Mechanism of Staphylococcal Resistance to Non-oxidative Antimicrobial Action of Neutrophils: Importance of pH and Ionic Strength in Determining the Bactericidal Action of Cathepsin G

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The staphylococcal action of highly purified, enzymically inactive human lysosomal cathepsin G was studied. The bactericidal action of cathepsin G was optimal at pH 7.5 and was inhibited by NaCl; concentrations greater than 0.15 M NaCl completely inhibited killing of *Staphylococcus aureus*. Under optimal conditions (pH, temperature and NaCl concentration) the ED₅₀ (effective dose) of cathepsin G against *S. aureus* strain 8325-4 was about 3.1 µg ml⁻¹. Polymeric teichoic acid may serve as a binding site for cathepsin G by promoting electrostatic interactions since a mutant lacking this surface component exhibited enhanced resistance to the lethal action of cathepsin G, compared to the teichoic-acid-positive parental strain. These results suggest that (i) the ability of cathepsin G to kill intraphagosomal staphylococci may be regulated in part by the ionic strength of the environment and the pH of the maturing phagolysosome, and (ii) that strategies which retard acidification of the developing phagolysosome would promote the staphylococcal action of cathepsin G.

INTRODUCTION

Patients with phagocytic disorders are susceptible to recurrent, often life-threatening bacterial infections (Hill, 1984). Perhaps the best documented and studied disorder is that of chronic granulomatous disease (CGD). Leukocytes from patients with this condition have reduced microbicidal activity due to their inability to generate toxic oxygen radicals (Holmes, 1968). While these polymorphonuclear leukocytes (PMN) have reduced phagocytic killing capacity against *S. aureus* (Johnston & Newman, 1977) they readily kill other bacteria, e.g. gonococci (Rest *et al.*, 1982). The ability of these PMNs, clearly deficient in oxidative killing activity, to kill bacteria has been attributed to the action of cationic granule proteins. The cationic antimicrobial proteins (CAPs) mediate bactericidal action independent of the reduction of molecular oxygen (Spitznagel & Shafer, 1985).

One of the first human CAPs described (Odeberg & Olsson, 1975, 1976), cathepsin G (previously termed chymotrypsin-like cationic protein), has been studied in this laboratory for its lethal effects against *Neisseria gonorrhoeae* (Shafer *et al.*, 1986*a, b*). On a molar basis, cathepsin G appeared to be the most active CAP against gonococci *in vitro*. In our studies and those of Odeberg & Olsson (1975, 1976), the antimicrobial action of cathepsin G was independent of proteolytic activity since the latter could be inhibited by heat or diisopropyl fluorophosphate (DFP) treatment without influencing bactericidal activity. The studies of Odeberg & Olsson (1975, 1976) suggested that cathepsin G was a potential mediator of non-oxidative bactericidal activity against *S. aureus*; we have re-investigated the

Abbreviations: CAP, cationic antimicrobial protein; CGD, chronic granulomatous disease; DFP, diisopropyl fluorophosphate; HBSS, Hanks' balanced salt solution; PMN, polymorphonuclear leukocyte; TSA, trypticase soy agar.

staphylococcal action of cathepsin G because no satisfactory explanation has been advanced as to why this pathogen resists killing by CGD PMNs yet is killed by a mediator of the non-oxidative arsenal.

METHODS

Strains of S. aureus used. Strain 8325-4 (kindly provided by J. Iandolo, Kansas State University, Manhattan, Kan., USA) was the primary test strain used in this study. In other experiments we also studied phage group II strains H and 52A5, a polymeric teichoic-acid-deficient mutant of strain H (Chatterjee *et al.*, 1969). These isogenic strains were kindly provided by M. Cooper (Southern Illinois University, Springfield, Ill., USA). All strains were maintained on trypticase soy agar (TSA) slants at 4 °C.

Preparation of cathepsin G from lysosomal extracts of human PMNs. Cathepsin G was prepared from crude granule extracts obtained from normal human PMNs (Rest *et al.*, 1977) essentially as described previously (Travis *et al.*, 1980). Protein concentrations were determined as described by Bradford (1976) using chicken egg white lysozyme as a standard. The cathepsin G preparations were iodinated using Iodogen (Pierce Chemical Co.) as described by Farley *et al.* (1987). The specific activity of these iodinated preparations was typically 3×10^5 c.p.m. ($\mu\text{g protein}^{-1}$). After iodination the protein was treated with DFP as described by Shafer *et al.* (1984). The purity of the DFP-treated and untreated preparations of cathepsin G was evaluated by SDS-PAGE (Laemmli, 1970) using proteins solubilized under reducing conditions (Shafer & Morse, 1987). After electrophoresis in a 12.5% (w/v) polyacrylamide gel the proteins were fixed using 10% (w/v) trichloroacetic acid. After overnight fixation the gel was washed with distilled water, soaked in 1% (v/v) glycerol and dried on filter paper for autoradiography. Iodinated proteins were detected using Kodak X-OMAT AR-5 X-ray film after overnight exposure at -80 °C.

Bactericidal and binding assays. The bactericidal activity of cathepsin G was determined using mid-exponential phase cultures grown in trypticase soy broth at 37 °C with shaking as described by Rest *et al.* (1977). Prior to use in bactericidal assays, the bacteria were collected by centrifugation and washed twice in Hanks' balanced salts solution (HBSS; Difco) that had been adjusted to various pH values or NaCl concentrations. All cultures were incubated in sterile microtitre trays (Casey *et al.*, 1985) except those in which the buffering capacity of HBSS was checked at the end of each experiment. In this assay 1 ml cultures in sterile microfuge tubes were used. Prior to incorporation in the bactericidal assay the cathepsin G preparation was dialysed against distilled water at 4 °C. The bacteria to be tested were diluted to 10^4 c.f.u. ml^{-1} in HBSS. The total volume of the incubation mixture was 200 or 1000 μl , with each assay done in triplicate. The values for each sample did not vary by more than 5%. After incubation at 37 °C for 60 min, 10 and 100 μl samples were plated on TSA for analysis of viability. All plates were incubated at 37 °C for 18–24 h. In control experiments without cathepsin G, we found that the changes in either pH or NaCl concentration had no influence on survival of the test strain. All survival data, however, were calculated on the number of c.f.u. in control suspensions after 60 min. It is important to note that microscopic examination of strain 8325-4 in the various preparations of HBSS with or without cathepsin G showed that the mid-exponential phase cultures were consistently and largely single cocci with a minority population consisting of two to four cocci in clumps.

The binding of ^{125}I -labelled cathepsin G ($100 \mu\text{g ml}^{-1}$) to suspensions of staphylococci (10^8 c.f.u. ml^{-1}) was done using HBSS supplemented with various concentrations of NaCl essentially as described by Farley *et al.* (1987); controls consisted of the iodinated cathepsin G incubated in HBSS alone. After incubation in microfuge tubes at 37 °C for 60 min, the bacteria were collected by centrifugation for 2 min in a model 5414 Eppendorf table-top centrifuge. After careful drainage of the supernatant fluid the amount of radioactivity in the cell pellets and on the sides of the control tubes (<10% of experimental) was determined using a Beckman LS 4000 gamma scintillation counter.

RESULTS

Staphylococcal action of purified cathepsin G

SDS-PAGE revealed that iodinated cathepsin G migrated as three protein species with M_r values of 24000–25500 as described recently by us (Shafer, 1988). This was consistent with the reported M_r of the three isoenzymes of cathepsin G (Travis *et al.*, 1980).

Since the antimicrobial capacity of cathepsin G is independent of enzymic activity (Odeberg & Olsson, 1975; Shafer *et al.*, 1986a, c) all of the experiments described below used DFP-treated cathepsin G. When tested against *S. aureus* strain 8325-4 in the tryptone saline broth described previously (Rest *et al.*, 1977) we noted that about $200 \mu\text{g cathepsin G ml}^{-1}$ was required to achieve an ED_{50} ; this was similar to data obtained by Odeberg & Olsson (1975, 1976). However, since this value was at least 200-fold greater than that required for killing gonococci (ED_{50} $1 \mu\text{g ml}^{-1}$) (Shafer *et al.*, 1986b) we sought to optimize the bactericidal assay in order to better evaluate the capacity of cathepsin G to kill *S. aureus*.

Fig. 1

Fig. 2

Fig. 1. Bactericidal activity of 5.0 µg cathepsin G ml⁻¹ against *S. aureus* in HBSS containing increasing concentrations of NaCl. The standard bactericidal assay was done at 37 °C in HBSS (pH 7.5) containing the indicated concentration of NaCl.

Fig. 2. Effect of pH on the staphylococcal activity of cathepsin G. The HBSS was modified with respect to pH and used in bactericidal assays containing cathepsin G (5.0 µg ml⁻¹) and tested against *S. aureus* at 37 °C (—). The pH was monitored at the end of each experiment (.....).

Modulation of the staphylococcal action of cathepsin G by pH and NaCl

Since the tryptone saline broth used previously (Rest *et al.*, 1977; Shafer *et al.*, 1984) had a poor buffering capacity (data not presented) we used HBSS in the bactericidal assays described below. The bactericidal action of a number of granule proteins is influenced by temperature, pH and ionic strength (Weiss *et al.*, 1978; Ganz *et al.*, 1985; Shafer *et al.*, 1986*b*; Greenwald & Ganz, 1987). The bactericidal action of granule proteins appears to be restricted by temperature with optimal action at 37 °C. When tested against *S. aureus* at 4, 22 and 37 °C we found that cathepsin G exerted bactericidal action only at 37 °C (data not shown).

The influence of ionic strength on the bactericidal action of antimicrobial proteins has been studied by us and others in order to relate bactericidal activity *in vitro* to presumed changes in the ionic strength of the phagolysosome resulting from Ca²⁺ and Na⁺ influx from serum. Consistent with the earlier work of Odeberg & Olsson (1975) we found that the bactericidal action of cathepsin G was greatly reduced with increasing concentrations of NaCl (Fig. 1).

Certain granule proteins function best at neutral pH while others (e.g. CAP, *M*_r 37 000, Shafer *et al.*, 1986*b*) have slightly acidic pH optima. When we tested the susceptibility of strain 8325-4 to the lethal action of cathepsin G over a pH range of 5.0–7.5, bactericidal action was more evident at the alkaline end of this range (Fig. 2).

Having optimized the bactericidal assay with respect to temperature, pH and ionic strength we sought to determine the ED₅₀ of cathepsin G against *S. aureus* under optimal conditions. This value corresponded to 3.1 µg ml⁻¹ (Fig. 3), a value 64-fold less than that observed previously (see above) using the tryptone saline broth described by Rest *et al.* (1977). At five times the ED₅₀, cathepsin G exhibited a delayed bactericidal action (Fig. 4) seen earlier in our work with *N. gonorrhoeae* (Shafer *et al.*, 1986*b*).

NaCl inhibits the binding of ¹²⁵I-labelled cathepsin G to staphylococci

Our finding that the bactericidal action of cathepsin G was diminished in the presence of increasing concentrations of NaCl was consistent with the hypothesis advanced by Odeberg & Olsson (1976) that cathepsin G binds to bacterial surfaces via ionic interactions. To test this hypothesis the binding of ¹²⁵I-labelled cathepsin G to suspensions of staphylococci with increasing ionic strength was evaluated. We found that the total binding of cathepsin G to

Fig. 3

Fig. 4

Fig. 3. Bactericidal action of various concentrations of cathepsin G under optimal conditions; the ED_{50} and ED_{90} are indicated.

Fig. 4. Time-course of killing of *S. aureus* by cathepsin G. *S. aureus* 8325 was incubated in HBSS containing $15 \mu\text{g cathepsin G ml}^{-1}$. Samples were removed at the indicated intervals and analysed for survival.

staphylococci decreased with increasing ionic strength. However, even at the highest concentration of NaCl (0.21 M), which totally abrogated bactericidal action, the binding of cathepsin G to *S. aureus* was reduced by only 55% (data not presented); in the absence of added NaCl we detected 4.9×10^4 c.p.m. of ^{125}I -labelled cathepsin G bound per 300 c.f.u., while in the presence of 0.21 M-NaCl we detected 2.2×10^4 c.p.m. of ^{125}I -labelled cathepsin G bound per 300 c.f.u.

Importance of teichoic acid in determining staphylococci susceptibility to cathepsin G

While the above data indicated a role for ionic bonding of cathepsin G to the surface of *S. aureus* it was unclear as to which cell surface structures might participate in binding. Since cathepsin G has a $\text{pI} > 12.5$ (Travis *et al.*, 1980), we considered the possibility that negatively charged surface structures might contribute to the total binding of cathepsin G. Accordingly, we tested whether a mutant strain of phage group II *S. aureus* deficient in polymeric teichoic acid (Chatterjee *et al.*, 1969) differed from its parental strain in susceptibility to cathepsin G. In these experiments the parental strain H was readily killed by cathepsin G ($\text{ED}_{50} 1.25 \mu\text{g ml}^{-1}$) while the mutant strain (52A5) exhibited decreased susceptibility ($\text{ED}_{50} 25 \mu\text{g ml}^{-1}$). However, the mutation resulting in the loss of polymeric teichoic acid has pleiotrophic effects, including increased clumping of staphylococci. This precluded unequivocal interpretation of both bactericidal and binding data obtained for cathepsin G in experiments similar to those described above. Nevertheless, the loss of polymeric teichoic acid and increased staphylococcal resistance to cathepsin G is consistent with the notion that this granule protein interacts with microbial cell surfaces via ionic interactions.

DISCUSSION

Our finding that cathepsin G exerts bactericidal action against *S. aureus* and that such activity is modulated by ionic strength and pH confirms and extends the earlier work of Odeberg & Olsson (1975, 1976). Importantly, we found that when tested under optimal conditions, the bactericidal activity of cathepsin G was substantially greater than earlier appreciated. Showing an ED_{50} of $3.1 \mu\text{g ml}^{-1}$, cathepsin G was as potent as other granule proteins known to exert bactericidal action against Gram-negative bacteria (Weiss *et al.*, 1978; Shafer *et al.*, 1984, 1986b) and was as active against *S. aureus* as against gonococci.

The impetus for this work was, however, to gain an insight into why cathepsin G, a proposed mediator of non-oxidative killing of bacteria (Spitznagel & Shafer, 1985), exerts bactericidal action *in vitro* yet may be ineffectual *in vivo*. This was considered important since *S. aureus*, but not other bacteria (e.g. gonococci) displaying susceptibility to cathepsin G *in vitro*, resists intraphagosomal killing by PMNs obtained from patients with CGD. We considered the possibility that conditions within the phagolysosome, which are thought to change during its maturation might modulate the bactericidal action of cathepsin G. Although the pH of the developing phagolysosome in both normal and CGD PMNs is thought to become mildly acidic (Segal *et al.*, 1981) we found that the bactericidal action of cathepsin G was best at pH 7.5. However, this value is at or near the initial values of vacuolar pH which Segal *et al.* (1981) observed in their studies with normal PMNs. They proposed that the initial rise in pH was due to the respiratory burst. CGD PMNs did not display this initial rise in pH since they were unable to generate the burst. Subsequent to this rise in pH, the acidification of the vacuole, also described by Segal *et al.* (1981), may retard the bactericidal action of cathepsin G.

A second possible reason why cathepsin G may have a reduced capacity to kill staphylococci in phagolysosomes is that the ionic strength of the environment may not be suitable. Our data suggests that the antibacterial property of cathepsin G could be retarded in the developing phagolysosome if the ionic strength is increased, perhaps due to ion influx from serum. Mechanistically, an increase in ionic strength could antagonize ionic bonding between cationic domains of cathepsin G and electronegative bacterial surface groups (e.g. teichoic acid). This hypothesis is consistent with the known ability of CAPs (Shafer *et al.*, 1984, 1986c; Farley *et al.*, 1988) and bactericidal/permeability-increasing proteins (Weiss *et al.*, 1978) to bind to negatively charged bacterial surface structures. It is important to stress, however, that we could completely inhibit bactericidal activity but not binding of cathepsin G by the addition of NaCl. This may have been due to salt interference with post-binding events. Alternatively, the number of molecules bound may have been insufficient to kill *S. aureus* or were bound in a nonspecific manner precluding bactericidal action. Clearly, additional experiments will be needed to address both the binding and post-binding events that are needed to achieve killing of *S. aureus*.

It is relevant to question, however, whether the results obtained in this study have a bearing on the intraphagosomal fate of *S. aureus* given that the studied concentrations of cathepsin G are far below those which might be expected in the phagolysosome. Specifically, it is possible that the achievable concentration of cathepsin G, like other granule proteins, is sufficient to overcome the constraints of ionic strength seen in our *in vitro* experiments. If so, this may explain, in part, why Segal *et al.* (1981) observed killing of *S. aureus* in the phagolysosomes of CGD PMNs when methylamine was added to artificially create an alkaline environment. In this case, the slightly alkaline environment achieved by the addition of methylamine may have promoted the antimicrobial action of cathepsin G or defensins (Ganz *et al.*, 1985). Hence, the pH of the vacuole and not ionic strength may be the more important determinant of intraphagosomal killing of *S. aureus* by cathepsin G.

Taken together, our data verify the staphylococcal action of cathepsin G *in vitro* and the importance of pH in determining its antibacterial action. We also suggest that binding of cathepsin G to *S. aureus* is aided by electrostatic interactions between cathepsin G and teichoic acid. More importantly, however, our work also suggests that intraphagosomal killing of *S. aureus* by cathepsin G may be increased by therapeutic interventions which retard acidification of the maturing phagolysosome.

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