

Increase in Perforin-positive Peripheral Blood Lymphocytes in Extrinsic and Intrinsic Asthma

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The cause of asthma, which has been linked to a chronic, T-cell-mediated bronchial inflammation, remains unclear. A number of other T-lymphocyte-mediated, chronic inflammatory disorders have been associated with autoimmunity and there are data indicating that autoimmune phenomena might also be present in asthma. Expression of perforin, a cytotoxic molecule produced by lymphocytes, has been implicated in the pathogenesis of autoimmune diseases. We therefore tested the hypothesis that allergic and intrinsic asthma might be associated with an increase in lymphocytes producing perforin by comparing the expression of intracellular perforin in peripheral blood lymphocytes of patients with extrinsic asthma (n = 13), intrinsic asthma (n = 7), and healthy control subjects (n = 18). Lymphocytes were identified using flow cytometry and subdivided into CD3⁺, CD4⁺, CD8⁺, CD16⁺, and CD56⁺ subpopulations after staining with appropriate monoclonal antibodies. The percentage of perforin-positive total lymphocytes was significantly elevated in patients with allergic as well as intrinsic asthma when compared with normal control subjects. Analysis of lymphocyte subpopulations also revealed a significant increase in the percentage of CD3⁺, CD4⁺, CD8⁺, and CD56⁺ cells expressing perforin in allergic asthma and a significant increase in the percentage of CD4⁺ and CD56⁺ cells in intrinsic asthma when compared with healthy control subjects. Perforin expression in CD4⁺ cells in intrinsic asthma was also significantly elevated compared with allergic asthma. We conclude that allergic and intrinsic asthma is associated with increased expression of perforin in T-lymphocyte subsets. Arnold V, Balkow S, Staats R, Matthys H, Luttmann W, Virchow JC, Jr. Increase in perforin-positive peripheral blood lymphocytes in extrinsic and intrinsic asthma.

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Bronchial asthma is a chronic inflammatory disorder of unknown origin. Clinically, asthma can be divided into extrinsic/atopic asthma and an intrinsic/nonallergic variant. Whereas in extrinsic asthma allergens have been implicated in the development of airway inflammation and bronchospasm, the pathogenesis of intrinsic asthma remains unclear. Both allergic and intrinsic asthma have been linked to a chronic, T-cell- and eosinophil-mediated bronchial inflammation (1, 2) but the underlying etiologic mechanisms are still elusive. Based on clinical observations it has been hypothesized that asthma might have an autoimmune component (3). Several studies have reported organ- and nonorgan-specific autoantibodies in allergic and/or intrinsic asthma. Circulating autoantibodies directed against smooth muscle, thyroid, parietal cells, mitochondria, as well as antinuclear antibodies and IgG-anti-IgG antibodies have been described (4-7). In some of these studies autoanti-

body concentrations were more frequently detected in patients with intrinsic asthma (4, 6). In addition, the frequency of antinuclear antibodies was increased in patients with asthma and aspirin intolerance and these patients were more likely to suffer from clinical signs of autoimmunity (3). Other studies reported elevated concentrations of autoantibodies in both allergic and intrinsic asthma (4, 8). Yet, a pathogenetic role for these antibodies in asthmatic inflammation has never been conclusively demonstrated.

However, to our knowledge there are no studies that have investigated cell-mediated autoimmune phenomena in asthma. The chronic persistent nature of chronic allergic asthma and the chronic relentless course of intrinsic asthma have been associated with the accumulation of inflammatory cells in the airways, some of which have cytolytic potential. In addition to eosinophils (9), activated CD8⁺ T lymphocytes (1) and natural killer (NK) cells (10) have been located in allergic and/or intrinsic asthma, and the CD4/CD8 ratio in peripheral blood of patients with intrinsic asthma was elevated compared with allergic asthmatics and normal control subjects (1). The mechanisms, however, by which these cells might contribute to the pathogenesis of asthma have not been studied further.

Perforin, a 60-kD pore-forming protein stored intracellularly which is produced by NK cells, gamma/delta (γ/δ) cells, cytotoxic CD8⁺ T lymphocytes, and a small population of CD4⁺ T lymphocytes (11-16) has been reported in elevated concentrations in several chronic inflammatory disorders with

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autoimmune features such as multiple sclerosis (17, 18), Takayasu's arteritis (19), or autoimmune thyroid disease (20). Perforin can induce apoptosis in a number of cells including T lymphocytes and thus might also play a role in the resolution of inflammatory immune responses by eliminating inflammatory (21–23) or virally infected cells (24, 25). Because several clinical (26) as well as immunological (1) features of intrinsic asthma are compatible with an autoimmune process, we hypothesized that there might be evidence for an increase in perforin expression in lymphocytes in patients with asthma.

METHODS

Subjects

Patients attending the local chest clinics and who were diagnosed as suffering from allergic or intrinsic asthma as previously described (1) and published (27) were randomly selected to participate in this study. There were 13 subjects with extrinsic asthma (7 male, 6 female) with a mean age of 43 yr (range, 22 to 74 yr) and a mean FEV₁ of 77.6% $\pm$ 5.9% of predicted (range, 44 to 105%). The seven patients with intrinsic asthma (1 male, 6 female) had a mean age of 55 yr (range, 41 to 64 yr) and a mean FEV₁ of 62.4% $\pm$ 8.2% of predicted (31 to 89%). Eighteen healthy volunteers (14 female, 4 male) with a mean age of 40 yr (range, 25 to 65 yr) and a normal FEV₁ served as control subjects. Further patient characteristics are listed in Table 1. All patients gave their informed consent and the study protocol was approved by the local ethics committee.

Cell Preparation of Mononuclear Cells

Mononuclear cells were isolated from 10 ml of venous blood containing 0.2% ethylenediaminetetraacetic acid (EDTA) obtained from patients with extrinsic and intrinsic bronchial asthma as well as from

healthy volunteers on a gradient of Ficoll with a density of 1.077 g/L (Seromed, Berlin, Germany) as previously reported (28). Isolated peripheral blood mononuclear cells (PBMCs) were washed twice and resuspended in phosphate-buffered saline (PBS) supplemented with 2% heat-inactivated fetal calf serum (FCS; GIBCO, New York) at a concentration of 2×10^6 per milliliter.

Monoclonal Antibodies

Specific staining of the respective cell surface molecules was performed by anti-human CD3-phycoerythrin (PE) (clone UCHT1; Dako, Hamburg Germany), anti-human CD4-PE (clone, EDU-2; Cymbus Biotechnology, Hants, UK), anti-human CD8-PE (clone DK25; Dako), anti-human CD16-PE (clone 3G8; Immunotech, Hamburg, Germany), and anti-human CD56-PE (clone B-A19; Diaclone, Besancon, France). Anti-human perforin-fluorescein isothiocyanate (FITC) (clone δ G9; Hölzel Diagnostika, Köln, Germany) was used to analyze intracellular perforin. IgG-FITC and IgG-PE served as isotype-specific controls (both from Dako).

Intracellular Perforin Staining

After incubation of mononuclear cells with either anti-CD3, anti-CD4, anti-CD8, anti-CD16, or anti-CD56 antibodies, cells were fixed in paraformaldehyde (4% in PBS) for 15 min on ice, washed twice, and permeabilized with saponin (0.1% in PBS). Subsequently cells were incubated with FITC-conjugated antiperforin antibodies for 30 min, washed twice again, and then analyzed by flow cytometry.

Statistical Analysis

Results are expressed as arithmetic means $\pm$ SEM. Differences between groups were analyzed using the Mann-Whitney sum rank test. Differences with *p* values < 0.05 were considered statistically significant.

TABLE 1
CHARACTERISTICS OF PATIENTS

Sex	Age (yr)	Duration of Asthma (yr)	FEV ₁ Baseline (L)	FEV ₁ (% pred)	IVC (% pred)	IgE (kU/L)	Medication
Characteristics of patients with allergic asthma							
Male	24	4	4.4	98	83	93.2	ICS, β
Female	22	2	3.4	105	90	ND	ICS, β
Male	29	20	2.7	61	86	ND	ICS, β
Male	47	1	4.0	95	84	ND	ICS, β
Male	45	6	2.9	70	86	98.2	ICS, β
Female	59	27	2.1	75	87	194	ICS, β
Male	55	2	4.1	103	90	57.4	ICS, β
Female	64	2	1.3	47	59	7.4	ICS, β , L
Female	46	13	1.3	44	74	137	ICS, β , T
Male	26	3	2.0	53	60	1,009	ICS, β , L, T
Female	49	—	2.4	82	92	ND	ICS, β , T
Male	74	54	3.1	89	88	170	ICS, β , T
Female	23	16	3.0	87	74	ND	ICS, β
Mean	43.3	12.5	2.8	77.6	81.0	220.8	
SEM	4.8	4.5	0.3	5.9	3.1	114.6	
Characteristics of patients with intrinsic asthma							
Female	47	5	1.4	49	62	ND	C, ICS, β
Female	63	10	2.2	82	85	35	ICS
Female	55	19	2.4	89	93	48	ICS
Female	63	40	2.1	81	92	ND	ICS, β , T
Female	41	31	0.8	31	43	29	C, ICS, β , T
Male	64	4	1.6	49	56	61.3	ICS, β , T
Female	53	1	1.4	56	67	16.0	ICS, β
Mean	55.1	15.7	1.7	62.4	71.1	66.7	
SEM	3.4	5.6	0.2	8.2	7.3	24.0	

Definition of abbreviations: β = β_2 -agonists; C = oral corticosteroids; ICS = inhaled corticosteroids; L = leukotriene receptor antagonist; ND = not determined; T = theophylline.

RESULTS

Distribution of Lymphocyte Subpopulations in Peripheral Blood of Normal Control Subjects and Patients with Allergic and Intrinsic Asthma

The distribution of lymphocyte subpopulations was analyzed after incubation of cells with fluorescence-labeled antibodies against CD3, CD4, CD8, CD16, or CD56 and their subsequent differentiation by flow cytometry. As depicted in Figure 1 comparison of these lymphocyte subpopulations revealed no statistically significant differences in the percentage of CD3⁺, CD4⁺, CD8⁺, CD16⁺, and CD56⁺ subpopulations between patients with extrinsic or intrinsic asthma and healthy control subjects (Figure 1).

Perforin-Positive Lymphocytes

In contrast, when Ficoll separated mononuclear cells were fixed with paraformaldehyde and permeabilized by saponin, and then incubated with monoclonal antibodies against perforin there was a significantly higher percentage of cells expressing perforin in patients with extrinsic (35.3 ± 3.5%) and intrinsic asthma (37.7 ± 3.9%) compared with normal control subjects (21.7 ± 2.5%; $p < 0.05$ compared with allergic as well as intrinsic asthma) (Figure 2). However, there was no significant difference in the percentage of perforin-positive cells when cells from allergic and intrinsic asthma were compared.

Perforin Expression in Lymphocyte Subsets

To determine perforin expression in lymphocyte subsets, Ficoll-separated lymphocytes were incubated with monoclonal antibodies against CD3, CD4, CD8, CD16, or CD56 before fixation and permeabilization and then stained intracellularly with specific antibodies against perforin. Although the percentage of perforin-positive lymphocytes was highest in the CD16⁺ and CD56⁺ (NK cell) population, perforin⁺/CD3⁺, -CD4⁺, and -CD8⁺ cells were clearly detectable in all asthmatic patients. While there was no difference in the expression of perforin in the CD16⁺ lymphocyte population, perforin expression in CD3⁺, CD4⁺, CD8⁺, and CD56⁺ lymphocytes in patients with allergic asthma was significantly elevated compared with normal control subjects (each $p < 0.05$). Similar results were obtained for CD4⁺ and CD56⁺ cells expressing perforin in patients with intrinsic asthma ($p < 0.01$). Although the percentage of CD3⁺ and CD8⁺ lymphocytes expressing per-

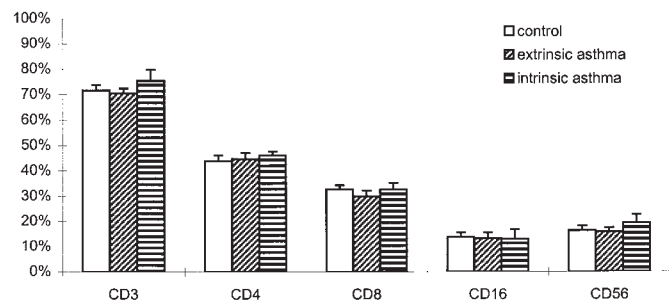


Figure 1. Composition of blood lymphocytes. Peripheral blood lymphocyte subpopulations from extrinsic ($n = 13$) and intrinsic asthma ($n = 7$) and healthy volunteers ($n = 18$) in percentage (%) of all lymphocytes. There were no significant differences in the distribution between the groups examined. Analyses were performed in whole blood. Values are mean ± SEM.

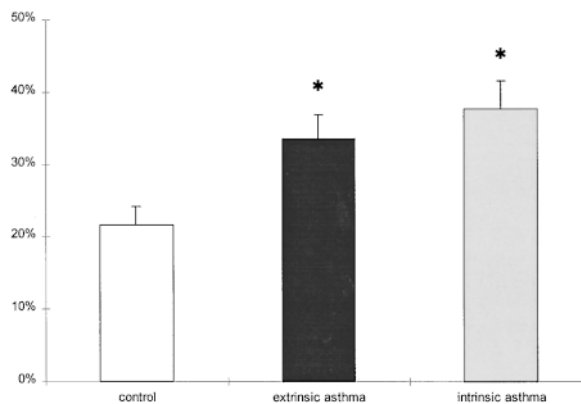


Figure 2. Percentage of perforin-containing lymphocytes. Comparison of the percentage of peripheral blood lymphocytes expressing intracellular perforin in healthy volunteers ($n = 18$), patients with extrinsic ($n = 13$) and intrinsic asthma ($n = 7$). As indicated by asterisks, extrinsic as well as intrinsic asthma had significantly increased percentages of perforin-expressing cells compared with normal control subjects. Values are mean ± SEM; * $p < 0.05$.

forin was elevated in patients with intrinsic asthma compared with normal control subjects, this difference failed to reach statistical significance. Interestingly, perforin expression in CD4⁺ lymphocytes in intrinsic asthma was also significantly elevated compared with patients with allergic asthma ($p < 0.05$) (Figure 3).

Correlation with Clinical Parameters

There was no correlation between parameters of airflow limitation, duration of asthma, or concomitant medication and expression of perforin in peripheral blood lymphocytes and lymphocyte subpopulations in all the groups studied. Although the percentage of perforin⁺/CD3⁺ and perforin⁺/CD8⁺ lymphocytes

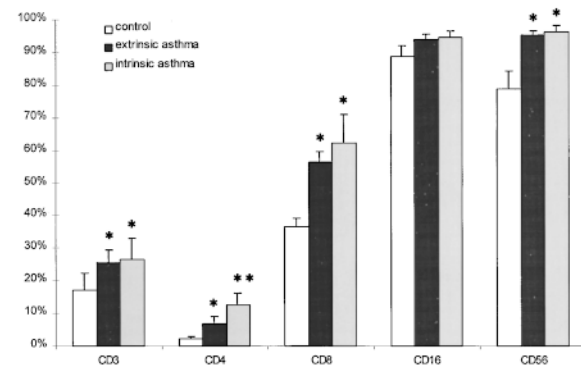


Figure 3. Percentage of perforin-containing subpopulations. Percentages of perforin-positive lymphocyte subpopulations from extrinsic and intrinsic asthma and normal control subjects. Both allergic and intrinsic asthma had significantly elevated percentages of perforin-positive cells in the total lymphocyte gate expressing perforin. All values are mean ± SEM. * $p < 0.05$. In contrast to the other lymphocyte subpopulations there was no significant difference in the percentage of CD16⁺/perforin⁺ lymphocytes between patients with asthma and normal control subjects. ** $p < 0.05$ compared with allergic asthma.

phocytes were related to the age of the patients in the intrinsic asthmatic subgroup ($r = 0.8$ and $r = 0.77$), this failed to reach statistical significance when corrected for multiple correlations.

DISCUSSION

The etiology of bronchial asthma is still incompletely understood. Despite increasing evidence that allergen-dependent augmentation of bronchial inflammation plays an important role in maintaining the cellular infiltrate in bronchi of allergic asthma, a number of features in the chronic course of this disease remains unexplained. Current hypotheses fail to explain why asthma often persists in the absence of allergen, and at present the mechanisms underlying the pathogenesis of intrinsic asthma are unclear. The clinical observation that asthma often takes a chronic, protracted course, its association with activated T lymphocytes in allergic as well as intrinsic asthma (1, 2), its association with cell-mediated damage to the bronchial mucosa and processes of tissue remodeling (29), its responsiveness to corticosteroids, the demonstration of an increased frequency of autoantibodies (3–7) together with the increased incidence of intrinsic asthma in female patients (30), and its unpredictable course with exacerbations and remissions but sometimes purely progressive forms lend support to the hypothesis that bronchial asthma has an autoimmune background to its pathogenesis.

However, cellular events associated with autoimmunity have not been assessed in asthma. In this study we provide evidence that the percentage of perforin-positive lymphocytes in peripheral blood is increased in both allergic and intrinsic asthma as compared with normal control subjects. The observed difference in perforin expression between asthmatics and normal control subjects persisted when CD3⁺, CD4⁺, CD8⁺, and CD56⁺ lymphocyte subpopulations were investigated separately, suggesting that perforin expression in these lymphocyte subpopulations is upregulated in bronchial asthma. The observed difference in perforin expression by lymphocytes between patients with asthma and normal control subjects is unlikely the result of a selective redistribution of lymphocyte subpopulations because these were basically identical between the three groups (Figure 3). Thus, our data suggest that in the presence of a similar distribution of CD3⁺, CD4⁺, CD8⁺, CD16⁺, and CD56⁺ cells, the elevated, perforin-positive fraction of each of these subpopulations reflects a true increase in the peripheral blood of patients with asthma.

It has recently been reported that the percentages of CD3⁺/perforin⁺, CD4⁺/perforin⁺, and CD8⁺/perforin⁺ cells decline with increasing age (31). This observation is contrasted by our findings of markedly elevated perforin-positive lymphocytes and lymphocyte subpopulations, especially in intrinsic asthma, despite the fact that this patient population was somewhat older than the normal control subjects. Thus, in view of the findings of Rukavina and coworkers (31) the differences in perforin expression observed in our study might have been even more pronounced in age-matched populations. On the other hand, our observation of different percentages of perforin-expressing lymphocyte subpopulations in allergic asthmatics and normal control subjects despite a similar age distribution in these groups suggests that the observed differences in perforin expression cannot be attributed to the age of our study subjects alone.

Although this is the first study to report an increase in the percentage of perforin-expressing lymphocytes in patients with asthma, our study cannot provide conclusive evidence for the functional or clinical significance of the increased percentage of perforin-expressing cells in asthma. At present their rel-

ative cytotoxic potential must remain unclear since to date there are no studies that have satisfactorily addressed this question in human diseases.

In normal volunteers perforin expression has been detected intracellularly in peripheral blood NK and CD8⁺ T cells but only in a small percentage of CD4⁺ T lymphocytes (32). Using immunocytochemistry Nakata and coworkers even failed to detect any perforin expression in unstimulated CD4⁺ T lymphocytes of healthy control subjects (16) which is in contrast to our findings where a small percentage of perforin-positive cells was detectable in healthy control subjects. An increase in the percentage of perforin⁺/CD4⁺ cells has been reported in infectious mononucleosis (16) and after treatment for Hodgkin's disease (33). Elevated percentages of CD4⁺/perforin⁺ T cells were also present in peripheral blood lymphocytes of patients with Wegener's granulomatosis (M. Schlesier, personal communication). Interestingly, the highest percentage of CD4⁺/perforin⁺ T lymphocytes was observed in patients with intrinsic asthma. Because little is known about the physiologic role and the putative cytotoxic functions of CD4⁺ major histocompatibility complex (MHC) class II-restricted T cells (34), we can only speculate about the role of perforin⁺/CD4⁺ T cells in bronchial asthma. Increased perforin expression has been reported in several other chronic inflammatory disorders with autoimmune phenomena such as multiple sclerosis (17, 18), Takayasu's arteritis (19), or autoimmune thyroid disease (20) and Crohn's disease (35) in which it was localized to mononuclear cells, CD4⁺, CD8⁺, CD16⁺, γ/δ T cells, or NK cells. Our findings of elevated numbers of potentially cytolytic CD4⁺ and CD8⁺ T lymphocytes in peripheral blood of these patients are therefore in line with our hypothesis of an autoimmune component to the pathology of asthma.

There are several possibilities by which the increased percentage of perforin-positive T lymphocytes might be associated with inflammation in asthma. Systemic interleukin-2 (IL-2) immunotherapy has been associated with an increased percentage of perforin⁺/CD4⁺ T cells, suggesting that the effects of IL-2 immunotherapy are also mediated by cytolytic lymphocytes (36). Similarly, perforin-mediated cytotoxicity has been associated with the vascular leak syndrome induced by IL-2 suggesting that IL-2 upregulates perforin expression (37). Elevated concentrations of IL-2 have been detected in bronchoalveolar lavage fluid of patients with intrinsic asthma (1) as well as following segmental allergen provocation in allergic asthma (2). Therefore, the elevated concentrations of IL-2 measured in patients with asthma might contribute to the increase in perforin-positive lymphocytes in our study population.

On the other hand, in an animal model of lupus erythematosus, perforin-deficient animals had more severe disease, suggesting that cytolytic lymphoid regulation plays a critical role in the immune homeostasis of these animals (38). Whether the increased percentage of perforin-positive lymphocytes observed in our patients reflects an increase in cytolytic activity inherent to the pathogenesis of asthma or instead a reaction of the immune system to remove abundant inflammatory cells remains at present unclear.

In conclusion, we provide evidence that bronchial asthma is associated with an increase in the percentage of cytolytic, perforin-positive lymphocytes. Whether these cells have a functional relevance in the pathogenesis of asthma requires further studies, and although there is circumstantial evidence linking perforin expression to the pathology of asthma, any causal relationship between asthma and perforin expression remains to be established.

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