

Comparative Antimicrobial Activity of Granulysin against Bacterial Biothreat Agents

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Abstract: Granulysin is a cationic protein produced by human T cells and natural killer cells that can kill bacterial pathogens through disruption of microbial membrane integrity. Herein we demonstrate antimicrobial activity of the granulysin peptide derived from the active site against *Bacillus anthracis*, *Yersinia pestis*, *Francisella tularensis*, and *Burkholderia mallei*, and show pathogen-specific differences in granulysin peptide effects. The susceptibility of *Y. pestis* to granulysin is temperature dependent, being less susceptible when grown at the flea arthropod vector temperature (26°C) than when grown at human body temperature. These studies suggest that augmentation of granulysin expression by cytotoxic lymphocytes, or therapeutic application of granulysin peptides, could constitute important strategies for protection against select agent bacterial pathogens. Investigations of the microbial surface molecules that determine susceptibility to granulysin may identify important mechanisms that contribute to pathogenesis.

Keywords: Granulysin, antimicrobial, *B. anthracis*, *B. mallei*, *Y. pestis*, *F. tularensis*.

INTRODUCTION

Bacteria that evade the host immune response by surviving phagocytosis comprise some of the most serious microbial threats to human health. Among these are *Mycobacterium tuberculosis* (*Mtb*) and some of the pathogens classified by the Center for Disease Control and Prevention as bacterial select agents, *Bacillus anthracis*, *Yersinia pestis*, *Burkholderia mallei*, and *Francisella tularensis*. The tropism for phagocytic cells by these pathogens is an important factor for their successful evasion of the immune system and poses significant challenges for immunization or therapeutic intervention. Characterizing protective immune responses to these pathogens, then, is paramount to development of prophylactic or early therapeutic intervention strategies. Cytotoxic T cells (T_c) and NK cells control intracellular pathogens through multiple killing mechanisms, including death receptor activation and release of cytolytic granule proteins. In human cytotoxic lymphocytes, granulysin is an important component of the lytic repertoire and is stored in cytotoxic granules along with perforin and granzymes [1-5]. Perforin and granzymes kill the infected cell, whereas granulysin has the unique capacity to kill the intracellular pathogen by disruption of microbial membranes [5-7]. The lytic activity due to granulysin has been described against several important pathogens, including *Mtb*, *Plasmodium falciparum*, *Cryptococcus neoformans*, *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*), *Escherichia coli* 0157:H7 and *Staphylococcus aureus* [5-10].

The ability of granulysin to kill intracellular *Mtb* supports an important role for this molecule in the cytotoxic lymphocyte (T_c and NK cells) response to intracellular bacterial pathogens that evade the immune response through residence in macrophages. To investigate a potential role for granulysin in the immune response to an expanded list of intracellular bacterial pathogens for which interventions are urgently needed, we utilized a peptide derived from the active site of human granulysin and a negative control peptide. The active site peptide has been previously shown to reproduce the effects of the recombinant granulysin molecule, and is considered to have potential as an antibacterial therapeutic [5-7, 10, 11]. In this study, we demonstrate and compare the antibacterial activity of granulysin against *B. anthracis* (Ames), *Y. pestis* (CO 92), *F. tularensis* (SHU S4 and LVS), and *B. mallei* (ATCC 23344), using a peptide derived from the active site of granulysin. These studies support the need to further characterize the role of granulysin in the innate and acquired CTL response to these important pathogens.

MATERIALS AND METHODS

Synthetic custom peptides >95% pure by HPLC were purchased from New England Peptide LLC (Gardner, MA, USA). A peptide corresponding to amino acid residues 34-55 of human granulysin (CRTGRSRWRDVCNFMRRYQSR) was synthesized. As a negative control, a peptide corresponding to amino acids 2-22 (RDYRTCLTIVQKLKKMVDKPT) of human granulysin was also synthesized. Lyophilized peptide was stored in desiccant at -20 °C prior to use. Peptide stocks (5 mM) were solubilized in 0.1 N acetic acid solution, and then further diluted in sterile PBS to 1 mM, prior to use. Polypropylene tubes were used to store,

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aliquot, or perform experiments with peptides to prevent loss of peptides due to binding to tubes. All experimentation with select agents was performed under level 2 or 3 biosafety conditions according to protocols approved by the University of Texas Medical Branch Environmental Health and Safety. *S. Typhimurium*, *F. tularensis*, *B. anthracis* and *B. mallei* were grown in brain heart infusion broth (BHI), Muller Hinton media with Isovitale X, Luria-Bertani (LB) media, and LB media supplemented with 4% glucose (LBG), respectively. Cultures of *S. Typhimurium*, *F. tularensis* (LVS, SHU S4), *B. anthracis* (Ames strain) and *B. mallei* (ATCC 23344) were grown to exponential phase and diluted in growth medium to approximately 10^5 colony forming units (CFU)/ml. A 90 μ l aliquot of diluted microorganism was pre-incubated with 10 μ l of PBS or 10 μ l of individual granulysin peptides serially diluted in PBS to final concentrations of 1, 10, and 100 μ M. After 3 hours of incubation at room temperature, 100 μ l aliquots of *S. Typhimurium*, *F. tularensis*, and *B. mallei* were plated on LB or LBG agar plates, and 100 μ l aliquots of peptide and *B. anthracis* was plated on trypticase soy agar with 5% sheep blood. CFU's were counted after overnight incubation (48h for *B. mallei*) at 37°C. Exponential growth phase cultures of *Y. pestis* CO92 or *Y. pestis* CO92 *caf* were grown overnight at 26°C versus 37°C in Heart-Infusion Broth (HIB, Difco). Cultures were diluted 1:100 after overnight growth and grown at 26°C and 37°C to log phase in HIB medium. Cultures were harvested by centrifugation and washed once with Phosphate buffered saline buffer (PBS, pH 7.4). *Y. pestis* (2×10^6 CFU/ml) were incubated with various concentration of granulysin as described above and plated on HIB agar plates. CFU's were counted after 48h incubation at 26°C. Results for all isolates were verified by three (*B. mallei*, *B. anthracis*), four (*S. Typhimurium*, *F. tularensis*), or five (*Y. pestis*) independent experiments. A total of five independent experiments were performed with *Y. pestis* to firmly establish the temperature-dependent differences in susceptibility to peptide. Data were analyzed by one-way analysis of variance (ANOVA) followed by a Tukey's pair wise comparison test (GraphPad Software v4.0).

RESULTS

In this study, we tested the antimicrobial activity of the granulysin peptide against four aerosol-acquired select agent bacterial pathogens, *B. anthracis*, *Y. pestis*, *F. tularensis*, and *B. mallei*. *S. Typhimurium* was used as a positive control to compare the relative antibacterial activity of the peptide observed in earlier studies [7, 10, 12]. To normalize data across experiments and organisms, results in Fig. (1) are shown as percentage of control growth from each individual experiment. Statistically significant differences due to treatment, however, were determined using actual CFU relative to peptide concentrations. A peptide derived from a region in granulysin previously determined to lack antimicrobial activity (helix 1) was used as a negative control [6, 7, 10].

In support of previous studies with peptide and recombinant protein [7, 10, 12], we observed that granulysin peptide had potent antimicrobial activity against *S. Typhimurium* with a significant, dose dependent, increase in activity from 1 to 100 μ M peptide concentration (Fig. 1). A significant reduction of *B. anthracis*, *F. tularensis*, and *B. mallei* was evident at 10 and 100 μ M of peptide (Fig. 1), though *B. anthracis* and *F. tularensis* were less susceptible to 100 μ M levels than *B. mallei* or *S. Typhimurium*. The concentration of peptide required to reduce CFU of *B. anthracis* and *F. tularensis* was comparable to peptide concentrations required to reduce *Mtb* and *M. bovis*, in similar studies [5, 7, 12]. The killing activity of granulysin against *F. tularensis* SHU S4 (virulent) and LVS (attenuated vaccine strain) were similar (data not shown) indicating that susceptibility to granulysin peptide is not a likely variable in pathogenesis. Surprisingly, the negative control peptide derived from helix 1 had moderate activity against *B. anthracis* at a concentration of 100 μ M (38% reduction of CFU's as compared to the no peptide control; data not shown). The growth of *S. Typhimurium*, *Y. pestis*, *F. tularensis*, or *B. mallei*, was not affected by the helix 1 peptide when compared to growth in the absence of peptide. Due to the effects of the helix 1 peptide on *B. anthracis*, the no peptide control CFUs were used to calculate the percentage of control growth shown in Fig. (1). As shown in Fig. (2), *Y. pestis* growth was also

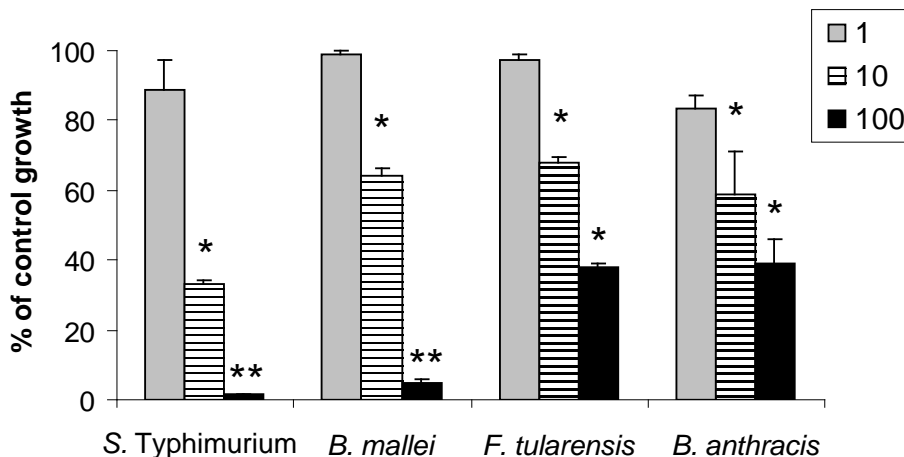


Fig. (1). Antimicrobial activity of granulysin peptide against select agent pathogens. Reduction of *S. Typhimurium*, *B. mallei*, *F. tularensis* (SHU S4), and *B. anthracis* (Ames) by a peptide derived from the active site of granulysin (1, 10, 100 μ M) displayed as a percentage of control (no peptide) growth. Data shown are mean \pm SEM of three to four independent experiments performed in triplicate. * $p < 0.05$; ** $p < 0.01$, indicate statistically significant differences between peptide treatment and negative control.

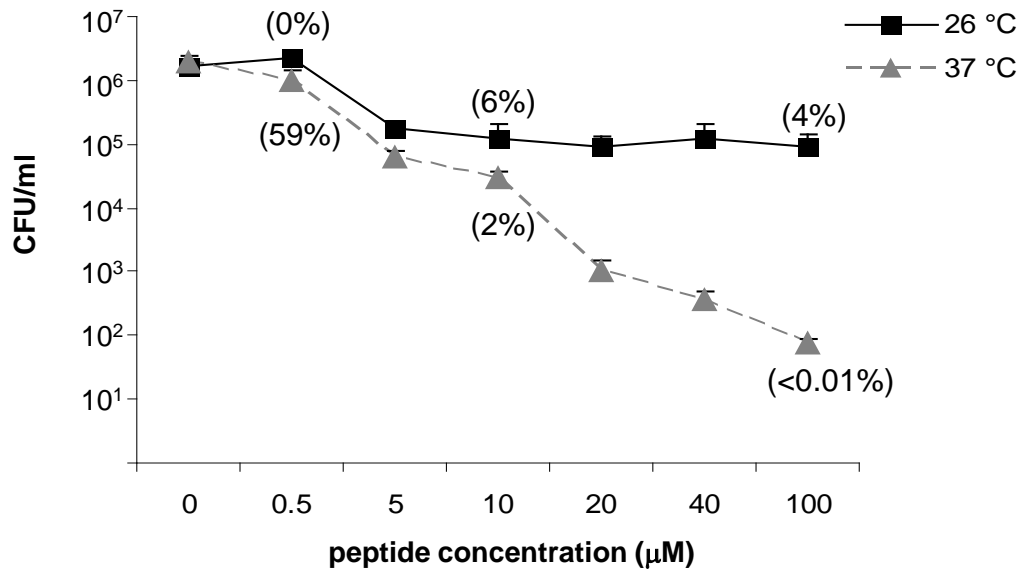


Fig. (2). Activity of granulysin peptide against *Y. pestis* is growth temperature-dependent. CFU reduction of *Y. pestis* (CO 92) across granulysin peptide concentration when grown at 26°C and 37°C. Reduction of CFU following 3 h incubation with peptide was determined by overnight growth on HIB agar plates. Percentage reduction from control growth at representative peptide concentrations is shown in parenthesis. Data shown are mean \pm SEM of five independent experiments. Compared to negative control, growth was significantly ($p < 0.01$) reduced by 5 to 100 μ M concentration of granulysin peptide at both 26 and 37°C. Effects of peptide on growth reduction were significantly different ($p < 0.05$) at 26°C compared to 37°C from 5 to 100 μ M concentration.

significantly reduced by the granulysin active site peptide in a dose-dependent manner. A growth temperature-dependent susceptibility of *Y. pestis* to cationic peptides has previously been characterized [13-15]. To determine if this effect could be observed with granulysin, *Y. pestis* was incubated with the peptide after growth at ambient temperature, generally corresponding to that in the flea arthropod vector (26°C), or human body temperature (37°C). When grown at ambient temperature to exponential growth phase, *Y. pestis* was much more resistant to the effects of granulysin peptide. In fact, the reduction of *Y. pestis* growth by granulysin peptide was several orders of magnitude greater when grown at 37°C, as compared to growth at 26°C (Fig. 2). As a percentage of the negative control, however, significant reduction of *Y. pestis* by 5 to 100 μ M concentrations of granulysin peptide was evident at both temperatures (Fig. 2, numbers in parenthesis). Recently, surface-exposed bacterial molecules (capsular antigen fraction 1) have been shown to modulate the susceptibility of *Y. pestis* to antimicrobial molecules in the respiratory epithelium [16]. To evaluate the role of the capsule substance in the susceptibility of *Y. pestis* to granulysin, a capsule-negative mutant of *Y. pestis* CO 92 (*Y. pestis* CO 92 *caf*) was also grown at both temperatures prior to incubation with peptide. The mutant contains a 1,176 bp deletion in the *caf*-operon eliminating synthesis of Caf1 capsular subunit and Caf1A usher proteins (V. Motin, unpublished). A temperature-dependent difference in killing activity by *Y. pestis* CO 92 *caf* was observed similar to the wild-type strain shown in Fig. (2), while the presence or absence of the capsule did not affect the susceptibility (data not shown). These differences in susceptibility to the antimicrobial effects of granulysin peptide, due to temperature, were not caused by altered growth patterns as organisms grew to similar density at both temperatures (Fig. 2).

DISCUSSION

The identification of immune mechanisms that can protect against bacterial select agent pathogens is a critical component in the development of control measures for these potential biotreats. The important role of both innate and acquired cell-mediated immune (CMI) responses to intracellular pathogens strongly supports the need to characterize CMI mechanisms that can be targeted to prevent disease or improve clinical outcome. In this study we showed that granulysin, a cytotoxic lymphocyte-derived antimicrobial, may have an important role in immunity to several important intracellular bacteria with select agent status.

Granulysin is a cationic molecule that consists of five compact α -helical segments. Homologues of granulysin in multiple mammalian species, fish, and birds indicates conservation of this antimicrobial mechanism [2, 12, 17-21]. Curiously, a granulysin homologue has not been identified in mice, hampering studies of the effects of gene deletion. In contrast to other antimicrobial molecules of the immune system, granulysin expression is limited to NK cells and antigen-specific T cells, the cytotoxic component of the CMI response. Expression is constitutive in NK cells and inducible in antigen specific T cells following activation with specific antigen or cytokines[1-3]. Native granulysin has chemoattractant capabilities [22] and has recently been implicated in adverse drug reactions that affect epidermal cells [23]. Normally, granulysin does not have cytotoxic effects on non-transformed human cells, while induction of apoptosis of tumor cells has been shown by several groups [10, 24, 25]. Peptide mapping in three separate species has characterized a core amino acid region including residues in helix 2 through helix 3 as the lytic site [6, 7, 10, 12]. The localization of the antimicrobial activity of granulysin to a short amino acid segment indicates the potential for use as a

therapeutic peptide, as has been described for other small "killer" peptides [26]. Peptides derived from the lytic site of granulysin are able to reproduce the antimicrobial effects of recombinant granulysin. The activity of the derived peptide can exceed the recombinant molecule [5, 7], an effect that may be due to the harsh denaturing conditions required to purify the recombinant molecule or to a membrane contact advantage of the peptide. Nonetheless, this peptide is an important screening tool to identify the antibacterial potential of granulysin against different bacterial pathogens and may also have utility as a bactericidal therapeutic. Granulysin-derived peptides have been shown to neutralize LPS simultaneously with direct bacterial killing and may have potential as a treatment for septic shock [11]. In the current study we also observed antimicrobial effects of the peptide derived from helix 1 of granulysin against *B. anthracis*. This result was unexpected, as peptides corresponding to the helix 1 region of granulysin have previously been reported to have no antimicrobial activity [6, 7, 10]. Following elucidation of the crystal structure of granulysin, however, the helix 1 region was predicted to contribute to the antimicrobial activity of granulysin due to the distribution and orientation of positively charged amino acids in this region [27]. Further studies with peptides and site specific amino acid substitution in recombinant protein are needed to fully characterize the lytic sites utilized by granulysin against various classes of microbial pathogens.

Our results demonstrate that granulysin may have very potent lytic effects on *Y. pestis* and *B. mallei* and can reduce the growth of *F. tularensis* and *B. anthracis* at higher concentrations. The relative potency of granulysin to reduce bacterial numbers in the current study varied by organism, and in regards to *Y. pestis*, the effect is temperature dependent. Collectively, the results from these and other studies suggest that the surface-exposed structures on microbial membranes that determine susceptibility to granulysin could differ among microorganisms, or vary in response to environmental signals. Peptides derived from granulysin are able to bind LPS and neutralize LPS-induced secretion of TNF α by peripheral blood mononuclear cells [11]. Differences in acylation of the LPS lipid A moiety due to environmental temperature is a defined mechanism for bacterial resistance to antimicrobial peptides [28]. Antimicrobial peptides have a significant role in insect host defense and are an important selective pressure for survival of bacteria that utilize insect reservoirs. With regard to *Y. pestis*, growth at environmental temperature increases the resistance of *Y. pestis* to cationic molecules with antimicrobial activity [13-15]. These differences are frequently attributed to temperature-induced alterations in lipid A acylation [13-15, 29], though acyl transferase deficiency has no effect on *Y. pestis* susceptibility to polymyxin B [30]. Very recently, bacterial capsule polysaccharides from several species were demonstrated to mediate resistance to both polymyxin B and alpha-defensin from human neutrophils and were proposed to act as bacterial decoys for antibacterial peptides [31]. Susceptibility of *Y. pestis* to beta-defensin and cathelicidin was also recently shown to be mediated by the capsular antigen fraction [16]. In our studies, a *Y. pestis* variant deficient for the capsule substance was not more susceptible to granulysin peptide effects. Thus, the role of capsule polysaccharides as bacterial

decoys may be an important difference between granulysin and other cationic antimicrobial molecules.

As previously observed, high concentrations of granulysin are required to kill micro-organisms in bulk culture compared to antibiotics or many synthetic antimicrobials [5, 7, 12]. An important difference between granulysin and other antimicrobials is that NK cells and antigen-specific T cells deliver granulysin to individual target cells at sites of infection. These cytotoxic immune cells are able to methodically kill one infected target at a time using only a portion of available granules while continually replenishing the granule armament. Perforin functions to increase membrane permeability of target cells to facilitate entry and activity of granulysin and granzymes [5, 32, 33]. Granulysin molecules are able to directly disrupt microbial membranes by electrostatic charge disruption [7, 27, 34]. Granulysin can also elicit extra-cellular microbicidal effects against pathogens [5, 9]. In this regard, both virulent *Francisella* and LVS were shown to have a significant extra-cellular phase in their *in vivo* life cycle [35].

The antibacterial effects of the granulysin peptide observed in the current study support continued investigation of granulysin as an important cytotoxic effector molecule contributing to the protective CMI responses to these and other serious biothreats. Promoting activation of granulysin expression by NK cells and T cells could represent an important avenue for immune modulation to protect susceptible populations from weaponized or naturally occurring bacterial pathogens. Strategies to promote activation of NK cell antimicrobial activity may be very effective in the early innate immune response to several pathogens. Promoting antimicrobial protein expression as part of the effector repertoire of antigen-specific T cells may also improve vaccine efficacy. In summary, induction of granulysin by NK cells or antigen-specific T cells should be further characterized as a mechanism to augment protective immune function against intracellular bacterial pathogens.

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