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Soluble Granzymes Are Released during Human Endotoxemia and in Patients with Severe Infection Due to Gram-Negative Bacteria

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Extracellular release of granzymes is considered to reflect the involvement of cytotoxic T lymphocytes and NK cells in various disease states. To obtain insight into granzyme release during bacterial infection, granzyme levels were measured during experimental human endotoxemia and in patients with melioidosis, a severe infection due to gram-negative bacteria. Plasma concentrations of granzyme A (GrA) and GrB increased transiently after endotoxin administration, peaking after 2–6 h. 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 *Burkholderia pseudomallei*, neutralization of tumor necrosis factor, interleukin-12, or interleukin-18 inhibited granzyme secretion, which was independent of interferon- 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.

Cytotoxic CD8 T lymphocytes (CTLs) and NK cells are important effector cells of cell-mediated immunity and are involved in, respectively, adaptive and innate immune responses. Together, these cells are often referred to as cytotoxic lymphocytes (CLs) [1]. CLs protect the host by lysing cells infected by viruses, intracellular bacteria, or parasites and by destroying 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 granzyme A (GrA) and GrB are important for the initiation of apoptosis in the target cell [5–7]. Although GrB rapidly induces target cell apoptosis, GrA acts slowly via a different pathway and can be detected only if the activity of GrB is inhibited, thus serving as a critical backup system [3].

Previous studies have demonstrated that granzymes are also released extracellularly during degranulation of CLs [8]. In addition, CLs constitutively secrete part of the 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]. Increased concentrations of soluble granzymes are 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 human immunodeficiency virus type 1 (HIV-1) infection and during primary cytomegalovirus infection [10–12]. We were interested in 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 infection caused by the gram-negative bacterium *Burkholderia pseudomallei*, in which there is extensive proinflammatory cytokine production [14]. In addition, during

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Written informed consent for the endotoxemia study was obtained from all study subjects, and the study was approved by the research and ethical committees of the Academic Medical Center. Informed consent for the melioidosis study was obtained from all patients or attending relatives, and ethical approval was obtained from the Thai Ministry of Public Health.

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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) were admitted to the Clinical Research Unit of the Academic Medical Center. Medical history, physical and routine laboratory examination, chest radiography, and electrocardiography 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 1 min 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 done. In addition, blood for cell cytometric analysis (FACScan; Becton Dickinson, Rutherford, NJ) was obtained at selected time points (0, 3, 6, and 24 h) in heparin-containing Vacutainer tubes (Becton Dickinson).

FACScan analysis. Peripheral blood mononuclear cells were isolated by Ficoll-Paque density gradient centrifugation (Pharmacia Biotech, Uppsala, Sweden) at room temperature for 20 min at 1000 *g*. Peripheral blood mononuclear cells were collected in the interphase, washed twice with PBS, and resuspended in FACS buffer (PBS supplemented with 0.01% [wt/vol] NaN_3 , 0.5% [wt/vol] bovine serum albumin, and 0.3 mM EDTA). For staining of cell surface markers, 0.5×10^6 cells/tube were incubated with the following mouse monoclonal antibodies (MAbs): phycoerythrin-cyanin 5.1 (PC5)-labeled anti-CD3 MAb (Immunotech, Marseilles, France), fluorescein isothiocyanate-labeled anti-CD8 (CLB, Amsterdam, the Netherlands), and/or Simultest CD3/CD16 CD56 (Becton Dickinson). Appropriate isotype controls (all Immunotech) were included in all experiments. After incubation, cells were washed twice and resuspended in FACS buffer. Lymphocytes were gated by forward and side scatter by use of a FACScan (Becton Dickinson), and 5000 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 for suspected severe melioidosis [15, 16]. Clinical outcome did not differ between treatment groups, and therefore data were combined for the present investigation. The patients (ages >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, when 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 (time 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 residing in Ubon Ratchathani or the surrounding provinces).

Plasma was separated immediately and stored at -70°C until assays were done.

Whole blood stimulation. Heat-killed *B. pseudomallei*, *Pseudomonas aeruginosa*, *E. coli*, *Streptococcus pneumoniae*, and *Staphylococcus aureus* were prepared from clinical isolates. Isolates were suspended in culture medium and incubated overnight in 5% CO_2 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 counts. Bacteria were harvested by centrifugation, washed twice in pyrogen-free 0.9% NaCl, resuspended in 20 mL of 0.9% NaCl, and heat-inactivated for 60 min 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 persons by means of a sterile collecting system consisting of a butterfly needle connected to a syringe (Becton Dickinson). Anticoagulation was done with endotoxin-free heparin (Leo Pharmaceutical Products, Weesp, The Netherlands; final concentration, 10 U/mL of blood). Whole blood, diluted 1:1 in pyrogen-free RPMI 1640 (Bio Witteraker, Verviers, Belgium), was stimulated for 24 h at 37°C with 10^7 cfu/mL heat-killed bacteria or LPS (final concentration, 10 ng/mL; from *E. coli* serotype O111:B4; Sigma, St. Louis). Incubations with heat-killed *B. pseudomallei* or LPS were also done in the presence or absence of mouse anti-human tumor necrosis factor (TNF; MAK 195; final concentration, 10 $\mu\text{g}/\text{mL}$), anti-interleukin-12 (IL-12), anti-IL-18, anti-interferon- γ (IFN- γ), or anti-IL-15 (all mouse IgG MAbs; R&D Systems, Abingdon, UK; final concentration for each, 10 $\mu\text{g}/\text{mL}$). MAK 195F was provided by Knoll (Ludwigshafen, Germany). During *in vitro* cell stimulation, these concentrations of the anti-cytokine MAbs used completely neutralize the activity of recombinant human TNF, IL-12, IL-18, IFN- γ , and IL-15 when added at levels 1–2 log higher than are detected after whole blood stimulation with heat-killed *B. pseudomallei* [17] (information on the neutralizing capacities of the MAbs used was 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 done.

Assays. Leukocyte counts and differentials were determined in EDTA-anticoagulated blood by means of flow cytometry. Concentrations of GrA and GrB were measured by specific ELISAs as described before [10]. In short, purified MAb GA29 or GB11 (both 1 $\mu\text{g}/\text{mL}$) were used as coating antibodies, biotinylated GA28 or GB10 (both 1:1000) as detecting antibodies, and purified GrB and lymphokine-activated killer cell lysate as standards. Detection limits of the assays were 3.2 pg/mL for GrA and 5 pg/mL for 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; 8 pg/mL) were measured according to the instructions of the manufacturer.

Statistical analysis. Data for the endotoxemia study are expressed as mean \pm SE of 8 subjects. Changes in time were analyzed by 1-way analysis of variance. Differences from baseline were assessed by Dunnett's *t* test for multiple comparisons and the Wilcoxon test. Values for 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

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

Cell type	Time, h after LPS injection			
	0	3	6	24
Leukocytes, $\times 10^9/L$	5.03 \pm 0.43	4.10 \pm 0.50 ^a	10.79 \pm 0.79 ^a	11.4 \pm 0.51 ^a
Lymphocytes, $\times 10^9/L$	1.65 \pm 0.10	0.38 \pm 0.03 ^a	0.29 \pm 0.02 ^a	1.55 \pm 0.12
CD3 CD8 lymphocytes, %	22.51 \pm 2.41	14.21 \pm 1.56 ^a	13.83 \pm 2.44 ^a	18.42 \pm 2.81
CD3 CD8 lymphocytes, $\times 10^9/L$	0.37 \pm 0.04	0.06 \pm 0.01 ^a	0.04 \pm 0.01 ^a	0.29 \pm 0.06
NK cells, %	7.64 \pm 1.29	4.71 \pm 1.24	4.19 \pm 0.97 ^a	7.09 \pm 1.08
NK cells, $\times 10^9/L$	0.12 \pm 0.02	0.02 \pm 0.01 ^a	0.01 \pm 0.00 ^a	0.11 \pm 0.01

NOTE. Data are mean \pm SE of 8 healthy subjects. LPS (4 ng/kg) was given as intravenous bolus injection at 0 h. Analysis was done by flow cytometry and FACScan analysis. Data are expressed as cell counts or % of positive cells within lymphocyte population.

^a $P < .05$ vs. baseline (Wilcoxon test).

treatment were analyzed by 1-way analysis of variance. These two tests were done after log transformation of the data. Spearman's ρ was used to determine correlation coefficients. Data for the in vitro stimulations are expressed as mean \pm SE of 6 donors. Statistical analysis was done by the Wilcoxon test. $P < .05$ was considered significant.

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 ($38.6 \pm 0.3^\circ\text{C}$; $P < .05$).

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.

Granzyme concentrations. Plasma levels of GrA and GrB were detectable at low levels before LPS administration (10.75 ± 1.25 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; $P < .001$) (figure 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; $P < .001$).

Melioidosis Study

Patients. A total of 86 consecutive patients were studied, 43 each male and female. The median age was 50 years (range, 16–85). Cultures were positive for *B. pseudomallei* for 64 patients. Blood cultures were positive for 34 (53%), of whom 16 died (47%); for the other 30 patients, *B. pseudomallei* was isolated from sites other than blood, and 2 of these patients died (7%). For 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 (*E. coli*, *Klebsiella pneumoniae*, *P. aeruginosa*, and

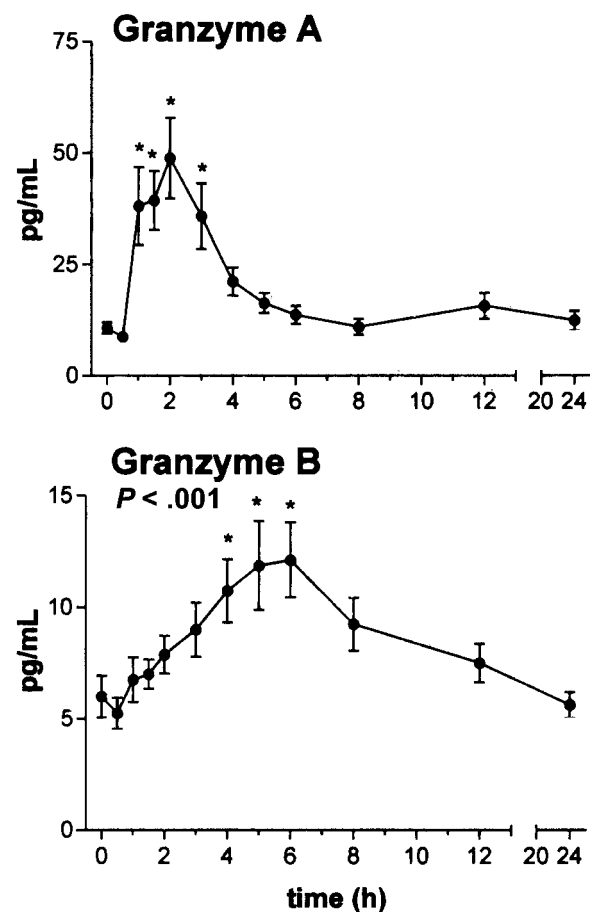


Figure 1. Plasma levels of soluble granzyme A and B after intravenous bolus injection of endotoxin (4 ng/kg) in healthy male subjects. Data are expressed as mean \pm SE of 8 subjects. P reflects changes in time analyzed by 1-way analysis of variance. * $P < .05$ vs. baseline by Dunnett's t test.

S. 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 with hepatocellular carcinoma, and 3 had no final diagnosis made (1 died). This group of 22 patients is further referred to as patients with diseases other than melioidosis. The median APACHE II score in the total patient population was 13 (range, 1–30). Detailed patient characteristics are shown in table 2.

Granzyme concentrations on admission. The median plasma level of GrA in healthy controls was 53.2 pg/mL (range, 42.7–95.0) (figure 2). GrA levels were significantly elevated in patients with bacteremic melioidosis (median, 107.3 pg/mL [range, 39.1–1472.0]) compared with controls ($P < .001$) and were higher in patients who died (median, 142.6 pg/mL [range, 42.3–1472.0]) than in patients who survived (median, 107.3 pg/mL [range, 39.1–289.8]), although this difference was not significant. GrA concentrations were slightly increased in patients with nonbacteremic melioidosis (median, 77.7 pg/mL [range, 23.1–1854.0]) compared with controls (not significant) but to a lesser extent than for bacteremic melioidosis patients ($P = .006$ for difference between bacteremic and nonbacteremic patients). Levels of GrA were also slightly increased in patients with diseases other than melioidosis (median, 76.0 pg/mL [range, 11.6–976.3]), but the difference compared with healthy controls was not significant. GrA levels showed no correlation with APACHE II scores.

The median concentration of GrB in plasma of healthy controls was 19.3 pg/mL (range, ≤ 5.0 –24.8). GrB levels in bacteremic melioidosis patients (median, 27.3 pg/mL [range, ≤ 5.0 –571.3]) were elevated compared with controls ($P < .01$). GrB levels were similar in survivors and nonsurvivors. Concentrations of GrB in patients with nonbacteremic melioidosis (median, 13.6 pg/mL [range, ≤ 5.0 –628.9]) and in patients with diseases other than melioidosis (median, 19.4 pg/mL [range, ≤ 5.0 –654.6]) were not elevated compared with 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 ($\rho = .46$; $P < .001$) and in the total patient population ($\rho = .53$; $P < .001$).

Granzymes during follow-up. Patients with culture-proven melioidosis were followed during 72 h of antibiotic treatment with either ceftazidime or imipenem. Because granzyme levels in patients with nonbacteremic melioidosis were not significantly elevated compared with controls, we concentrated on patients with bacteremic melioidosis. We have previously reported that after the first dose of treatment, administration of ceftazidime was associated with higher peak levels of endotoxin (within 6 h after the start of therapy) than resulted from treatment with imipenem, although it did not differentially influence

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

Patient group	Age, years	Sex, male/female	APACHE II score
Melioidosis (blood culture positive)			
Survivors ($n = 18$)	49 (21–72)	5/13	12.5 (4–25)
Nonsurvivors ($n = 16$)	41.5 (18–76)	7/9	19 (7–30)
Melioidosis (blood culture negative)			
Survivors ($n = 28$)	50 (24–71)	23/5	9 (1–24)
Nonsurvivors ($n = 2$)	44 (32–56)	0/2	14.5 (9–20)
Diseases other than melioidosis			
Survivors ($n = 16$)	55.5 (27–85)	6/10	10.5 (5–24)
Nonsurvivors ($n = 6$)	42.5 (16–62)	2/4	18.5 (11–22)

NOTE. Data are median (range).

cytokine concentrations (TNF, IL-6, and IL-10) [16]. Also, the type of antibiotic treatment did not influence plasma levels of granzymes (data not shown), and therefore, data from the 2 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: median, 107.1 pg/mL [range, 40.5–242.8]; GrB: median, 24.6 pg/mL [range, ≤ 5.0 –135.8]) (figure 3).

Whole blood stimulation. Melioidosis is associated with elevated plasma levels of a number of proinflammatory cytokines [17, 18]. 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 that have been demonstrated to play a role in the pathogenesis of melioidosis and/or in the activation of CTL and NK cells: TNF, IFN- γ , IL-12, IL-18, and IL-15 [17–20]. Incubation of whole blood for 24 h without *B. pseudomallei* resulted in low levels of GrA (28 ± 4 pg/mL), whereas GrB levels remained below the detection limit of the assay. Incubation with *B. pseudomallei* resulted in GrA release (1037 ± 242 pg/mL) and in remarkably high concentrations of GrB ($16,395 \pm 5737$ pg/mL). Release of GrA and GrB was 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 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 recombinant human IFN- γ (10 ng/mL) with or without anti-IFN- γ (10 μ g/mL) and measured the production of IP-10 and Mig, two CXC chemokines whose production is strongly IFN- γ dependent. In these experiments, the anti-IFN-

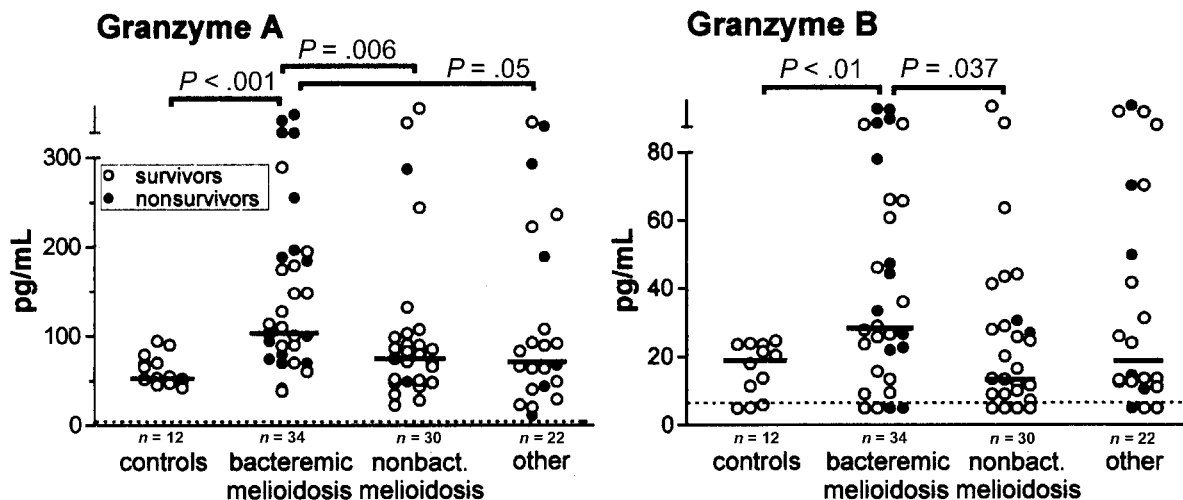


Figure 2. Plasma concentrations of soluble granzymes on admission in patients with melioidosis (bacteremic and nonbacteremic), patients with diseases other than melioidosis, and healthy controls. Horizontal lines represent medians. Dotted lines represent detection limits of assays. *P* reflects differences between groups by Mann-Whitney *U* test.

γ MAb completely neutralized IFN- γ activity, because the production of IP-10 and Mig induced by IFN- γ at concentrations comparable to those found after whole blood stimulation with heat-killed *B. pseudomallei* [17] was totally blocked when anti-IFN- γ was added (data not shown). Combination of anti-IL-12 and/or anti-IL-18 with anti-IFN- γ or anti-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 (for GrA: anti-TNF, 45.1% \pm 2.3%; anti-IL-12, 73.3% \pm 8.2%; for GrB: anti-TNF, 65.6% \pm 2.4%; anti-IL-12, 72.1% \pm 6.6%; all *P* < .05), whereas anti-IFN- γ had no effect. Although 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).

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—heat-killed *P. aeruginosa* and *E. coli* (both gram-negative bacteria) and *S. pneumoniae* and *S. aureus* (both gram-positive bacteria; final concentrations, 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.

Discussion

Granzymes are released from cytoplasmic granules of CTL or NK cells on 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 granzymes have previously been reported in patients with Epstein-Barr virus or HIV-1 infection [10] and in patients with primary cytomegalovirus infection after renal transplantation [11]. In addition, in patients with rheumatoid arthritis, elevated granzyme concentrations have been found in plasma and synovial fluid [12]. The present study is the first to demonstrate that bacterial stimuli induce extracellular release of granzymes. 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 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,

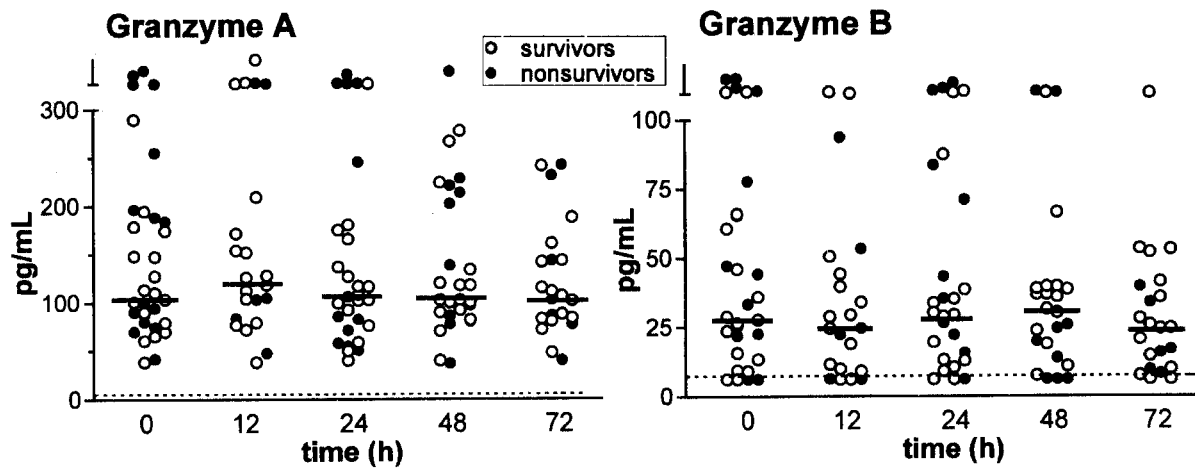


Figure 3. Plasma levels of soluble granzymes in patients with bacteremic melioidosis during antibiotic treatment. Horizontal lines represent medians. Dotted lines represent detection limits of assay.

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 with those in healthy controls, but 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 bloodstream are potent inducers of granzyme release. Remarkably, the healthy Thai controls had higher plasma concentrations of granzymes than did the healthy volunteers from the endotoxin study at baseline. An explanation could be found in the fact that the Thai volunteers are already primed or preactivated for the release of granzymes, which can be the result of exposure to environmental pathogens. However, because the Thai controls were all from the same surroundings as the melioidosis patients, this likely does not influence the interpretation of the results or might even have masked greater differences in plasma concentrations of granzymes that can be found between healthy controls and patients during severe (systemic) bacterial infection.

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 [17, 18]. Because these cytokines can influence CL functions [2, 19, 20], 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, whereas 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 recombinant

human IL-12 [21]. IL-12 and IL-18 are both potent inducers of IFN- γ production, and many biologic effects of IL-12 and IL-18 are considered to be mediated by IFN- γ [19, 20]. 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 that have reported that IL-12 and IL-18 can have antitumor effects independent of IFN- γ [22, 23]. In addition, IL-12 can still induce an inflammatory response in IFN- γ receptor-knockout mice [24]. Since IL-12 and IL-18 have synergistic effects on IFN- γ production [25], 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 with ad-

Table 3. Effects of neutralizing monoclonal antibodies (MAbs) against proinflammatory cytokines on granzyme (Gr) A and GrB release during whole blood stimulation with heat-killed *Burkholderia pseudomallei*.

MAb	GrA	GrB
Anti-TNF	44.8 \pm 1.8 ^a	39.9 \pm 6.1 ^a
Anti-IL-12	60.0 \pm 4.9 ^a	51.9 \pm 1.9 ^a
Anti-IL-18	14.8 \pm 4.4 ^a	10.8 \pm 4.0 ^a
Anti-IFN- γ	5.5 \pm 6.9	4.2 \pm 7.9
Anti-IL-15	5.4 \pm 5.9	8.2 \pm 2.6
Anti-IL-12 + anti-IL-18	69.4 \pm 4.3 ^{a,b}	59.2 \pm 4.2 ^{a,b}

NOTE. Data are mean \pm SE of 6 healthy donors and are expressed as % of inhibition relative to incubation with heat-killed *B. pseudomallei* only (GrA, 1038 \pm 242 pg/mL; GrB, 16,395 \pm 5737 pg/mL). Whole blood, diluted 1:1 in RPMI, was stimulated for 24 h at 37°C with 10⁷ cfu/mL heat-killed *B. pseudomallei* in presence or absence of neutralizing MAbs to tumor necrosis factor (TNF), interleukin (IL)-12, IL-18, interferon (IFN)- γ , or IL-15 (final concentration for each, 10 mg/mL).

^a P < .05 vs. control.

^b P < .05 vs. anti-IL-12.

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

Stimulus	Granzyme A	Granzyme B
Heat-killed bacteria, 10 ⁷ cfu/mL		
<i>Burkholderia pseudomallei</i>	812 ± 209	11,358 ± 2370
<i>Pseudomonas aeruginosa</i>	505 ± 115	7321 ± 1576
<i>Escherichia coli</i>	954 ± 301	13,446 ± 3660
<i>Streptococcus pneumoniae</i>	93 ± 33	1056 ± 502
<i>Staphylococcus aureus</i>	646 ± 112	7287 ± 1351
Lipopolysaccharide, 10 ng/mL	494 ± 174	4204 ± 1275

NOTE. Data are mean ± SE for 6 healthy subjects. Whole blood, diluted 1:1 in RPMI, was stimulated for 24 h at 37°C with different heat-killed bacteria or lipopolysaccharide. Levels of granzyme A and B were below detection limit when no bacteria or lipopolysaccharide were added.

dition 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 *B. pseudomallei* stimulation [17, 26]. These data indicate that TNF and IL-12, and to a lesser extent IL-18, play a role in the activation of CLs and subsequent release of granzymes after bacterial stimulation. The release of granzymes in vitro was not a unique property of *B. pseudomallei*, because 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 [27, 28]. 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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