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Granzyme Activity in the Inflamed Lung Is Not Controlled by Endogenous Serine Proteinase Inhibitors[1]

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Numerous lung diseases, such as hypersensitivity pneumonitis (HP), are characterized by the presence of activated alveolar CTL and NK cells. Since these cells produce granzymes, granzyme A and B levels in bronchoalveolar lavage (BAL) fluids from 14 normal subjects and 12 patients with HP were measured by ELISA. Median (range) BAL granzyme A and B levels were 4 (0–37) and 0 (0–6) pg/ml in normal subjects. BAL granzyme levels were significantly higher in HP patients, being at 74 (0–1889) and 10 (0–78) pg/ml for granzymes A and B, respectively. In vitro, neither of the three main serine protease inhibitors of the lung, namely α_1-antitrypsin, secretory leukocyte protease inhibitor, and elafin, showed any effect on granzyme A or B activity. In addition, granzyme A was shown to be fully active in BAL fluids. Hence, these data show that granzyme activity may be poorly controlled by protease inhibitors in inflamed tissues. Thus, granzymes could contribute to tissue remodeling and inflammation characterizing HP. ***The Journal of Immunology*****, 2000, 165: 3966–3969.**

Granzymes A and B belong to a family of serine proteases found in the granules of activated CTL and NK cells (1). Granzyme B induces apoptosis by processing and activating members of the caspase family, while granzyme A does not play a primary role in cell-mediated cytotoxicity. In vitro, both granzymes may be released extracellularly following degranulation (2). This observation led Spaeny-Dekking and colleague to hypothesize that the levels of granzyme in biological fluids could reflect the presence of activated CTL and NK cells (3). Indeed, elevated levels of granzymes A and B were found in the plasma of patients with EBV, HIV-1, or posttransplant primary CMV infection, and in the plasma and synovial fluid of patients with rheumatoid arthritis (3–5).

The presence of granzymes in the extracellular milieu could significantly modulate the inflammatory response. For example, granzyme A is believed to participate in target cell lysis, migration, and extravasation of T lymphocytes; activation of prourokinase-type plasminogen activator; regulation of B cell growth; and control of viral infection (6). Moreover, granzyme A stimulates the production of different cytokines (7, 8) and is an IL-1β-converting enzyme (9). Finally, extracellular granzymes A and B may also contribute to tissue destruction and remodeling by degrading various extracellular matrix proteins (10–12).

Numerous inflammatory lung diseases such as hypersensitivity pneumonitis (HP)[3] are characterized by the presence of increased numbers of activated CTL and NK cells in the alveolar space (13). Alveolar lymphocytes are able to show increased granzyme B gene expression (14). Therefore, increased levels of granzymes could be generated into the alveolar space during a lymphocytic pulmonary inflammation and contribute to the disease process.

The lung is protected from the detrimental destructive potential of proteases by protease inhibitors (15). The main serine protease inhibitors present in the lung are α_1-antitrypsin, secretory leukocyte protease inhibitor (SLPI), and elafin (15, 16). It is not yet clear whether these inhibitors can inhibit granzymes. Some have reported that α_1-antitrypsin inhibits granzyme B (17), while we (C.E.H., unpublished observations) and others (P. Bird, personal communication) have not observed such inhibition. Since elafin suppresses by 75% the degradation of insoluble elastin by purified peripheral blood T cells (18), and granzyme A is elastolytic (19), it has been suggested that elafin inhibits granzyme A (18). To our knowledge, there are no data available on granzyme inhibition by SLPI.

The present study was done to measure levels of granzymes A and B in bronchoalveolar lavage (BAL) fluids obtained from normal subjects and patients with HP. Finally, we verified whether granzymes are inhibited by α_1-antitrypsin, SLPI, and elafin, and whether they are active in BAL fluids.

Materials and Methods

Subjects and sample procedures

BAL fluid samples were obtained from 14 unexposed healthy subjects and 12 patients with active HP. All subjects were nonsmokers. The diagnostic of HP (including farmer's lung, peat moss lung, humidifier lung, and pigeon breeder's disease) was based on published criteria that include a documented history of exposure to sensitizing Ag, symptomatic acute febrile episodes, dyspnea, and abnormal pulmonary functions characterizing this disease (13).

BAL was done with a 300-ml (6 × 50 ml) instillation of isotonic saline (0.9% NaCl). Total cell count was determined with a hemocytometer and differential cell count on Diff-Quik (Dade Diagnostics, Aguada, PR)-stained cytocentrifuge cell preparations. After centrifugation, cell-free aliquots of BAL fluids were frozen at −70°C until analysis. For two normal and nine HP subjects, serum was collected from venous blood and stored at −70°C until analysis.

Measurement of granzyme A and B levels

BAL fluid levels of granzymes A and B were measured with two previously described and validated ELISA using specific anti-granzyme mAbs showing no cross-reactivity with other serine proteases (3).

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[3] Abbreviations used in this paper: HP, hypersensitivity pneumonitis; BAL, bronchoalveolar lavage; SLPI, secretory leukocyte protease inhibitor.

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Inhibition of granzymes by serine protease inhibitors and determination of active granzyme levels in BAL samples

Effect of serine protease inhibitors on granzyme A activity was done using a method based on the interaction of active granzyme A with antithrombin III (20). Briefly, granzyme A, purified as described previously (20), was bound to anti-granzyme A mAb-28 immobilized onto an ELISA plate at 3 ng/ml, and incubated with increasing concentrations of up to 10,000-fold molar excess of α_1-antitrypsin (Calbiochem, La Jolla, CA), SLPI (R&D Systems, Minneapolis, MN), or elafin (Peptides International, Louisville, KY), with biotinylated antithrombin III in the presence of heparin. Bound biotinylated antithrombin III was detected with streptavidin-coupled polymerized HRP. Purified antithrombin III (Sigma-Aldrich Chemicals, St. Louis, MO) was tested as a positive control.

Effect of serine protease inhibitors on granzyme B activity was performed according to a protocol from Enzyme Systems Products (Livermore, CA). Briefly, granzyme B (Enzyme Systems Products) and inhibitors were resuspended in 100 mM Tris, 500 mM NaCl, pH 7.5, at a working concentration of 150 nM. Then, 5 pmol of granzyme B was incubated with 5, 20, or 80 pmol of α_1-antitrypsin, SLPI, or elafin for 30 min at 37°C in a final volume of 50 μl. This volume was added to 150 μl of 270 μM BOC-Ala-Ala-Asp-S-Bzl (Enzyme Systems Products) in 200 mM HEPES, 300 mM NaCl, 1 mM EDTA, 0.05% (v/v) Triton X-100, pH 7, containing 410 μM of 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent). Kinetic changes in absorbance were measured at 405 nm with a *THERMO*$_{max}$ microplate reader (Molecular Devices, Sunnyvale, CA).

These two methods for granzyme A and B activity were also used to determine the presence of active granzyme A and B concentrations in the BAL fluid samples.

Statistical analysis

BAL total and differential cell counts are expressed as mean ± SEM and were analyzed using a paired Student's *t* test. Levels of granzymes A and B are expressed as median (range) and were analyzed by a Mann-Whitney test. Spearman's coefficient of rank correlation was used to assess the degree of association between BAL cell numbers and serum granzyme levels. In all cases, $p < 0.05$ was considered as significant (21).

Results

BAL fluid cell numbers

BAL fluid recovery was similar for each group of subjects. Of the 300-ml instilled, 181 ± 21 and 170 ± 14 ml were recovered from normal and HP subjects, respectively. Total cell number in BAL fluid was 97.1 ± 12.2 × 10^3 cells/ml for normal subjects. Cell number was significantly increased in HP patients to 785.3 ± 103.5 × 10^3 cells/ml (*t* test, $p = 0.0001$). All BAL cell types were increased in HP patients compared with control subjects (Table I). Macrophages represented about 85% of BAL cells in normal subjects. In contrast, lymphocytes were the predominant cells (>60%) in HP patients.

BAL fluid granzyme levels

BAL fluids from normal subjects showed minimal levels of granzymes A and B (Figs. 1 and 2). In these subjects, the median (range) granzyme A and B concentrations were 4 (0–37) and 0 (0–6) pg/ml of BAL fluid, respectively. It is noteworthy that, of 14 normal subjects studied, 5 and 10 had no detectable levels of granzymes A and B, respectively.

BAL fluids from patients with HP contained 74 (0–1889) pg/ml of granzyme A (Fig. 1) and 10 (0–78) pg/ml of granzyme B (Fig. 2). These values were significantly higher than those of the normal subjects (Mann-Whitney test, $p < 0.001$ and $p = 0.007$ for granzymes A and B, respectively). Granzyme A and B levels were correlated with each other (r_s = 0.69, $p = 0.004$). In addition, both granzyme levels were correlated with the number of lymphocytes/ml ($r_s = 0.794$, $p < 0.001$ and $r_s = 0.735$, $p = 0.002$ for granzymes A and B, respectively).

Table I. *BAL differential cell counts*[a]

	Normal Subjects	HP Patients
Macrophages	82.6 ± 12.4	214.9 ± 38.2*
Lymphocytes	11.8 ± 2.8	507.5 ± 100.2*
Neutrophils	1.5 ± 0.7	47.0 ± 15.2*
Eosinophils	0.6 ± 0.5	11.7 ± 3.9*

[a] Data are presented as thousands of cells/milliliter (mean ± SEM). *, Significantly different from normal subjects, $p < 0.05$ using a paired Student *t* test.

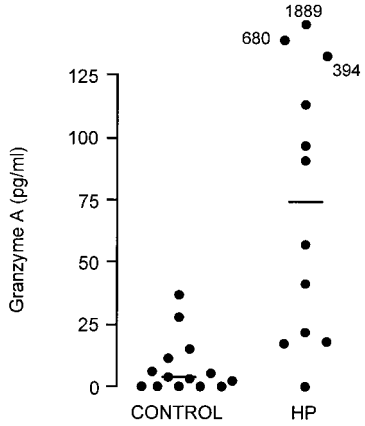

FIGURE 1. Median and individual values of granzyme A in BAL fluids (picograms/milliliter) for the two groups of subjects. *, Significantly different from control subjects (Mann-Whitney test, $p = 0.0006$).

Serum concentrations of granzymes A and B

Granzymes A and B were only measured in serum from two normal and nine HP subjects; samples were not available from the other subjects. In the two normal subjects, concentrations of granzymes A and B were 37 and 134 pg/ml, and 10 and 13 pg/ml, respectively. In HP patients, serum levels of granzymes A and B were 50 (27–262) and 6 (4–368) pg/ml, respectively. These concentrations are similar to previously published serum concentrations of granzymes (3). There was no correlation between BAL and serum levels of granzymes A or B (Fig. 3).

Inhibition of granzymes A and B by serine protease inhibitors

Neither α_1-antitrypsin, SLPI, nor elafin, even in molar excess, showed any inhibitory activity against granzyme A or B. To ensure that the three inhibitor preparations were fully active, they were shown to inhibit human sputum elastase (Elastin Products Company, Owensville, MO) using the chromogenic substrate *N*-methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide (Sigma-Aldrich Chemicals), according to a procedure described at www.elastin.com.

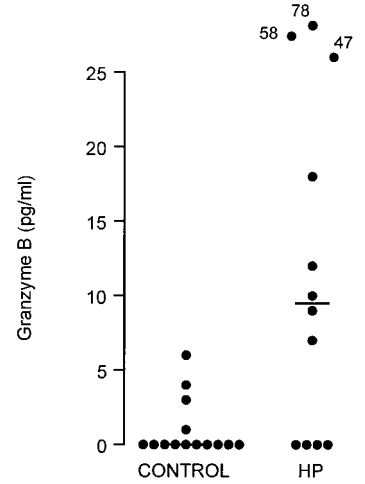

FIGURE 2. Median and individual values of granzyme B in BAL fluids (picograms/milliliter) for the two groups of subjects. *, Significantly different from control subjects (Mann-Whitney test, $p = 0.0072$).

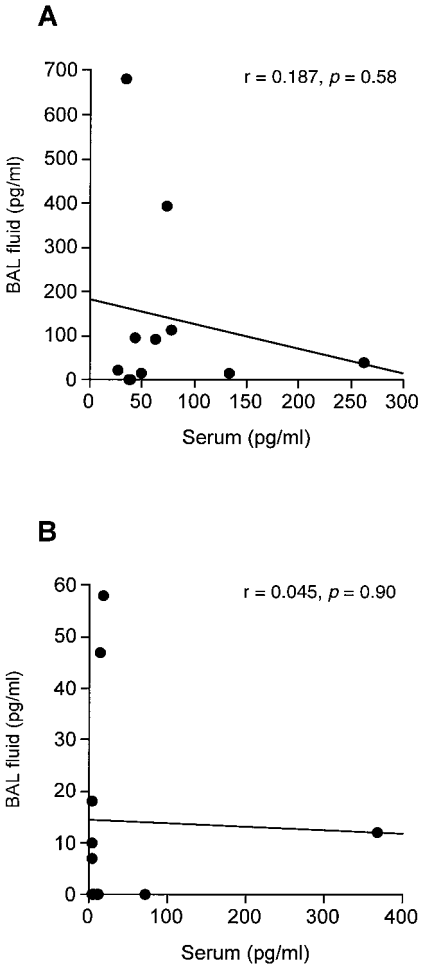

FIGURE 3. Absence of correlation between BAL fluid and serum concentrations of granzymes A (*A*) and B (*B*).

Determination of granzyme A and B activities in BAL fluid samples

Having shown the presence of granzymes A and B in BAL fluid samples and that serine protease inhibitors, present in BAL fluid in vivo, do not inhibit both granzymes in vitro, we next addressed the question as to whether granzymes A and B in BAL fluids were active. Usually, the BLT assay is used to measure active granzyme A levels. However, in our hands, this assay has a sensitivity of about 3 ng/ml. Therefore, we determined the levels of active granzyme A with a novel and more sensitive assay, which is based on the binding of antithrombin III to active granzyme A in the presence of heparin (20). The sensitivity of this assay, as it was run, was approximately 50 pg/ml of active granzyme A. Since only seven HP patients had more than 50 pg/ml of granzyme A, as measured by ELISA, granzyme A activity was also determined in subjects with other lung conditions having high levels of granzyme for a total of 15 subjects. There was a highly significant correlation between BAL granzyme A levels determined by ELISA, on one hand, and active granzyme A levels, on the other (Fig. 4). The slope of the regression line was 0.962, a value quite close to 1. Taken together, these results show that all granzyme A present in BAL fluid is fully active. Accordingly, we did not measure complexes between granzyme A and its natural inhibitor antithrombin III in any of the BAL fluid samples. Finally, we were unable to show any active granzyme B in BAL fluids, even in the samples with the highest concentrations of this granzyme. However, this negative result could be due to the lack of sensitivity of the esterolytic assay used to detect granzyme B activity. Considering this lack of sensitivity, the presence of active granzyme B in BAL fluid samples cannot be ruled out.

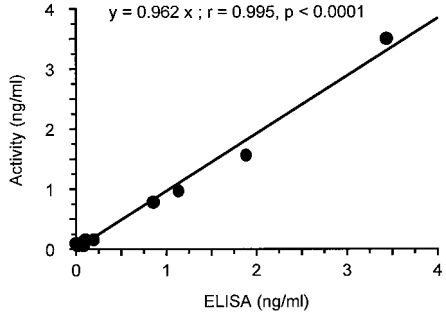

FIGURE 4. Correlation between BAL fluid granzyme A levels, as measured by ELISA, and granzyme A activity levels.

Discussion

The four main observations of the present study are that 1) granzymes A and B are present and measurable in BAL fluids even in some normal subjects, 2) granzymes A and B are locally increased in BAL fluids in HP, a lymphocytic alveolitis, 3) granzymes are not inhibited by α_1-antitrypsin, SLPI, and elafin, and 4) granzyme A is fully active in BAL fluid.

Normal BAL fluid is characterized by a proportion of lymphocytes of 5–15% (22), which includes activated (23, 24) and granzyme B gene-expressing (14) lymphocytes. The presence of such activated alveolar lymphocytes in the normal setting could explain the presence of low levels of granzymes in some healthy subjects.

There was a correlation between BAL levels of granzymes A and B. This makes sense considering that both granzymes are stored in the same cytoplasmic granules and are released together following exocytosis (6, 25). The increase in granzyme concentrations in HP is more than likely locally derived since there was a correlation between granzyme A and B levels, on one hand, and lymphocyte numbers, on the other hand. Also, the fact that serum granzyme levels were within normal range in HP patients (except for one subject) and were not correlated to BAL levels supports the concept that granzyme release is restricted to the lung and that serum levels are probably not influenced by local lung production. Published normal plasmatic concentrations of granzymes A and B are 33.5 (1–121) and 11.5 (1–113) pg/ml, respectively (3). In contrast, circulating granzyme concentrations can increase well over 1000 pg/ml in patients with EBV or HIV-1 infection (3), or rheumatoid arthritis (5).

In vitro, we did not observe any significant inhibitory activity of α_1-antitrypsin, SLPI, or elafin on granzyme A or B. The present results, as well as previous results from our group (20), are in contradiction with those of others who have reported that α_1-antitrypsin inhibits both granzyme A (6) and B (17), but are in accordance with those of one group that was unable to substantiate the inhibition of granzyme B by α_1-antitrypsin (P. Bird, personal communication). Regarding SLPI and elafin, our results clearly show that they do not inhibit either granzyme A or B. Based on indirect observations, Cowan and colleagues suggested that elafin could inhibit granzyme A (18). Our results do not support this concept.

An important observation from the present study is that granzyme A activity strongly correlated with antigenic levels in BAL fluid, indicating that all granzyme A in the lung is active. In support for this, we did not measure a detectable inhibitory effect of α_1-antitrypsin, SLPI, or elafin, the main serine protease inhibitors in the lungs (15, 16), on granzyme A or B activity in vitro. We did not measure any active granzyme B in the BAL fluids tested. Considering the low concentrations of granzyme B in BAL fluids, the lack of sensitivity of the assay used to determined granzyme B activity, and that neither α_1-antitrypsin, nor SLPI, nor elafin inhibits granzyme B in vitro, the presence of active granzyme B in the alveolar space cannot be ruled out.

Beside α_1-antitrypsin, SLPI, and elafin, other serine protease inhibitors obviously do not contribute significantly to the inactivation of active granzyme A in the lung since this granzyme is fully active in BAL fluids. α_2-Macroglobulin, present in small concentration (0.01 μM) in the normal alveolar epithelial lining fluid (15), is reported to inhibit both granzymes A and B (6, 17). We recently showed that α_2-macroglobulin is a major inhibitor of granzyme A in the blood compartment (20), in which this inhibitor is present at much higher concentrations than in the alveolar space, i.e., 2.5–5 μM (15). The assay we used to determine granzyme A activity in the present study, based on the interaction of the protease with antithrombin III, is the same as we previously used to quantify the same activity in the blood (20). It is important to point out that such assay excludes the possibility that granzyme A is entrapped, and therefore inhibited, by α_2-macroglobulin (20), since entrapped proteases are not able to interact with high m.w. substrates such as antithrombin III in the case of granzyme A. Moreover, we have clearly shown that all granzyme A in BAL fluid is active. From this, we can conclude that α_2-macroglobulin does not play any important regulatory role in granzyme A activity in the lung alveolar space. α_1-Antichymotrypsin is also present in the alveolar space, but it does not inhibit granzyme A (6). Finally, proteinase inhibitor 9 (PI-9), a member of the OVA serpin family, is an efficient inhibitor of granzyme B, but this inhibitor is not secreted by cells and restricts its action to the cytoplasm of lymphocytes, protecting these cells from granzyme B-induced apoptosis (26).

The presence of increased levels of active granzyme A in the alveolar space in HP is of particular interest. Indeed, granzyme A stimulates the production of TNF-α, IL-6, and IL-8 (7, 8). These cytokines are important signal molecules coordinating the lung inflammatory and immune responses (27). Not surprisingly, BAL levels of these cytokines are increased in HP (28). Therefore, it is tempting to speculate that granzyme A contributes, at least in part, to their up-regulation. Moreover, being an IL-1β-converting enzyme (9), granzyme A could further contribute to the local inflammatory response characterizing HP. Last but not least, the proteolytic action of granzymes A and B on collagen and proteoglycans (10–12) may significantly contribute not only to tissue remodeling, but also to the migration of lymphocytes into the alveolar space in HP, perpetuating, therefore, the chronic state of this lung disease. It remains to clarify whether increased BAL levels of granzymes are specific to HP or if this is also a characteristic of other lymphocytic lung diseases.

In conclusion, we provide evidence that BAL fluid from patients with HP contains high levels of granzymes A and B that are not inhibited by the main serine protease inhibitors of the lung, and that granzyme A is fully active in the alveolar space. These data not only suggest that these proteases may contribute to the pathogenesis of HP, but also show that high concentrations of soluble active granzymes may occur locally in inflamed tissues.

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