IL-12, IL-18 and IFN-gamma in the immune response to bacterial infection
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Soluble granzymes are released during human endotoxemia and in patients with severe gram-negative bacterial infection

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Abstract

Extracellular release of granzymes is considered to reflect the involvement of cytotoxic T lymphocytes and natural killer cells in various disease states. To obtain insight into granzyme release during bacterial infection, we measured granzyme levels during experimental human endotoxemia and in patients with melioidosis, a severe gram-negative infection. Granzyme(Gr)A and GrB plasma concentrations increased transiently after endotoxin administration, peaking after 2-6h. In patients with bacteremic melioidosis, GrA and GrB levels were elevated on admission and remained high during the 72-h study period. In whole blood stimulated with heat-killed *B. pseudomallei*, neutralization of either TNF, IL-12 or IL-18 inhibited granzyme secretion, which was independent of IFN-γ. Stimulation with endotoxin and other gram-negative and gram-positive bacteria also strongly induced the secretion of granzymes, suggesting that granzyme release is a general immune response during bacterial infection. The interaction between the cytokine network and granzymes may play an important immunoregulatory role during bacterial infections.
Introduction

Cytotoxic CD8+ T lymphocytes (CTL) and natural killer (NK) cells are the effector cells of cell-mediated immune responses. Together, these cells are often referred to as cytotoxic lymphocytes (CL) [1]. CL protect the host by lysing cells infected by viruses, intracellular bacteria or parasites, and by destroying of tumor cells [2]. CL-induced cytotoxicity is mediated either by Fas ligation or by the granule exocytosis pathway, which involves the release of perforin and a family of serine proteases called granzymes, into the intercellular space between CL and the target cell [1, 2]. Perforin facilitates target cell entry and/or trafficking of granzymes [3, 4]. Various studies have indicated that granzymes (Gr)A and B are important for the initiation of apoptosis in the target cell [5-7]. While GrB rapidly induces target cell apoptosis, GrA acts slowly via a different pathway and can only be detected if the activity of GrB is inhibited, thus serving as a critical back-up system [3].

Previous studies have demonstrated that granzymes are also released extracellularly during degranulation of CL [8]. In addition, it was found that CL constitutively secrete a part of newly synthesized granzymes by a non-granule dependent pathway [9]. Recently, specific ELISAs have been developed to measure soluble granzymes in vivo in humans [10]. Measurement of concentrations of soluble granzymes is considered to reflect the involvement of CTL and NK cells in various disease states. Indeed, elevated plasma levels of granzymes have been reported in patients with rheumatoid arthritis, Epstein-Barr virus or HIV-1 infection, and during primary cytomegalovirus infection [10-12]. We were interested whether granzymes are also secreted during bacterial infections. Therefore, we studied the levels of soluble granzymes during experimental human endotoxemia, a well accepted model of systemic inflammation in humans [13], and in patients with melioidosis, a severe gram-negative infection caused by Burkholderia pseudomallei, in which there is extensive proinflammatory cytokine production [14]. In addition, during whole blood stimulation in vitro, we determined the role of several proinflammatory cytokines in the activation of CL and subsequent release of granzymes.

Materials and methods

Experimental human endotoxemia

Eight healthy male volunteers (mean age 22 years, range 19-25 years) were admitted to the Clinical Research Unit of the Academic Medical Center. Written informed consent was obtained from all study objects. The study was approved by the research and ethical
committees of the Academic Medical Center. Medical history, physical and routine laboratory examination, chest X-ray and electrocardiogram of all volunteers were normal. Each volunteer received a bolus intravenous injection of *Escherichia coli* endotoxin (LPS), lot G (United States Pharmacopeial Convention, Rockville, MD) administered over one minute in an antecubital vein at a dose of 4 ng/kg. Blood was collected in EDTA tubes directly before LPS injection and at 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 12 and 24 h thereafter. Plasma was obtained after centrifugation, and stored at -20°C until assays were performed. In addition, blood for FACScan analysis was obtained at selected timepoints (t=0, 3, 6 and 24 h) in heparin containing vacutainer tubes.

**FACScan analysis**

Peripheral blood mononuclear cells (PBMC’s) were isolated by Ficoll-Hypaque density gradient centrifugation (Ficoll Paque, Pharmacia Biotech, Uppsala, Sweden) at room temperature for 20 minutes at 1000 x g. PBMC’s were collected in the inter-phase, washed twice with PBS and resuspended in FACS buffer (PBS supplemented with 0.01% (w/v) NaN₃, 0.5% BSA (w/v) and 0.3 mM EDTA). For staining of cell surface markers, 0.5 x 10⁶ cells/ tube were incubated with the following mouse monoclonal Abs: Cy-Chrome5-labeled anti-CD3 mAb (Immunotech, Marseille, France), FITC-labeled anti-CD8 (CLB) and/or Simultest CD3/CD16+CD56 (Becton Dickinson & Co, Rutherford, NJ). The appropriate isotype controls (all Immunotech) were included in all experiments. After incubation, cell were washed twice and resuspended in FACS buffer. Lymphocytes were gated by forward and side scatter using a FACScan (Becton Dickinson) and 5,000 cells were counted.

**Melioidosis patients and study design**

The patients included in the present study were part of a clinical trial comparing the efficacy of intravenous imipenem and ceftazidime in suspected severe melioidosis [15]. Clinical outcome did not differ between the two treatment groups and therefore data were combined for the present investigation. Informed consent was obtained from all patients or attending relatives. Ethical approval for this clinical trial was obtained from the Thai Ministry of Public Health. The patients (aged over 14 years) included in this study were all admitted to the Sappasitprasong Hospital, Ubon Ratchathani, Thailand. Melioidosis was considered in all patients admitted during the rainy season with symptoms and/or signs of community-acquired sepsis or pneumonia, particularly if underlying diabetes or renal disease was present. From all patients with possible melioidosis, blood, urine and throat swab specimens, plus, where available, specimens of sputum and pus, were collected for culture. Clinical data (and baseline APACHE II score) were recorded at study entry. Blood samples (EDTA-anticoagulated) were collected directly before the start of antibiotic treatment (t=0), and at 12,
24, 48, and 72 h thereafter. In addition, blood was collected from 12 healthy adult volunteers (patients' relatives or hospital staff, all resident in Ubon Ratchathani or the surrounding provinces). Plasma was separated immediately and stored at -70°C until assays were performed.

**Whole blood stimulation**

Heat-killed *B. pseudomallei, Pseudomonas aeruginosa, Escherichia coli, Streptococcus pneumoniae* and *Staphylococcus aureus* were prepared from clinical isolates. The isolate was suspended in culture medium and incubated overnight in 5% CO₂ at 37°C. This suspension was diluted in fresh medium the next morning and incubated until log-phase growth was obtained. Thereafter, 10-fold dilutions of this suspension were made and plated on blood agar plates for colony-forming unit (CFU) counts. Bacteria were harvested by centrifugation, washed twice in pyrogen-free 0.9% NaCl, resuspended in 20 ml 0.9% NaCl, and heat inactivated for 60 minutes at 80°C. A 500-μl sample on a blood agar plate did not show growth of bacteria.

Whole blood was collected aseptically from 6 healthy individuals using a sterile collecting system consisting of a butterfly needle connected to a syringe (Becton Dickinson). Anticoagulation was obtained using endotoxin-free heparin (Leo Pharmaceutical Products B.V., Weesp, the Netherlands; final concentration 10 U/ml blood). Whole blood, diluted 1:1 in pyrogen-free RPMI 1640 (Bio Wittaker, Verviers, Belgium), was stimulated for 24 h at 37°C with 10⁷ CFU/ml heat-killed bacteria or LPS (final concentration 10 ng/ml; from *E. coli* serotype 0111:B4; Sigma, St. Louis, MO). Incubations with heat-killed *B. pseudomallei* or LPS were also performed in the presence or absence of mouse anti-human TNF (MAK 195; final concentration 10 μg/ml), anti-IL-12, anti-IL-18, anti-IFN-γ or anti-IL-15 (all mouse IgG mAbs, R&D Systems, Abingdon, United Kingdom; final concentration all 10 μg/ml). MAK 195F was generously provided by Knoll AG, Ludwigshafen, Germany. During in vitro cell stimulation, these concentrations of the anti-cytokine mAbs used completely neutralize the activity of recombinant human TNF (rhTNF), rhIL-12, rhIL-18, rhIFN-γ and rhIL-15 when added at 1-2 log higher concentrations compared to levels detected after whole blood stimulation with heat-killed *B. pseudomallei* [16] (information on the neutralizing capacities of the mAbs used provided by the manufacturer). Control mouse IgG (R&D Systems) was used in the appropriate concentrations. After the incubation, supernatant was obtained after centrifugation and stored at -20°C until assays were performed.

**Assays**

Leukocyte counts and differentials were determined in EDTA-anticoagulated blood using flow cytometry. Concentrations of granzymes A and B were measured by specific
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ELISA's as described before [10]. In short, purified mAb GA29 or GB11 (both 1 µg/ml) were used as coating Abs, biotinylated GA28 or GB10 (both 1:1000) as detecting Abs, and purified GrB and lymphokine-activated killer (LAK) cell lysate as standards. Detection limits of the assays were 3.2 pg/ml (GrA) and 5 pg/ml (GrB). IFN-γ-inducible protein-10 (IP-10) (R&D Systems; detection limit 20 pg/ml) and monokine induced by IFN-γ (Mig) (PharMingen, San Diego, CA; 8 pg/ml) were measured according to the instructions of the manufacturer.

Statistical analysis
Data of the endotoxemia study are expressed as mean ± SE of 8 subjects. Changes in time were analyzed by one-way analysis of variance (ANOVA). Differences from baseline were assessed by Dunnett's t test for multiple comparisons and Wilcoxon test. Values in patients are given as medians and ranges. Differences between controls and/or patient groups were analyzed by the Mann-Whitney U test. Changes in time during antibiotic treatment were analyzed by one-way ANOVA. These two tests were performed after log transformation of the data. Spearman's ρ was used to determine correlation coefficients. Data of the in vitro stimulations are expressed as mean ± SE of six donors. Statistical analysis was performed by Wilcoxon test. P < 0.05 was considered to represent a significant difference.

Results

Human endotoxemia study

Clinical response to LPS
Intravenous injection of LPS was associated with transient influenza-like symptoms, including headache, nausea, myalgia and chills, starting 1-2 h after LPS administration, and lasting no longer than 3-4 h. In addition, a rise in body temperature was recorded, peaking at 3-4 h after LPS (38.6 ± 0.3°C; P < 0.05).

Effect of LPS on lymphocyte counts
After an initial decline, leukocyte counts strongly increased after LPS injection, and remained high until 24 h (Table 1). LPS administration induced a marked lymphopenia with lowest cell numbers after 6 h. This decrease in lymphocyte counts was associated with a strong decrease in the number of CD3+/CD8+ lymphocytes and the number of NK cells.

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Table 1. Effect of LPS administration in vivo on cell counts and differentials.

<table>
<thead>
<tr>
<th></th>
<th>Time (h after LPS injection)</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Leukocytes (x 10^9/L)</td>
<td>5.03 ± 0.43</td>
</tr>
<tr>
<td>Lymphocytes (x 10^5/L)</td>
<td>1.65 ± 0.10</td>
</tr>
<tr>
<td>CD3+/CD8+ lymphocytes (%)</td>
<td>22.51 ± 2.41</td>
</tr>
<tr>
<td>CD3+/CD8+ lymphocytes (x 10^5/L)</td>
<td>0.37 ± 0.04</td>
</tr>
<tr>
<td>NK cells (%)</td>
<td>7.64 ± 1.29</td>
</tr>
<tr>
<td>NK cells (x 10^6/L)</td>
<td>0.12 ± 0.02</td>
</tr>
</tbody>
</table>

Values are mean ± SE of 8 healthy subjects. LPS (4 ng/kg) was given as an i.v. bolus injection at t= 0 h. Analysis was performed by flow cytometry and FACSscan analysis. Data are expressed as cell counts or percentage positive cells within the lymphocyte population. * indicates P < 0.05 versus baseline by Wilcoxon.

Granzyme concentrations

Plasma levels of GrA and GrB were detectable at low levels before LPS administration (10.75 ± 1.25 pg/ml and 6.00 ± 0.93 pg/ml respectively). LPS injection resulted in a transient increase in GrA levels, peaking at 2 h (48.88 ± 9.04 pg/mL; P < 0.001) (Fig. 1). Levels of GrB showed a gradual increase after LPS administration with peak levels after 5-6 h (6 h: 12.13 ± 1.67 pg/mL; P < 0.001).

Figure 1. Plasma levels of soluble granzyme A and B after an i.v. bolus injection of endotoxin (4 ng/kg) in healthy male subjects. Data are expressed as mean ± SE of 8 individuals. P-value reflects changes in time analyzed by one way ANOVA. * indicates P < 0.05 versus baseline by Dunnett t-test.
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Melioidosis study

Patients
A total of 86 consecutive patients were studied; 43 were male and 43 female. The median age was 50 years (range 16-85 years). Positive cultures for *B. pseudomallei* were found in 64 patients, with positive blood cultures in 34 patients (53 %), of whom 16 patients died (47 %). In the other 30 patients, *B. pseudomallei* was isolated from sites other than blood; 2 of these patients died (7 %). In the remaining 22 patients, no positive cultures for *B. pseudomallei* were found. The majority of these patients (15) were diagnosed with infections other than melioidosis: clinical sepsis in 9 patients (of whom 4 died) with positive blood cultures in 4 patients (*Escherichia coli*, *Klebsiella pneumoniae*, *P. aeruginosa* and *Staphylococcus aureus*), pneumonia in 2 patients (positive cultures for *S. aureus* in 1 patient, who died), urinary tract infection in 1 patient and tuberculosis in 3 patients. In 3 patients, liver and/or splenic abscesses without positive cultures were found, 1 patient was diagnosed for hepatocellular carcinoma, while in 3 patients no final diagnosis was made (1 died). This group of 22 patients is further referred to as the group of patients with diseases other than melioidosis. Median APACHE II score in the total patient population was 13 (range 1-30). Detailed patient characteristics are shown in Table 2.

Table 2. Clinical characteristics on admission in patients with clinically suspected melioidosis.

<table>
<thead>
<tr>
<th></th>
<th>Melioidosis</th>
<th>Disease other than melioidosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blood culture positive</td>
<td>Blood culture negative</td>
</tr>
<tr>
<td></td>
<td>Survivors</td>
<td>Non-survivors</td>
</tr>
<tr>
<td>Age (years)</td>
<td>(18)</td>
<td>(16)</td>
</tr>
<tr>
<td>Sex (M / F)</td>
<td>5/13</td>
<td>7/9</td>
</tr>
<tr>
<td>APACHE II score</td>
<td>12.5 (4-25)</td>
<td>19 (7-30)</td>
</tr>
</tbody>
</table>

Data are median (range) values.

Granzyme concentrations on admission
Plasma levels of GrA in healthy controls were 53.2 (range 42.7-95.0) pg/ml (Fig. 2). GrA levels were significantly elevated in patients with bacteremic melioidosis (107.3 (39.1-1472.0) pg/ml) compared to controls (P < 0.001) and were higher in patients who died (142.6 (42.3-1472.0) pg/ml) then in patients who survived (107.3 (39.1-289.8) pg/ml), although this difference was not significant. GrA concentrations were slightly increased
Granzyme release in melioidosis

Figure 2. Plasma concentrations of soluble granzymes on admission in patients with culture proven melioidosis, in patients included under the clinical suspicion of melioidosis and in healthy controls. Horizontal lines represent median. Dotted lines represent the detection limits of the assays. P-values reflect differences between groups by Mann-Whitney U test.

in patients with non-bacteremic melioidosis (77.7 (23.1-1854.0) pg/ml) compared to controls (NS), but to a lesser extent compared to bacteremic melioidosis patients (P = 0.006 for difference between bacteremic and non-bacteremic patients). Levels of GrA were also slightly increased in patients with diseases other than melioidosis (76.0 (11.6-976.3) pg/ml), but the difference compared to healthy controls was not significant. GrA levels showed no correlation with APACHE II scores.

Concentrations of GrB in plasma of healthy controls were 19.3 (≤5.0-24.8) pg/ml. GrB levels in bacteremic melioidosis patients (27.3 (≤5.0-571.3) pg/ml) were elevated compared to controls (P < 0.01). GrB levels were similar in survivors and non-survivors. Concentrations of GrB in patients with non-bacteremic melioidosis (13.6 (≤5.0-628.9) pg/ml) and in patients with diseases other than melioidosis (19.4 (≤5.0-654.6) pg/ml) were not elevated compared to controls. There was no correlation between GrB concentrations and APACHE II scores.

GrA and GrB levels showed a positive correlation with each other both in patients with culture-proven melioidosis (ρ = 0.46; P < 0.001) and in the total patient population (ρ = 0.53; P < 0.001).
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Granzymes during follow-up

Patients with culture-proven melioidosis were followed during 72 h antibiotic treatment with either ceftazidime or imipenem. Since granzyme levels in patients with non-bacteremic melioidosis were not significantly elevated compared to controls, we concentrated on patients with bacteremic melioidosis. The type of antibiotic treatment did not influence plasma levels of granzymes (data not shown) and therefore, data from the two treatment groups were combined. Plasma levels of both GrA and GrB did not decrease during antibiotic therapy, and remained elevated until the end of the 72-h study period (GrA: 107.1 (40.5-242.8) pg/ml and GrB: 24.6 (≤5.0-135.8) pg/ml (Fig. 3).

![Figure 3. Plasma levels of soluble granzymes in patients with bacteremic melioidosis during antibiotic treatment. Horizontal lines represent median. Dotted lines represent the detection limits of the assays.](image)

Whole blood stimulation

Melioidosis is associated with elevated plasma levels of a number of proinflammatory cytokines [16, 17]. To obtain insight into the role of endogenous cytokines in the release of soluble granzymes during melioidosis, we incubated whole blood with heat-killed *B. pseudomallei* (amount equivalent to $10^7$ CFU/ml) in the presence or absence of neutralizing mAbs against a number of cytokines which have been demonstrated to play a role in the pathogenesis of melioidosis and/or in the activation of CTL and NK cells, i.e. TNF, IFN-γ, IL-12, IL-18 and IL-15 [16-19]. Incubation of whole blood for 24 h without *B. pseudomallei* resulted in low levels of GrA (28 ± 4 pg/ml), while GrB levels remained below the detection limit of the assay. Incubation with *B. pseudomallei* resulted in GrA release (1,037 ± 242 pg/ml) and in remarkably high concentrations of GrB (16,395 ± 5,737 pg/ml). GrA and GrB release were already strong at 8 h after incubation, and further increased until 24 h after incubation, indicating that GrA and GrB concentrations measured after 24 h are the result of both degranulation (early release) and secretion of
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newly synthesized granzymes (subsequent release). Addition of mouse IgG did not influence granzyme levels. Addition of mAbs against TNF, IL-12 or IL-18 resulted in a significant decrease in both GrA and GrB secretion, which was most pronounced for anti-IL-12 (Table 3). The combination of anti-IL-12 and anti-IL-18 resulted in a further decrease of granzyme release. In contrast, addition of anti-IFN-γ or anti-IL-15 did not significantly inhibit granzyme release. To confirm the neutralizing capacity of anti-IFN-γ in our experiments, we incubated whole blood with rhIFN-γ (10 ng/ml) with or without anti-IFN-γ (10 μg/ml), and measured the production of IP-10 and Mig, two CXC chemokines of which the production is strongly IFN-γ dependent. In these experiments, the anti-IFN-γ mAb completely neutralized IFN-γ activity, since the production of IP-10 and Mig induced by IFN-γ added at comparable concentrations as found after whole blood stimulation with heat-killed *B. pseudomallei* [16], was totally blocked when anti-IFN-γ was added (data not shown). Combination of anti-IL-12 and/or IL-18 with anti-IFN-γ or IL-15 had no additional inhibitory effect on granzyme release (data not shown).

In line with our observations in humans injected with LPS in vivo, incubation of whole blood with LPS also induced the secretion of granzymes. Similar to results obtained with heat-killed *B. pseudomallei*, addition of anti-TNF or anti-IL-12 strongly attenuated the LPS-induced release of granzymes (GrA: % inhibition anti-TNF: 45.1 ± 2.3 % and anti-IL-12: 73.3 ± 8.2; GrB: % inhibition anti-TNF: 65.6 ± 2.4 % and anti-IL-12: 72.1 ± 6.6 %; all both P < 0.05), while anti-IFN-γ had no effect. While anti-IL-18 modestly inhibited *B. pseudomallei*-induced granzyme release, it did not influence granzyme release elicited by LPS, either alone or in combination with anti-IL-12 (data not shown).

Table 3. Effects of neutralizing mAbs against proinflammatory cytokines on granzyme A and B release during whole blood stimulation with heat-killed *B. pseudomallei*.

<table>
<thead>
<tr>
<th>mAb</th>
<th>GrA % inhibition</th>
<th>GrB % inhibition</th>
</tr>
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<tbody>
<tr>
<td>anti-TNF</td>
<td>44.8 ± 1.8 *</td>
<td>39.9 ± 6.1 *</td>
</tr>
<tr>
<td>anti-IL-12</td>
<td>60.0 ± 4.9 *</td>
<td>51.9 ± 1.9 *</td>
</tr>
<tr>
<td>anti-IL-18</td>
<td>14.8 ± 4.4 *</td>
<td>10.8 ± 4.0 *</td>
</tr>
<tr>
<td>anti-IFN-γ</td>
<td>5.5 ± 6.9</td>
<td>4.2 ± 7.9</td>
</tr>
<tr>
<td>anti-IL-15</td>
<td>5.4 ± 5.9</td>
<td>8.2 ± 2.6</td>
</tr>
<tr>
<td>anti-IL-12 + anti-IL-18</td>
<td>69.4 ± 4.3 * #</td>
<td>59.2 ± 4.2 * #</td>
</tr>
</tbody>
</table>

Data are mean ± SE of 6 healthy donors and expressed as percentage inhibition relative to incubation with heat-killed *B. pseudomallei* only. Whole blood, diluted 1:1 in RPMI, was stimulated for 24 h at 37°C with 10⁷ CFU/ml heat-killed *B. pseudomallei* in the presence or absence of neutralizing Abs against TNF, IL-12, IL-18, IFN-γ or IL-15 (final concentration all 10 μg/ml). GrA levels after stimulation with heat-killed *B. pseudomallei* only were 1,038 ± 242 pg/ml, GrB levels were 16,395 ± 5,737 pg/ml. * P < 0.05 vs. control. * < 0.05 vs. anti-IL-12.
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To determine whether the induction of granzyme release is specific for *B. pseudomallei* or whether other bacteria also stimulate granzyme secretion, we compared the effect of heat-killed *B. pseudomallei* with other bacterial stimuli, i.e. heat-killed *P. aeruginosa* and *E. coli* (both gram-negative bacteria), *Streptococcus pneumoniae* and *S. aureus* (both gram-positive bacteria) (final concentration all 10^7 CFU/ml), and with LPS (the common cell-wall component of gram-negative bacteria) (10 ng/ml) during whole blood stimulation in vitro. As shown in Table 4, all bacteria and LPS were potent inducers of both GrA and GrB release. GrB levels were higher than GrA levels after stimulation with all stimuli.

**Table 4.** Granzyme release during whole stimulation with different bacterial stimuli.

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>GrA (pg/ml)</th>
<th>GrB (pg/ml)</th>
</tr>
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<tbody>
<tr>
<td>Heat-killed bacteria (10^7 CFU/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. pseudomallei</em></td>
<td>812 ± 209</td>
<td>11,358 ± 2,370</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>505 ± 115</td>
<td>7,321 ± 1,576</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>954 ± 301</td>
<td>13,446 ± 3,660</td>
</tr>
<tr>
<td><em>S. pneumoniae</em></td>
<td>93 ± 33</td>
<td>1,056 ± 502</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>646 ± 112</td>
<td>7,287 ± 1,351</td>
</tr>
<tr>
<td>LPS (10 ng/ml)</td>
<td>494 ± 174</td>
<td>4,204 ± 1,275</td>
</tr>
</tbody>
</table>

Data are mean ± SE of 6 healthy subjects. Whole blood, diluted 1:1 in RPMI, was stimulated for 24 h at 37°C with different heat-killed bacteria (final concentration 10^7 CFU/ml) or LPS (10 ng/ml). GrA and GrB levels were below the detection limit when no bacteria or LPS were added.

**Discussion**

Granzymes are released from cytoplasmic granules of CTL or NK cells upon stimulation, and are delivered intracellularly into target cells, where they can activate pathways of apoptosis. Also, granzymes can be released into the extracellular environment, and the concentrations of these soluble granzymes are considered to reflect involvement of CL in various disease states. Elevated levels of soluble granzyme levels have previously been reported in patients with EBV or HIV-1 infection [10], and in patients during primary CMV infection after renal transplantation [11]. In addition, in patients with rheumatoid arthritis granzyme concentrations have been found elevated in plasma and synovial fluid [12]. The present study is the first to demonstrate that bacterial stimuli induce extracellular release of granzymes. First, we measured levels of soluble granzymes in healthy humans after a bolus intravenous injection of LPS, a component of the cell wall of gram-negative bacteria, which induces a transient activation of host inflammatory
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responses as found during clinical sepsis [13]. Plasma concentrations of both GrA and GrB are transiently elevated during human endotoxemia, peaking early after LPS administration, which was associated with a decrease in the number of CTL and NK cells in the circulation. These data suggest that LPS administration induces activation of CL in vivo, resulting in a quick release of granzymes, which is most likely the result of degranulation of cytoplasmic granules. To determine whether granzymes are also elevated during clinical bacterial infection in humans, we measured soluble granzymes in patients with melioidosis, a severe infection caused by the gram-negative bacillus B. pseudomallei [14]. Melioidosis is an important cause of illness and death in parts of Southeast Asia, and the clinical presentation varies from mild localized disease to acute fulminant septicemia. We found that in patients with bacteremic infection, levels of both GrA and GrB were elevated compared to healthy controls, while they were not increased in patients with localized melioidosis or in patients with diseases other than melioidosis, suggesting that in particular bacteria circulating in the blood stream are potent inducers of granzyme release.

Previously, markedly elevated plasma concentrations of a number of cytokines, including TNF, IL-12, IL-18 and IFN-γ, were found in patients with melioidosis. These were correlated with the severity of disease and remained elevated for prolonged periods [16, 17]. As these cytokines can influence CL functions [2, 18, 19], we wished to determine their role in granzyme release during whole blood stimulation with heat-killed B. pseudomallei or LPS. Neutralization of TNF, IL-12 and/or IL-18 resulted in a significant decrease in B. pseudomallei-induced granzyme release, while in LPS-stimulated blood only anti-TNF and anti-IL-12 reduced granzyme concentrations. The important role of IL-12 in granzyme secretion induced by bacterial stimuli is in line with our earlier study demonstrating increases in GrA and GrB plasma concentrations in chimpanzees intravenously injected with rhIL-12 [20]. IL-12 and IL-18 are both potent inducers of IFN-γ production, and many biological effects of IL-12 and IL-18 are considered to be mediated by IFN-γ [18, 19]. Anti-IFN-γ did not influence granzyme release induced by either heat-killed B. pseudomallei or LPS, which indicates that the effect of IL-12 and IL-18 on granzyme release is mediated through an IFN-γ-independent pathway. This is in line with previous studies which have reported that IL-12 and IL-18 can have antitumor effects independent from IFN-γ [21, 22]. In addition, IL-12 can still induce an inflammatory response in IFN-γ receptor knock-out mice [23]. Since IL-12 and IL-18 have synergistic effects on IFN-γ production [24], we studied whether they may have synergistic effects on granzyme release. In whole blood stimulation in vitro, the combination of anti-IL-12 and anti-IL-18 did not result in an additional decrease in B. pseudomallei or LPS-induced granzyme release compared to addition of anti-IL-12 or anti-IL-18 alone. Our finding that anti-IL-18 failed to affect LPS-induced granzyme
release may be the result of the relatively low levels of IL-18 found after LPS stimulation compared with \textit{B. pseudomallei} stimulation \cite{16, 25}. These data indicate that TNF and IL-12, and to a lesser effect IL-18, play a role in the activation of CL and subsequent release of granzymes after bacterial stimulation. The release of granzymes in vitro was not a unique property of \textit{B. pseudomallei}, since stimulation with other bacteria, both gram-negative and positive, also induced high concentrations of granzymes. Interestingly, secreted GrA has been shown to induce the production of TNF, IL-6 and IL-8 from fibroblast and epithelial cell lines, and from human monocytes in vitro, and GrA can enhance the phagocytic capacity of human monocytes \cite{26, 27}. Together, these data suggest that cytokines and granzymes can be reciprocally stimulatory during bacterial infection and inflammation, and that granzymes may have an additional immunoregulatory role besides their role in cell-mediated immunity.

In the present study, we demonstrate that activation of CTL and NK during experimental endotoxemia and gram-negative bacterial infection in humans results in the release of soluble granzymes. Several bacterial stimuli were found to induce the release of granzymes in whole blood in vitro, which was largely mediated by TNF and IL-12. Previous in vitro studies have indicated that granzymes can stimulate the production of cytokines. Therefore, the interaction between the cytokine network and granzymes may play an important role in the early immune response to bacterial infection.

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\textbf{References}

Granzyme release in melioidosis


Chapter 12


