

Increase in granzyme B⁺ lymphocytes and soluble granzyme B in bronchoalveolar lavage of allergen challenged patients with atopic asthma

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SUMMARY

Asthma has been linked to a chronic, T-cell-mediated bronchial inflammation. Because other T-lymphocyte-mediated, chronic inflammatory disorders have been associated with elevated granzyme B (grB) expression we tested the hypothesis that atopic asthma might be associated with elevated grB levels in the bronchoalveolar compartment. Therefore we performed intracellular grB staining in lymphocytes from bronchoalveolar lavage (BAL) collected 42 h after segmental allergen provocation (SAP) in allergic patients with bronchial asthma. There was a significant increase in CD3⁺, CD8⁺, and CD16/56⁺ lymphocytes expressing grB in BAL 42 h after SAP as compared to saline challenged controls. However, compared to peripheral blood the percentages of these lymphocyte subsets detected as grB⁺ in BAL remained significantly lower. Measurement of extracellular grB in BAL fluids by a particle immunoassay revealed significantly elevated grB levels in the allergen challenged bronchoalveolar compartment 42 h following SAP in six of the eight patients (range, <1.0–348.1 pg/ml) as compared to saline challenged controls (range, <1.0–70.5 pg/ml). We conclude that total cell numbers of grB⁺ lymphocyte subsets increase 42 h after SAP in the lower respiratory tract. In addition there is evidence to suggest that grB is released into the airways of asthmatic patients. This suggests a role for grB in the pathophysiological processes following SAP but its definitive role in allergic bronchial asthma needs to be established.

Keywords asthma cytotoxic lymphocytes granzyme B inflammation NK cell

INTRODUCTION

Atopic asthma is a complex and heterogenous disease that is characterized by intermittent reversible obstruction and chronic inflammation of the airways, bronchial hyperreactivity and an infiltration of eosinophils and lymphocytes into the airway submucosa [1–3]. However, the underlying aetiological mechanisms are still elusive. Based on clinical observations it has been hypothesized that asthma might have an autoimmune component [4]. Several studies have reported organ and nonorgan-specific autoantibodies in asthma. Circulating autoantibodies directed against smooth muscle, thyroid, parietal cells, mitochondria, as well as antinuclear antibodies and anti-IgG antibodies have been described by several groups [5–8]. In one study 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 [4]. Yet, the pathogenic significance of such autoantibodies remains unclear.

However, studies investigating cell-mediated autoimmune responses in allergic asthma are rare. Chronic allergic asthma has been associated with the accumulation of inflammatory cells in the airways, some of which can be cytotoxic. In addition to eosinophils [9], activated CD8⁺ T-lymphocytes and NK cells have been associated with the pathogenesis of bronchial asthma but their role in chronic asthma remains unclear [2,10]. In a previous study we reported an elevated percentage of perforin⁺ CD3⁺, CD4⁺, CD8⁺ and CD56⁺ lymphocytes in peripheral blood of patients suffering from allergic and intrinsic asthma [11]. Perforin which is a key protein of cell-mediated cytotoxicity is stored together with grB in secretory lysosomes of cytotoxic cells, such as cytotoxic T-cells and NK cells [12,13].

GrB, a glycosylated serine protease with a molecular mass of 32 kD has proapoptotic features. It can activate several caspases [14–17] and cleave caspase downstream substrates [18,19]. Elevated grB levels have been linked to the pathogenesis of

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inflammatory and/or autoimmune disorders like rheumatoid arthritis [20], acute generalized exanthematous pustulosis [21] and atopic dermatitis [22].

Because several clinical as well as immunological features of asthma are compatible with an inflammatory and/or autoimmune process, we hypothesized that there might be evidence for elevated grB levels in the bronchoalveolar compartment of patients with atopic asthma. The aim of this study was to investigate whether grB can be measured in BAL from atopic asthmatic patients in the model of segmental allergen provocation. Therefore, we performed SAP followed by BAL in patients suffering from allergic asthma and measured lymphocytes expressing intracellular grB, as well as soluble grB concentrations in BAL fluids 42 h after SAP.

MATERIALS AND METHODS

Subjects

Eight patients (3 female, 5 male) with a mean age of 25 years (range 18–30 years) who were diagnosed as suffering from allergic asthma as previously described [2] and published [23] were recruited from the participating institutions. Their mean FEV₁ was 96% ± 16% of predicted (range 75–116%). Further patient characteristics are listed in Table 1. Anti-asthmatic medication was withdrawn 7 days prior to the study except β_2 -agonists. All patients gave their written informed consent and the study protocol was approved by the local ethics committee.

Segmental allergen provocation

Bronchoscopies were performed as described in the guidelines of the American Thoracic Society [24]. Patients were pretreated with atropine and codeine (Dicodid®; Knoll, Ludwigshafen, Germany) and local anaesthesia was provided with Oxybuprocaine (Novesine®; Novartis, Basel, Switzerland). As a control 2.5 ml of saline were deposited into the anterobasal segment (S8) of the left lower lobe and one of the segments of the lingula (S4 or S5). After approximately 10 min the anterobasal segment of the left lower lobe was lavaged using 100 ml of prewarmed saline. Allergen which was dissolved in 2.5 ml of saline was then instilled into the

anterobasal segment (S8) of the right lower lobe and one of the segments of the middle lobe (S4 or S5) and the anterobasal segment (S8) was also lavaged with 100 ml prewarmed saline. After 42 h the patients were rebronchoscoped and the lingular or middle-lobe segments were lavaged in this sequence as described. BAL fluid was then filtered twice through sterile gauze and centrifuged with 400 g for 10 min at 12°C. The cell free supernatant was stored at –70°C for grB analysis. Cells were washed twice and resuspended in PBS supplemented with 2% heat-inactivated fetal calf serum (FCS; Gibco, New York) at a concentration of 2×10^6 per milliliter.

Preparation of mononuclear cells

Venous blood was drawn from all patients prior to allergen challenge into S-Monovettes (Sarstedt, Nümbrecht, Germany) containing 0.2% ethylenediaminetetraacetic acid (EDTA). Mononuclear cells were isolated on a Ficoll-gradient with a density of 1.077 g/l (Seromed, Berlin, Germany) as previously described [25]. Isolated peripheral blood mononuclear cells (PBMC) were washed twice and resuspended in phosphate buffered saline (PBS) supplemented with 2% heat-inactivated FCS at a concentration of 2×10^6 per milliliter.

Monoclonal antibodies

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 DJ130c; Dako), anti-human CD19-PE (clone HD37; Dako) anti-human CD56-PE (clone B-A19, Diaclone, Besancon, France), anti-human grB fluorescein-conjugate (clone HC4; Hoelzel Diagnostika, Köln, Germany). Fluorescein-conjugated IgG and IgG-PE served as isotype-specific controls (both from Dako).

Intracellular granzyme B staining

After incubation with specific antibodies against surface molecules (CD3, CD4, CD8, CD16, CD19, CD56), mononuclear cells from peripheral blood or BAL cells were fixed in paraformaldehyde (4% in PBS) for 15 min on ice, washed twice with PBS, and

Table 1. Patient characteristics

Sex	Age (years)	Duration of asthma (years)	FEV ₁ Baseline (l)	FEV ₁ (% pred)	IVC (% pred)	IgE (kU/l)	Allergen used for challenge	spec. IgE (kU/l)	Medication
Female	24	19	3.1	87	94	95.9	<i>B. verrucosa</i>	32.3	β
Male	30	22	5.1	107	98	229	<i>B. verrucosa</i>	5.7	ICS, β
Male	28	12	5.7	116	101	986	<i>S. cereale</i>	>100	β
Female	24	14	3.3	106	95	715	<i>D. pteronyssinus</i>	>100	ICS, β
Male	27	8	3.5	75	92	204	<i>B. verrucosa</i>	21.1	CR
Male	27	8	3.5	75	92	484	<i>D. pteronyssinus</i>	>100	ICS, β
Female	18	6	3.2	110	114	247	<i>D. pteronyssinus</i>	>100	ICS, β
Male	20	8	4.0	92	nd	314	<i>B. verrucosa</i>	nd	β
Mean	25	12	3.9	96	98	409			
SD	4	6	1.0	16	8	302			

β , β_2 -agonists; CR, cromoglycate; FEV₁, forced expiratory volume in one second; ICS, inhaled corticosteroids; IVC, inspiratory slow vital capacity; SD, standard deviation; nd, not determined. All medications except β_2 -agonists were withdrawn ≥ 7 days prior to the SAP. All patients showed a clear positive reaction in the skin prick test with the allergen used for allergen challenge.

permeabilized with saponin (0.1% in PBS) for 10 min on ice. Subsequently, cells were incubated with FITC-conjugated antibodies against grB for 30 min, washed twice with saponin (0.1% in PBS), and then analysed by flow cytometry.

Concentration of BAL fluids

Concentration of BAL fluids was performed using Centrprep YM-10 Filters (molecular weight cut off: 10 kD; Amicon, Millipore Co, USA) blocked with 3% bovine serum albumin (BSA) in PBS for 2 h. Centrifugation was carried out at 4°C with 1275 g until 10 ml BAL fluid were concentrated to about 1 ml. The weight of BAL fluids before and after concentration was measured to determine the correct concentration factor, which was used to calculate protein concentrations in unconcentrated BAL fluids.

Measurement of grB and interleukin-5 in BAL supernatants

Extracellular grB in BAL fluids was measured in 10fold concentrated BAL fluids using a commercial particle immunoassay (Hoelzel Diagnostika; Köln, Germany). As a control for protein stability in BAL fluid and an additional measure of the inflammatory asthmatic reaction we also measured IL-5 in BAL fluids using the TH1/TH2 cytokine cytometric bead array (BD Pharmingen, San Diego, USA) as described in the users manual. Experiments were analysed with a Becton Dickinson FACS Scan.

Statistical analysis

Results are expressed as arithmetic means $\pm$ SEM. Differences between groups were analysed using the students *t*-test. Differences with *P*-values < 0.05 were considered statistically significant.

RESULTS

BAL and peripheral blood total and differential cell counts

As shown in Table 2, total cell numbers, and numbers of eosinophils, neutrophils, and lymphocytes were significantly higher 42 h after SAP (each *P* < 0.05) compared to 10 min after SAP and control BALs. In peripheral blood only the number of eosinophils increased significantly (*P* < 0.05) 42 h after SAP.

Analysing lymphocyte subpopulations revealed a statistically significant (each *P* < 0.05) increase in the total number of CD3⁺, CD4⁺, CD8-dim⁺, CD8-bright⁺, CD19⁺, and CD16/56⁺ lymphocytes 42 h after SAP compared to 10 min after SAP and saline challenged controls (Table 3). Compared to BAL there was only a small change in cell numbers of the respective lymphocyte subpopulations in peripheral blood 42 h after SAP. Numbers of CD3⁺, CD4⁺ and CD19⁺ lymphocytes in the peripheral blood decreased slightly (Table 3). Although the number of CD8-dim⁺, CD8-bright⁺, and CD16/56⁺ lymphocytes were elevated after SAP

Table 2. Total and differential cell counts in BAL fluids and peripheral blood

Subjects	Total Cell Count ($\times 10^4/\text{ml}$)	Macrophages, Monocytes ($\times 10^4/\text{ml}$)	Neutrophils ($\times 10^4/\text{ml}$)	Eosinophils ($\times 10^4/\text{ml}$)	Lymphocytes ($\times 10^4/\text{ml}$)
BAL C 10 min	18.7 $\pm$ 5.8	15.9 $\pm$ 4.8	1.8 $\pm$ 1.4	0.2 $\pm$ 0.1	1.0 $\pm$ 0.4
BAL P 10 min	11.9 $\pm$ 3.1	10.0 $\pm$ 2.5	0.8 $\pm$ 0.6	0.4 $\pm$ 0.2	0.8 $\pm$ 0.4
BAL C 42 h	16.3 $\pm$ 3.4	14.6 $\pm$ 3.0	0.5 $\pm$ 0.2	0.5 $\pm$ 0.2	0.7 $\pm$ 0.1
BAL P 42 h	110.2 $\pm$ 32.7*	33.0 $\pm$ 11.2	10.2 $\pm$ 3.8*	61.0 $\pm$ 25.5*	7.5 $\pm$ 2.6*
Blood 0 h	569 $\pm$ 35	34 $\pm$ 2	356 $\pm$ 42	12 $\pm$ 3	164 $\pm$ 14
Blood 42 h	579 $\pm$ 32	37 $\pm$ 3	337 $\pm$ 39	41 $\pm$ 6**	159 $\pm$ 6

Data represent the arithmetic mean $\pm$ SEM. Total cell number and numbers of neutrophils, eosinophils and lymphocytes increased significantly (**P* < 0.05) in BAL 42 h after SAP (BAL P 42 h) compared to 10 min after SAP (BAL P 10 min) and saline challenged controls (BAL C 10 min, BAL C 42 h). In peripheral blood the number of eosinophils was significantly higher (***P* < 0.05) 42 h after SAP (Blood 42 h) then before SAP (Blood 0 h).

Table 3. Total counts of lymphocyte subpopulations in BAL fluids and peripheral blood

Subjects	CD3 ⁺ ($\times 10^3/\text{ml}$)	CD4 ⁺ ($\times 10^3/\text{ml}$)	CD8-dim ⁺ ($\times 10^3/\text{ml}$)	CD8-bright ⁺ ($\times 10^3/\text{ml}$)	CD16/56 ⁺ ($\times 10^3/\text{ml}$)	CD19 ⁺ ($\times 10^3/\text{ml}$)
BAL C 10 min	8.29 $\pm$ 0.29	4.42 $\pm$ 0.53	0.74 $\pm$ 0.10	2.36 $\pm$ 0.43	1.88 $\pm$ 0.36	0.15 $\pm$ 0.05
BAL P 10 min	6.66 $\pm$ 0.95	2.97 $\pm$ 0.52	0.59 $\pm$ 0.13	2.58 $\pm$ 0.52	1.44 $\pm$ 0.3	0.14 $\pm$ 0.05
BAL C 42 h	5.61 $\pm$ 0.29	2.94 $\pm$ 0.30	0.48 $\pm$ 0.06	1.80 $\pm$ 0.36	1.21 $\pm$ 0.23	0.12 $\pm$ 0.03
BAL P 42 h	46.99 $\pm$ 2.49*	33.71 $\pm$ 2.95*	3.73 $\pm$ 0.48*	8.82 $\pm$ 4.31*	8.72 $\pm$ 1.56*	2.76 $\pm$ 0.97*
Blood 0 h	1249 $\pm$ 62	789 $\pm$ 57	176 $\pm$ 15	298 $\pm$ 26	287 $\pm$ 5	120 $\pm$ 21
Blood 42 h	1170 $\pm$ 20	692 $\pm$ 57	220 $\pm$ 19	310 $\pm$ 36	346 $\pm$ 4**	94 $\pm$ 1

Data represent the arithmetic mean $\pm$ SEM. Total cell numbers of CD3⁺, CD4⁺, CD8-dim⁺, CD8-bright⁺, CD16/56⁺ and CD19⁺ lymphocyte subpopulations were significantly higher (**P* < 0.05) in BAL 42 h after SAP (BAL P 42 h) compared to 10 min after SAP (BAL P 10 min) and saline challenged controls (BAL C 10 min, BAL C 42 h). In peripheral blood the number of CD16/56⁺ cells increased significantly (***P* < 0.05) 42 h after SAP (Blood 42 h) compared to before SAP.

only the increase in CD16/56⁺ cells reached statistical significance ($P < 0.05$).

GrB expression in total lymphocytes and lymphocyte subpopulations from peripheral blood and BAL

Analysing grB expression, we found a statistically significant ($P < 0.05$) increase in total numbers of lymphocytes and CD3⁺, CD8-dim⁺, CD8-bright⁺, and CD16/56⁺ lymphocyte subsets expressing grB 42 h after SAP compared to 10 min after SAP and saline challenged control segments (Table 4). In peripheral blood cell numbers of the respective lymphocyte populations expressing grB were also elevated 42 h after SAP, but without reaching statistical significance (Table 4).

Comparing percentages of grB expressing lymphocytes and lymphocyte subsets in BAL with the respective cells in peripheral blood, we found much lower percentages of these cells expressing grB in all BAL samples.

As shown, in Fig. 1a, in peripheral blood $20.8 \pm 2.9\%$ (before SAP) and $24.2 \pm 4.6\%$ (42 h after SAP) of all lymphocytes were detected as grB-positive. 10 min after SAP $5.8 \pm 1.7\%$ of all lymphocytes recovered from the allergen challenged lung segment and $4.0 \pm 1.1\%$ of the respective cells from the saline challenged control segment were detected as grB-positive. 42 h after SAP there was a slight decrease in the percentage of grB expressing lymphocytes recovered from the allergen challenged lung segment ($4.4 \pm 1.1\%$) and the saline challenged control segment ($3.2 \pm 0.6\%$).

In peripheral blood we found over 80% of CD16/56⁺ lymphocytes expressing grB, but only between 5 and 12% of BAL CD16/56⁺ lymphocytes were grB-positive (Fig. 1b). 68% of CD8-dim⁺ cells expressed grB in peripheral blood but only between 1 and 9% of those obtained by BAL (Fig. 1c). And the percentage of CD8-bright⁺ and CD3⁺ lymphocytes expressing grB was two to threefold lower in BAL compared to peripheral blood (Fig. 1d,e). The percentage of CD4⁺ lymphocytes expressing grB was lower than 1% in all samples (data not shown).

Extracellular grB and Interleukin-5 in BAL supernatants

As depicted in Fig. 2a six of the eight asthmatic patients had higher grB concentrations in the BAL supernatant collected 42 h after SAP compared to BAL supernatants from saline challenged control segments, while in two patients concentrations in the allergen challenged segments were lower than in saline

challenged control segments (range, <1.0 – 348.1 pg/ml versus <1.0 – 70.5 pg/ml). IL-5 levels which were measured in the identical BAL supernatants (Fig. 2b) revealed that six of the eight asthmatic volunteers also had significantly elevated IL-5 concentrations 42 h after SAP compared to the saline challenged control segments (range, 0.6 – 893.7 pg/ml versus <0.3 – 3.95 pg/ml). Interestingly, those two patients who had the lowest increase in IL-5 between saline and allergen challenged segments were the same who had lower grB concentrations in the BAL fluid recovered from the allergen challenged segment.

Correlation with clinical parameters

There was no correlation found between parameters of airflow limitation, duration of asthma, or medication and expression of grB in BAL and peripheral blood lymphocytes 42 h after SAP. In the same way there was no correlation between these clinical parameters and soluble grB in BAL supernatants 42 h after SAP.

DISCUSSION

The pathophysiological basis underlying bronchial asthma is not fully understood. It is well established that airway inflammation in asthma is enhanced by allergen-dependent mechanisms, but many features of the chronic course of this disease remain unexplained. In order to separate our analysis of cytotoxic mechanisms following allergen challenge a time point of 42 h after SAP was chosen to increase the likelihood that our observations might be related to T-lymphocyte dependent inflammation rather than mast cell mediated effects. Clinical observations in chronic asthma indirectly support the hypothesis that bronchial asthma might have an autoimmune background to its pathogenesis: There is the chronic, protracted course, an association with activated T-lymphocytes [2,3], and cell-mediated damage to bronchial mucosa as well as chronic repair processes possibly leading to processes of tissue remodeling [26]. Furthermore, chronic asthma responds well to corticosteroids, and the demonstration of an increased frequency of autoantibodies in patients with bronchial asthma [4–8] is suggestive of an autoimmune background.

Cellular mechanisms associated with autoimmunity, however, have so far rarely been investigated in asthma. The attempt of our present study therefore was to investigate the expression and release of the proapoptotic protease grB in allergic asthma following allergen challenge. In agreement with other studies, where

Table 4. Total counts of grB⁺ lymphocyte subpopulations in BAL fluids and peripheral blood

Subjects	Total lymphocytes ($\times 10^2$ /ml)	CD3 ⁺ ($\times 10^2$ /ml)	CD8-dim ⁺ ($\times 10^2$ /ml)	CD8-bright ⁺ ($\times 10^2$ /ml)	CD16/56 ⁺ ($\times 10^2$ /ml)
BAL C 10 min	4.0 ± 1.1	3.8 ± 1.3	0.1 ± 0.1	1.7 ± 1.2	1.0 ± 0.2
BAL P 10 min	4.6 ± 1.4	5.0 ± 1.2	0.5 ± 1.2	2.2 ± 0.9	1.2 ± 0.4
BAL C 42 h	2.2 ± 0.4	2.2 ± 0.5	0.4 ± 0.1	1.5 ± 0.3	0.8 ± 0.2
BAL P 42 h	$33.0 \pm 8.3^*$	$26.8 \pm 5.6^*$	$3.5 \pm 2.0^*$	$11.7 \pm 4.5^*$	$3.4 \pm 1.8^*$
Blood 0 h	3411 ± 479	1374 ± 375	1197 ± 112	650 ± 164	2514 ± 353
Blood 42 h	3852 ± 727	1521 ± 480	1504 ± 223	697 ± 223	2937 ± 470

Data represent the arithmetic mean $\pm$ SEM. Total numbers of grB⁺ lymphocytes and grB⁺ CD3⁺, CD8-dim⁺, CD8-bright⁺ and CD16/56⁺ lymphocyte subsets were significantly higher ($*P < 0.05$) in BAL 42 h after SAP (BAL P 42 h) compared to 10 min after SAP (BAL P 10 min) and saline challenged controls (BAL C 10 min, BAL C 42 h). In peripheral blood total numbers of grB⁺ lymphocytes and grB⁺ CD3⁺, CD8-dim⁺, CD8-bright⁺ and CD16/56⁺ lymphocyte subsets increased slightly 42 h after SAP (Blood 42 h), but failed to reach statistical significance.

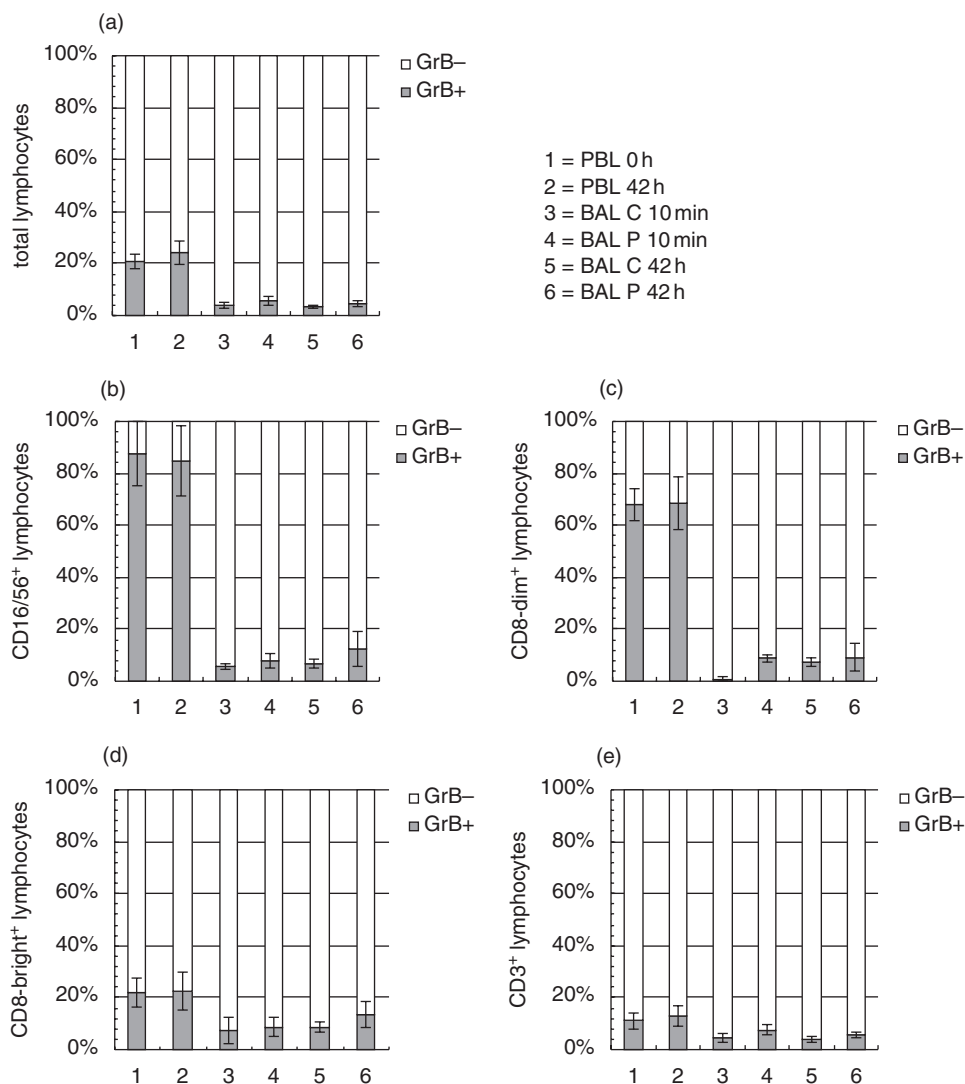


Fig. 1. Distribution of grB in peripheral blood lymphocytes (PBL) and BAL lymphocytes after SAP as indicated by flow cytometric analysis. Percentages are the mean $\pm$ SEM of eight different allergic asthmatic patients. ■ GrB+, □ GrB-. (a) Percentages of grB⁺ total lymphocytes in BAL and peripheral blood. Percentages of (b) CD16/56⁺, (c) CD8-dim⁺, (d) CD8-bright⁺ and (e) CD3⁺ lymphocytes expressing grB in BAL samples and peripheral blood. PBL 0 h, PBL before SAP; PBL 42 h, PBL 42 h after SAP; BAL C 10 min, BAL lymphocytes from saline challenged lung segments 10 min after SAP; BAL C 42 h, BAL lymphocytes from saline challenged lung segments 42 h after SAP; BAL P 10 min, BAL lymphocytes from allergen challenged lung segments 10 min after SAP; BAL P 42 h, BAL lymphocytes from allergen challenged lung segment 42 h after SAP.

repeated bronchoscopies were performed 18 h after SAP [2,3] we found significantly elevated numbers of eosinophils, neutrophils and CD3⁺, CD4⁺, CD8-dim⁺, CD8-bright⁺, CD19⁺ as well as CD16/56⁺ lymphocytes in BAL also 42 h after SAP (Tables 2 and 3). These data suggest that in this valid human model of allergic inflammation allergen induced mechanisms persist far beyond the clinically noticed late asthmatic reaction. To our knowledge this is the first study to address this question within this time frame after allergen challenge. But it is at present unclear whether these observations might already exist at earlier timepoints following allergen challenge.

Furthermore we were able to detect significantly higher numbers of grB⁺ lymphocytes in BAL 42 h after SAP (Table 4) although the percentages of these lymphocytes were almost similar in all BAL samples. Interestingly, however, the percentage of

grB⁺ lymphocytes was significantly reduced in BAL fluids compared to peripheral blood (Fig. 1). Analysis of lymphocyte subpopulations showed a marked difference in the percentage of grB⁺ CD16/56⁺ and CD8-dim⁺ cells in BALs compared to peripheral blood. In the case of CD3⁺ and CD8-bright⁺ BAL cells this difference was less pronounced but also significant (Fig. 1). Several studies have outlined that activated cytotoxic lymphocytes can migrate into the airways of asthmatic patients following allergen stimulation [2,27] where degranulation of these cells could occur after stimulation with several chemokines such as RANTES, MCP-1 and MIP-1 α [28] which are increased in allergic asthma [29].

In order to investigate whether grB was indeed released from cytotoxic lymphocytes or simply not synthesized from BAL cytotoxic lymphocytes extracellular grB was measured in BAL fluid.

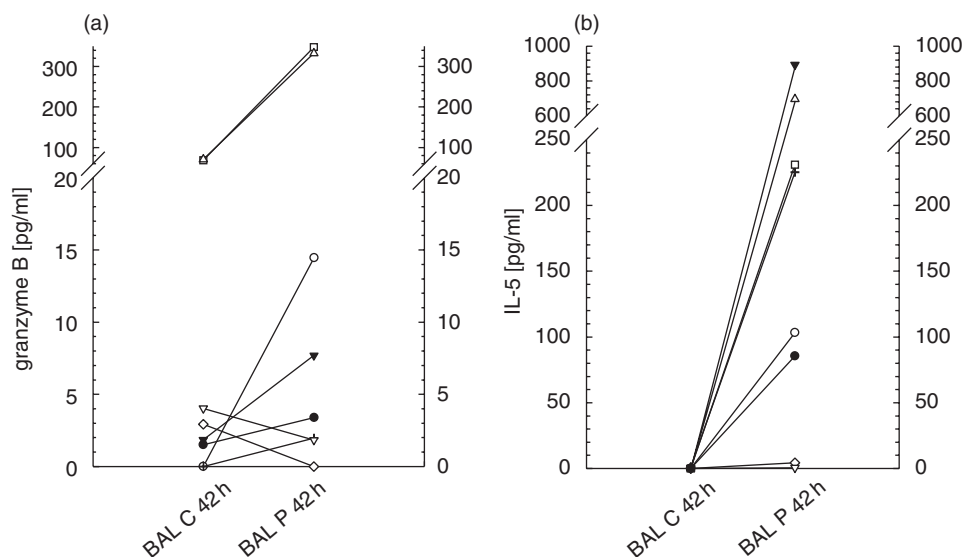


Fig. 2. Concentrations of extracellular (a) grB and (b) IL-5 in BAL fluid from eight different asthmatic patients 42 h after SAP. Six of eight patients showed significantly higher grB and IL-5 concentrations in the allergen challenged lung segment compared to saline challenged control. BAL C 42 h, BAL fluid from saline challenged lung segments 42 h after SAP; BAL P 42 h, BAL fluid from allergen challenged lung segment 42 h after SAP.

Compared with the saline challenged control segment extracellular grB concentrations were elevated in BAL fluid recovered 42 h after allergen challenge from six of the eight patients. The two patients whose extracellular grB concentrations were lower also showed a very low IL-5 concentration. We therefore cannot exclude that allergic inflammation 42 h after SAP in these patients was less severe which might also find its reflection in the lower grB concentrations. Another explanation for this phenomenon which cannot be ruled out at the present time might be a contamination of these BAL fluids with proteases resulting in protein degradation. Thus, the observed similarities in the low percentages of grB-positive cells in the different BAL samples might in part be due to a partial or complete release of grB from BAL lymphocytes 42 h after SAP.

Our observation of elevated extracellular grB levels 42 h after SAP is compatible with studies showing higher extracellular grB concentrations in other inflammatory diseases such as hypersensitivity pneumonitis [30] and rheumatoid arthritis [31]. Since Tremblay *et al.* [30] showed that grB activity is not inhibited by the three main serine protease inhibitors present in the lung, their is little evidence suggesting that extracellular grB measured in our study might not have been biologically active.

Nevertheless, the pathogenetic role of grB in chronic allergic asthma remains a matter of speculation. Resistance of T-cells to Fas-mediated apoptosis in asthma has been reported [32,33]. One speculation therefore assumes that grB is secreted in higher concentrations to compensate this phenomenon in order to regulate infiltrating cell numbers. Whether our observations are linked to the often debated association of asthma with viral and possibly bacterial infections [34] remains similarly unclear but both pathogens can initiate cytotoxic lymphocyte host defenses involving grB release [35]. Furthermore, it has been shown that grB is able to degrade proteoglycans indicating a role for this enzyme in joint destruction in rheumatoid arthritis [36,37]. Proteoglycans are one of the main constituents of the extracellular matrix and

extracellular grB could therefore participate in bronchial mucosal damage and airway remodeling.

In conclusion, our study provides evidence that allergen challenge in bronchial asthma might be associated with elevated levels of extracellular grB in the airways which is most likely secreted by activated NK cells and CD8⁺ lymphocytes. Because of the small number of subjects further investigations are required to confirm these findings and to elucidate the pathogenetic role of grB in this disease, but our data suggest that cytotoxic mechanisms might be another facet in chronic asthmatic inflammation.

REFERENCES

- Yssel H, Groux H. Characterization of T cell subpopulations involved in the pathogenesis of asthma and allergic diseases. *Int Arch Allergy Immunol* 2000; **121**:10–8.
- Walker C, Bode E, Boer L, Hansel TT, Blaser K, Virchow JC Jr. Allergic and nonallergic asthmatics have distinct patterns of T-cell activation and cytokine production in peripheral blood and bronchoalveolar lavage. *Am Rev Respir Dis* 1992; **146**:109–15.
- Virchow JC Jr, Walker C, Hafner D *et al.* T cells and cytokines in bronchoalveolar lavage fluid after segmental allergen provocation in atopic asthma. *Am J Respir Crit Care Med* 1995; **151**:960–8.
- Szeklik A, Nizankowska E, Serafin A, Dyczek A, Duplaga M, Musial J. Autoimmune phenomena in bronchial asthma with special reference to aspirin intolerance. *Am J Respir Crit Care Med* 1995; **152**:1753–6.
- Turner-Warwick M, Haslam P. Smooth muscle antibody in bronchial asthma. *Clin Exp Immunol* 1970; **7**:31–8.
- Egeskjold EM, Permin H, Nielsen I, Sorensen HJ, Osterballe O, Kallerup HE. Anti-IgG antibodies and antinuclear antibodies in allergic patients. *Eur J Allergy Clin Immunol* 1981; **36**:573–81.
- Menon P, Menon V, Hilman BC, Wolf R, Bairnsfather L. Antinuclear antibodies and anticytoplasmic antibodies in bronchial asthma. *J Allergy Clin Immunol* 1989; **84**:937–43.
- Venter JC, Fraser CM, Harrison LC. Autoantibodies to beta 2-adrenergic receptors: a possible cause of adrenergic hyporesponsiveness in allergic rhinitis and asthma. *Science* 1980; **207**:1361–3.

- 9 Bousquet J, Chanez P, Lacoste JY *et al.* Eosinophilic inflammation in asthma. *N Engl J Med* 1990; **323**:1033–9.
- 10 Krejsek J, Kral B, Vokurkova D *et al.* Decreased peripheral blood gamma delta T cells in patients with bronchial asthma. *Eur J Allergy Clin Immunol* 1998; **53**:73–7.
- 11 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. *Am J Respir Crit Care Med* 2000; **161**:182–6.
- 12 Trapani JA. Target cell apoptosis induced by cytotoxic T cells and natural killer cells involves synergy between the pore-forming protein perforin and the serine protease granzyme B. *Aust N Z J Medical* 1995; **25**:793–9.
- 13 Peters PJ, Borst J, Oorschot V *et al.* Cytotoxic T lymphocyte granules are secretory lysosomes, containing both perforin and granzymes. *J Exp Med* 1991; **173**:1099–109.
- 14 Darmon AJ, Nicholson DW, Bleackley RC. Activation of the apoptotic protease CPP32 by cytotoxic T-cell-derived granzyme B. *Nature* 1995; **377**:446–8.
- 15 Orth K, Chinnaiyan AM, Garg M, Froelich CJ, Dixit VM. The CED-3/ICE-like protease Mch2 is activated during apoptosis and cleaves the death substrate lamin A. *J Biol Chem* 1996; **271**:16443–6.
- 16 Gu Y, Sarnecki C, Fleming MA, Lippke JA, Bleackley RC, Su MS. Processing and activation of CMH-1 by granzyme B. *J Biol Chem* 1996; **271**:10816–20.
- 17 Duan H, Orth K, Chinnaiyan AM *et al.* ICE-LAP6, a novel member of the ICE/Ced-3 gene family, is activated by the cytotoxic T cell protease granzyme B. *J Biol Chem* 1996; **271**:16720–4.
- 18 Andrade F, Roy S, Nicholson D, Thornberry N, Rosen A, Casciola-Rosen L. Granzyme B directly and efficiently cleaves several downstream caspase substrates: implications for CTL-induced apoptosis. *Immunity* 1998; **8**:451–60.
- 19 Froelich CJ, Hanna WL, Poirier GG *et al.* Granzyme B/perforin-mediated apoptosis of Jurkat cells results in cleavage of poly (ADP-ribose) polymerase to the 89-kDa apoptotic fragment and less abundant 64-kDa fragment. *Biochem Biophys Res Commun* 1996; **227**:658–65.
- 20 Smeets TJM, Dolhain RJEM, Breedveld FC, Tak PP. Analysis of the cellular infiltrates and expression of cytokines in synovial tissue from patients with rheumatoid arthritis and reactive arthritis. *J Pathol* 1998; **186**:75–81.
- 21 Schmid S, Kuechler PC, Britschgi M *et al.* Acute generalized exanthematous pustulosis: role of cytotoxic T cells in pustule formation. *Am J Pathol* 2002; **161**:2079–86.
- 22 Yawalkar N, Schmid S, Braathen LR, Pichler WJ. Perforin and granzyme B may contribute to skin inflammation in atopic dermatitis and psoriasis. *Br J Dermatol* 2001; **144**:1133–9.
- 23 Anonymous. International consensus report on diagnosis and treatment of asthma. *Eur Respir J* 1992; **5**:601–41.
- 24 Anonymous. Guidelines for fiberoptic bronchoscopy in adults. American Thoracic Society. Med Section Am Lung Assoc Am Rev Respir Dis 1987; **136**:1066.
- 25 Luttmann W, Herzog V, Virchow JC Jr, Matthys H, Thierach KH, Kroegel C. Prostacyclin modulates granulocyte/macrophage colony-stimulating factor release by human blood mononuclear cells. *Pulm Pharmacol* 1996; **9**:43–8.
- 26 Leung DY. Atopic dermatitis: the skin as a window into the pathogenesis of chronic allergic diseases. *J Allergy Clin Immunol* 1995; **96**:302–18.
- 27 O'Sullivan S, Cormican L, Faul JL *et al.* Activated, cytotoxic CD8⁺ T lymphocytes contribute to the pathology of asthma death. *Am J Respir Crit Care Med* 2001; **164**:560–4.
- 28 Loetscher P, Seitz M, Clark-Lewis I, Baggiolini M, Moser B. Activation of NK cells by CC chemokines. Chemotaxis, Ca²⁺ mobilization, and enzyme release. *J Immunol* 1996; **156**:322–7.
- 29 Alam R, York J, Boyars M *et al.* Increased MCP-1, RANTES, and MIP-1alpha in bronchoalveolar lavage fluid of allergic asthmatic patients. *Am J Respir Crit Care Med* 1996; **153**:1398–404.
- 30 Tremblay GM, Wolbink AM, Cormier Y, Hack CE. Granzyme activity in the inflamed lung is not controlled by endogenous serine proteinase inhibitors. *J Immunol* 2000; **165**:3966–9.
- 31 Tak PP, Spaeny-Dekking L, Kraan MC, Breedveld FC, Froelich CJ, Hack CE. The levels of soluble granzyme A and B are elevated in plasma and synovial fluid of patients with rheumatoid arthritis (RA). *Clin Exp Immunol* 1999; **116**:366–70.
- 32 Jayaraman S, Castro M, O'Sullivan M, Bragdon MJ, Holtzman MJ. Resistance to Fas-mediated T cell apoptosis in asthma. *J Immunol* 1999; **162**:1717–22.
- 33 Spinozzi F. Onset and persistence of the allergic inflammation: a molecular explanation? *Arch Immunol Ther Exp* 1998; **46**:277–84.
- 34 Micillo E, Bianco A, D'Auria D, Mazzarella G, Abbate GF. Respiratory infections and asthma. *Allergy* 2000; **61**:42–5.
- 35 Spaeny-Dekking EHA, Hanna WL, Wolbink AM *et al.* Extracellular granzymes A and B in humans: detection of native species during CTL responses in vitro and in vivo. *J Immunol* 1998; **160**:3610–6.
- 36 Froelich CJ, Zhang X, Turbov J, Hudig D, Winkler U, Hanna WL. Human granzyme B degrades aggrecan proteoglycan in matrix synthesized by chondrocytes. *J Immunol* 1993; **151**:7161–71.
- 37 Ronday HK, van der Laan WH, Tak PP *et al.* Human granzyme B mediates cartilage proteoglycan degradation and is expressed at the invasive front of the synovium in rheumatoid arthritis. *Rheumatology* 2001; **40**:55–61.