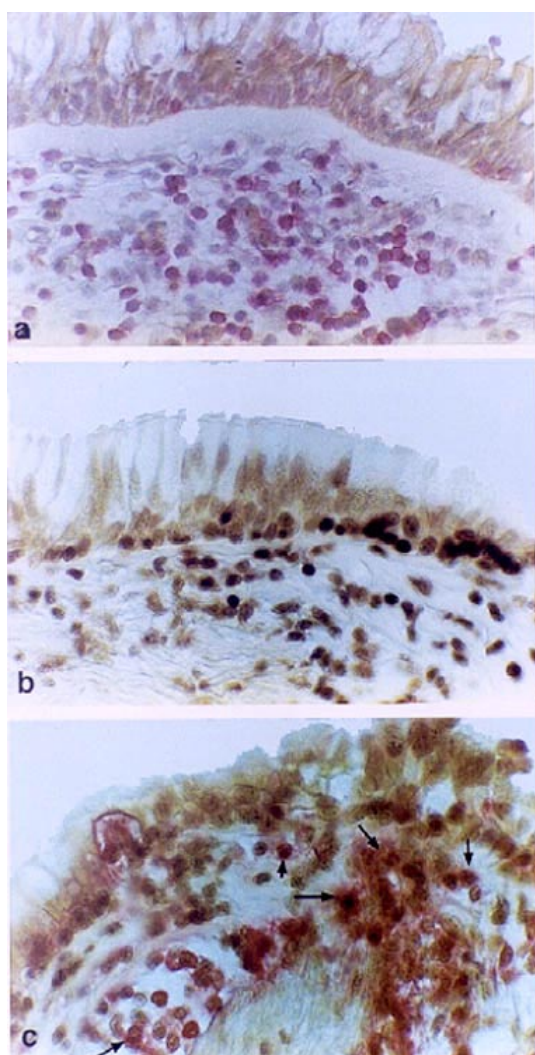


FIG 8. Correlation between the number of Bcl-2- and GM-CSF+ cells located in the submucosa of bronchial biopsy specimens of asthmatic patients and the clinical severity of asthma assessed by using the Aas score. Statistical analysis was done with Kendall's τ test.



the immune system, and in the enhancement of inflammatory cell survival.^{47,48} On the other hand the physiologic expression of P53 protein plays an important role in inducing cell apoptosis by blocking the cells in the G1 phase of the cell cycle.⁴⁹

The results of this study indicate an imbalance between the expression of Bcl-2 and P53, which appears to be more important in asthma than in chronic bronchitis. When compared with bronchial biopsy specimens from chronic bronchitis, specimens taken from asthmatic patients have an increased infiltration of cells expressing Bcl-2, which outnumbers that of cells expressing P53. The overexpression of Bcl-2 may play an important role in the reduced apoptosis of inflammatory cells in asthma. In this regard the correlation between the number of cells expressing Bcl-2 and T lymphocytes suggests that Bcl-2 may be involved in the enhancement of T-cell survival in the airways of asthmatic subjects. This hypothesis is also supported by the evidence that bcl-2 transcripts were found in lymphocytes but not in eosinophils and macrophages. Because of the pivotal role played by T lymphocytes in asthma, this latter evidence may have important clinical consequences and may indicate a possible mechanism by which the increased expression of Bcl-2 can influence the clinical severity of asthma, as shown in the present study.

With regard to P53, we acknowledge that our data do

FIG 9. Identification of lymphocytes bearing bcl-2 mRNA in bronchial biopsy specimens from an asthmatic subject. **a**, T lymphocytes stained with the anti-UCHL1 mAb. Cells are identified by the red staining caused by the APAAP technique. **b**, Cells bearing bcl-2 transcripts identified by using the in situ hybridization technique (dark brown staining). **c**, T lymphocytes bearing bcl-2 transcripts (arrows) identified combining the APAAP (red staining) and the in situ hybridization (dark brown staining) techniques (final magnification, 150 $\times$).

not indicate what cell types express this marker. However, it should be noted that in most of the biopsy specimens studied, P53 immunoreactivity was absent.

In conclusion, this study suggests that cell apoptosis can play a crucial role in the chronicity of airway inflammation associated with asthma and thus may represent a target for new and effective therapeutic strategies.

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