Human neutrophil lysosomal cathepsin G (cat G) exerts broad-spectrum antibacterial action in vitro against Gram-negative and -positive bacteria independent of its serine protease activity. We recently determined that an internal peptide of cat G (HPQYNQR), obtained after digestion of cat G with clostripain, possessed broad-spectrum antibacterial action in vitro, displaying an ED₅₀ of 5 × 10⁻⁸ M. In order to evaluate the structure-antibacterial properties of this peptide, synthetic variants with single alanine substitutions at each position were prepared and tested for antibacterial action. We found that alanine substitution for His-1 or Tyr-4, or certain modifications of the His-1 side chain, produced nonbactericidal peptides. A hexapeptide lacking the COOH-terminal Arg₇ but not a pentapeptide lacking both Gln-6 and Arg-7 possessed in vitro bactericidal activity. Interestingly, the cat G bactericidal peptide displays similarity to sequences within other serine proteases, notably the proposed cytotoxic granzymes present in the cytoplasmic granules of human and mouse cytotoxic T lymphocytes. We now report that an internal peptide of one human granzyme (granzyme B) with the sequence of HPAYNPK also displays bactericidal action in vitro. Our results suggest that an internal antibacterial domain among human serine proteases cat G and granzyme B has been functionally conserved through evolution perhaps for the purpose of host defense against microbial pathogens and targets of cytotoxic T lymphocyte killing.

The antibacterial action of cat G¹ and other azurophil granule proteins is thought to contribute significantly to the nonoxidative antibacterial capacity of human polymorphonuclear granulocytes (3, 4, 5, 9, 14–16, 18). This mechanism of killing phagocytosed bacteria is of presumed importance in the host's ability to respond to infection at sites of hypoxia or when oxygen-dependent antimicrobial systems are ineffec-

tual (15, 18). Although the precise mechanism(s) by which lysosomal proteins kill susceptible bacteria remains to be established, recent studies, which employed different antibacterial proteins and target microorganisms, collectively indicate that they damage the cytoplasmic membrane or inhibit biosynthetic reactions occurring at this intracellular site (3, 4, 7, 17).

Although cat G is a serine protease displaying chymotryptic-like activity (20), its capacity to kill bacteria is known (1, 8, 9, 14–18) to be independent of protease activity. To help understand the structure-antibacterial relationships of cat G, we isolated (1) and sequenced a peptide (HPQYNQR) from the enzyme with antibacterial action in vitro; the complete amino acid sequence for cat G has been deduced from the cDNA sequence (12) and the antibacterial peptide corresponds to residues 77–83. The antibacterial peptide HPQYNQR is likely to be important in cat G killing of target bacteria since an analogous sequence in chymase, which shows significant similarity to cat G, is thought to be positioned toward the surface of the enzyme (11). This surface orientation of HPQYNQR in cat G would enhance its interaction with microbial surfaces, a likely prerequisite for bactericidal action.

Sequences analogous to HPQYNQR have not been reported for other antibacterial peptides known to be present in polymorphonuclear granulocyte granules. However, certain serine proteases termed granzymes, which are present in the cytoplasmic granules of human and mouse CTLs, display similarity to cat G and possess internal peptides analogous to HPQYNQR (5, 6, 19). Thus, we sought to determine whether their analogous peptides might also display antibacterial properties in vitro similar to the cat G-derived peptide. We now report the amino acid sequence requirements for the bactericidal action of cat G peptide HPQYNQR and show that a similar peptide derived from the published sequence for human granzyme B also has bactericidal action in vitro. Our results raise the possibility that these similar sequences in the closely related serine proteases cat G and granzyme B are important for the bactericidal action and possible cytotoxicity of their full-length enzymes.

**EXPERIMENTAL PROCEDURES**

**Materials**

Cat G was purified from extracts of human polymorphonuclear granules as described previously (2) and protease activity was inactivated by the addition of diisopropylfluorophosphate (14). All reagents used for peptide synthesis were obtained from Applied Biosystems, Inc. LB and GCB agar and HBSS were purchased from Difco Laboratories. Other chemicals used were obtained from Sigma.
Human Lysosomal Cathepsin G and Granzyme B

Methods

Preparation of Synthetic Peptides—Peptides were synthesized on an Applied Biosystems model 430A peptide synthesizer (0.1-mmol scale) using phenylalanine, methionine, or p-methoxybenzylhydrazine polystyrene resins and tert-butylxycarbonyl (Boc)-protected amino acids (Applied Biosystems or Bachem Bioscience). Boc-N-methyl- Ala, Boc-Arg (4-toluenesulfonyl), Boc-Asp (benzyl), Boc-Cys (4-methoxybenzyl), Boc-Glu (benzyl), Boc-3-His (benzoylcarbonyl), Boc-His (2,4-dinitrophenyl), Boc-His (N-toluenesulfonyl), Boc-Lys (2-chlorobenzyloxycarbonyl), Boc-Thr (benzyl), and Boc-Tyr (2-bromobenzoxycarbonyl) were used for incorporation of N-methyl-Ala, Arg, Asp, Cys, His, Lys, Thr, and Tyr, respectively. All amino acids were in the L configuration (except for synthetic peptide d-HPQYNQR, see Table II). Boc-His (1-methyl) and Boc-His (3-methyl) or the D-stereoisomer of His was also used to ascertain the other amino acids which might be essential for bactericidal action against S. aureus. Bactericidal assays employed lo5 CFU/ml of diluted, mid-logarithmic phase cultures; 100 µl of such suspensions in HBSS alone. Incubation of bacteria with synthetic peptides was achieved by reverse-phase HPLC on an Aquapore RP-300 C18 silica column (1 × 10 cm, Applied Biosystems) using a linear gradient of acetonitrile in 0.1% aqueous trifluoroacetic acid. The purity of the synthetic peptides was confirmed by microbore HPLC on a C18 silica column (1 × 250 mm, Applied Biosystems) and by NH2-terminal sequencing in a model 477A Pulse Liquid Sequencer/120A phenylthiohydantoin Analyzer (Applied Biosystems). All peptides were stored in the lyophilised form at 4°C prior to use in antibacterial assays against Staphylococcus aureus as described in the text. They were dissolved in HBSS for use in antibacterial assays.

Antibacterial Assays—Antibacterial assays routinely employed S. aureus strain 8325-4, which has been described previously (16). In certain experiments, Neisseria gonorrhoeae strain FA102 (14) and Escherichia coli strain JF 568 were tested for susceptibility to the synthetic peptides. Bactericidal assays employed 106 CFU/ml of diluted, mid-logarithmic phase cultures; 100 µl of such suspensions in HBSS were incubated with several concentrations of synthetic peptides in 200-µl volumes in sterile microtiter trays. Controls consisted of bacteria incubated in HBSS alone. Incubation of bacteria with peptides lasted for 1 h at 37°C. After incubation, 100- and 10-µl samples were plated directly onto either LB or GCB agar. All plates were incubated at 37°C for 24 h; gonococci were incubated in the COOH terminus substitutions in a model 477A Pulse Liquid Sequencer/120A phenylthiohydantoin Analyzer (Applied Biosystems). All peptides were stored in the lyophilised form at 4°C prior to use in antibacterial assays. The data are expressed as % survival ± S.E. The number of individual assays for each peptide are shown as N. Percentage survival was calculated as follows:

\[
\frac{N \text{ of CFU in presence of peptide}}{N \text{ of CFU in absence of peptide}} \times 100
\]

RESULTS AND DISCUSSION

Influence of Single Alanine Substitutions in the Antibacterial Action of cat G Peptide HPQYNQR—We reported earlier (1) that substitution of the NH2-terminal histidine with alanine abrogated the bactericidal action of HPQYNQR. In order to ascertain the other amino acids which might be essential for the broad-spectrum antibacterial action of HPQYNQR, synthetic peptides with single alanine substitutions at each position were prepared and tested for their antibacterial action in vitro. When suspensions of 106 CFU ml-1 of S. aureus strain 8325-4 were incubated at 37°C in HBSS with 100 µg (~5 × 1011 M) of these synthetic peptides, it was observed that the parent peptide (HPQYNQR) and certain alanine-substituted derivatives (HAQYNQR, HPAYQVR, and HPQYNQA) exerted antibacterial action in vitro (Table I) while other substitutions reduced the activity (HPQYNAR and HPAYQNR) or undetectable (APQYNQR and HPQANQR) antibacterial activity. The bactericidal action of these peptides was not restricted to S. aureus, as exemplified by the activity of each antibacterial peptide to kill both E. coli and N. gonorrhoeae in vitro (data not shown).

The cationicity and hydrophobicity of lysosomal proteins suggested that the NH2-terminal histidine and the tyrosine residues at position 4 are essential residues for the antibacterial capacity of HPQYNQR. To more closely examine the structural requirements of HPQYNQR needed for bactericidal action, several synthetic peptides (Table II) (containing alterations in either His-1, substitutions at Tyr-4, or deletions at the COOH terminus) were prepared and examined for bactericidal action against S. aureus (Table II). Whereas methylation of either nitrogen-1 or -3 of the imidazole ring of His-1 had little effect on peptide activity, derivatives with the imidazole modified at nitrogen-3 with more bulky benzyol or dinitrophenyl groups were inactive. Thus, we suggest that major modifications of the imidazole ring of His-1, leading to loss of bactericidal activity, likely impaired its hydrogen bonding potential through steric hindrance. Moreover, a synthetic peptide containing the D-stereoisomer of His was also found to be inactive. While substitution of Tyr-4 with alanine abrogated bactericidal action (Table I), the addition of phenylalanine (Table II) partially restored activity, suggesting the need for an aromatic group-containing side chain at position 4.

The cationicity and hydrophobicity of lysosomal proteins

TABLE I

| Peptide sequence | % Survival of S. aureus |
|------------------|------------------------|
| HPQYNQR          | 28.6 ± 1.57            |
| APQYNQR          | 98.5 ± 2.1             |
| HAQYNQR          | 31.2 ± 2.4             |
| HPAYQNR          | 43.6 ± 2.54            |
| HPQANQR          | 96.7 ± 4.34            |
| HPQYNAR          | 30.6 ± 0.85            |
| HPQYNQA          | 51.2 ± 3.6             |
| HPQYNQA          | 39.8 ± 1.96            |

*The data are presented as % survival ± S.E. and represent results from at least three separate experiments for each synthetic peptide.

TABLE II

| Peptide sequence | % Survival of S. aureus |
|------------------|------------------------|
| Substitutions at position 1 |                 |
| HPQYNQR          | 28.6 ± 1.57            |
| HPQYNRA          | 96.7 ± 2.1             |
| HPQYNRA          | 37.3 ± 2.25            |
| HPQYNRA          | 18.3 ± 3.22            |
| HPQYNRA          | 102.2 ± 2.92           |
| HPQYNRA          | 95.9 ± 2.6             |
| HPQYNRA          | 31.0 ± 1.6             |
| HPQYNRA          | 98.7 ± 2.8             |
| d-HPQYNQR        | 98.6 ± 1.6             |

Substitutions at position 4 |                 |
| HPQANQR          | 96.7 ± 4.34            |
| HPQANQR          | 45.0 ± 4.91            |

COOH terminus substitutions |                 |
| HPQYNQA          | 39.8 ± 1.96            |
| HPQYNQA          | 32.8 ± 1.83            |
| HPQYNQA          | 95.8 ± 2.85            |

*All peptides were tested at 5 × 10-4 M as described in Table I. The data are expressed as % survival ± S.E., and N represents the number of experiments. Each experiment consisted of triplicate determinations.

Modified histidines are 1-methyl (1m), 3-methyl (3m), 2,4-dinitrophenyl (Dnp), benzyl (Bzl), acetyl (Ac), and heptanoyl (Hep), and the D-stereoisomer of His (d-HPQYNQR) as described in the text. All substitutions are underlined.
such as cat G have been suggested (18) to be important in their bactericidal action. To test these parameters, we prepared peptides lacking the Arg-7 residue and peptides with increased hydrophobicity (Table II). Surprisingly, replacement of Arg-7 with alanine or deletion of Arg-7 had no effect on bactericidal action. In addition, a synthetic peptide RRAI-RHPQYNQR (residues 72-83 of cat G) did not show any increase in bactericidal potency (data not presented), confirming that cationicity per se is not the underlying determinant for bactericidal action of this cat G-derived peptide. However, pentapeptide HPQYN-amide lacked bactericidal action, implying that the mechanism of killing of HPQYNQR is determined by structural considerations impaired by the first 6 residues. To learn whether the hydrophilic nature of HPQYNQR is important in determining its antibacterial property, either an acetyl or a heptanoyl group was attached to the NH2-terminal amino group. The small increase in hydrophobicity imparted by the acetyl group had no significant influence on antibacterial action, while the more bulky and hydrophobic heptanoyl group rendered HPQYNQR inactive. Thus, the presumed importance of cationicity and hydrophobicity of lysosomal proteins for bactericidal action does not apply for the cat G-derived antibacterial peptide HPQYNQR.

**Human Granzyme B Possesses an Internal Antibacterial Peptide Similar to HPQYNQR**—Comparison of the cat G sequence HPQYNQR with sequences of other serine proteases revealed some degree of similarity with the corresponding region of mouse and human granzymes (6). Since the granzymes have been implicated in lysis of target cells by CTLs, we synthesized peptides corresponding to all known sequences of mouse and human granzymes and tested them against *S. aureus* (Table III) and *N. gonorrhoeae*. As shown in Table III, synthetic mouse granzyme peptides located within granzymes A, C-G failed to exert any activity while the granzyme B-derived peptide was slightly bactericidal. In contrast to the mouse granzymes, the human granzyme B-derived peptide, but not granzyme A peptide displayed significant antibacterial action in vitro against *S. aureus* (Table III) and *N. gonorrhoeae* (data not shown).

The human granzyme B-derived peptide differs from the cat G peptide at positions 3, 6, and 7 (Table III). In order to test how these differences might influence antibacterial action, we synthesized and tested variants of the cat G and granzyme B peptides which contained some of these heterologous amino acids or had other substitutions at positions 3 and 6. Placement of the granzyme B Ala-3 residue in the cat G Gln-3 position suppressed antibacterial activity, suggesting that hydrogen bonding potential of bulky residues (e.g. Gln) at position 3 in the cat G peptide is important. Interestingly, however, placement of the cat G Gln-3 residue in the granzyme B peptide severely inhibited antibacterial action. Thus, for granzyme B the placement of alanine at position 3 appeared to be a crucial substitution for expression of its antibacterial activity. Proline, due to its cyclic imide character, is unique in imposing restrictions on peptide conformation, which in many instances is critical for biological activity. Although Pro-2 of the cat G peptide did not seem to have a significant role (Table I), Pro-6 in the granzyme B peptide appeared to be critical since its replacement by alanine-abrogated bactericidal activity (Table IV). Interestingly, however, replacement of the amide proton of the Ala-6 analog by a methyl group, i.e. substitution of Ala-6 with N-methyl alanine, giving an imino acid sharing with proline the propensity to cis-trans-isomerism of the peptide bond with the preceding residue (Asn-5), restored antibacterial potency. This indicates that the cis-peptide bond character in position 6 may be necessary for the active conformation of the granzyme B peptide sequence. The significance of the Pro-6 residue for the bactericidal action of the granzyme B-derived peptide was apparently unique for this peptide since its insertion in the cat G peptide sequence suppressed antibacterial activity. While deletion of the COOH-terminal Lys-7 residue had no effect on the bactericidal action of the granzyme B peptide, its replacement with arginine present in the cat G peptide severely briddled bactericidal action. Thus, these data collectively imply that, while the parent peptides have radical differences in at least two positions, none of which can be explained by single base changes at the DNA level (5, 6), they have collective compensating effects which maintain bactericidal action (Table IV). It is likely that such *en bloc* structural differences between these two bactericidal domains were, at the protein level, preserved for attaining active conformation at the site of action, a task not achievable through single-residue replacements.

**Parameters Influencing the Bactericidal Action of cat G and Granzyme B-derived Peptides**—Next, we examined whether certain environmental conditions (pH and ionic strength), thought to correspond to the intraphagolysosomal milieu (13,

**Table IV**

| Peptide       | Sequence       | % Survival of *S. aureus* | N  |
|---------------|----------------|--------------------------|----|
| Cat G         | HPQYNQR        | 28.6 ± 1.57              | 15*|
| Human granzyme A | YPCYDFPA      | 102 ± 2.6               | 3  |
| Human granzyme B | HPAYNPK        | 36.0 ± 2.5               | 9  |
| Mouse granzyme A | YPCYDEY      | 99.5 ± 1.7               | 3  |
| Mouse granzyme B | HPDYNPK        | 62 ± 3.4                | 3  |
| Mouse granzyme C | HPDYNPD       | 104 ± 3.8               | 4  |
| Mouse granzyme D, E* | HPDYNAT       | 98 ± 2.7                | 3  |
| Mouse granzyme F | HPAYDDK        | 98 ± 1.9                | 3  |
| Mouse granzyme G | HPAFDRK        | 102 ± 2.8               | 3  |

| Peptide       | Sequence       | % Survival of *S. aureus* | N  |
|---------------|----------------|--------------------------|----|
| Cat G         | HPQYNQR        | 28.6 ± 1.57              | 15*|
| Cat G (Ala-3)b | HPAYNPK        | 43.6 ± 2.54              | 6  |
| Cat G (Asn-3) | HPAYNQR        | 42.9 ± 2.51              | 3  |
| Cat G (Glu-3) | HPAYNQR        | 45.3 ± 1.78              | 3  |
| Cat G (Leu-3) | HPAYNQR        | 100.8 ± 0.69             | 3  |
| Cat G (Pro-6)b | HPAYNPK        | 78.7 ± 2.8              | 4  |
| Granzyme B    | HPAYNPK        | 36.0 ± 2.5               | 9  |
| Granzyme B (Gln-3)b | HPAYNPK      | 95.1 ± 3.4              | 5  |
| Granzyme B (Ala-6) | HPAYNK       | 97.0 ± 1.8              | 3  |
| Granzyme B (A*-6)c | HPAYN*K     | 40.5 ± 1.6              | 3  |
| Granzyme B (Arg-7)d | HPAYNPK   | 87.5 ± 4.3              | 5  |
| Granzyme B*   | HPAYN*mod     | 42.5 ± 3.6              | 5  |

* All peptides were tested at 5 × 10^-4 M as described in Table I. The data are expressed as % survival (±S.E.), and N represents the number of experiments.

* Peptide sequences for mouse granzymes D and E are identical (see Ref. 6).

* Analogus containing single heterologous replacement between cat G and granzyme B peptides.

* A*, N-methylalanine.

* HPAYN*mod; granzyme B derivative lacking the COOH-terminal Lys-7.
The bacterial action of cat G synthetic peptides (HPQYNQR, Ac-HPQYNQR) under different pH conditions was found to be optimal at slightly basic pH (Fig. 1A). Segal et al. (13) suggested this was near the final pH of the mature phagolysosome. Like full-length cat G, the antibacterial action of the human granzyme B peptide was sensitive to increasing concentrations of NaCl in the incubation solution (Fig. 1B) while surprisingly the cat G-derived peptide HPQYNQR and its acetylated derivative retained antibacterial action even at the highest NaCl concentration (0.21 M) tested. Having optimized the in vitro conditions for bacterial action of the cat G peptide and the human granzyme B peptide, we next determined their time course of killing S. aureus (Fig. 1C) and found that the human granzyme B peptide reduced its CFU capability within 30 min. In contrast, the cat G peptide HPQYNQR more rapidly reduced the CFU capability of the exposed bacteria and, after 120 min of exposure, <10% survival was noted; no demonstrable antibacterial action of the APQYNQR derivative peptide was noted even after 120 min of incubation.

It is of interest that two distinct human serine proteases, cat G and human granzyme B, localized within different cells (PMNs versus CTLs), possess a similar internal peptide sequence with broad-spectrum antibacterial action in vitro. That the antibacterial action of both the cat G and granzyme B-derived peptides is dependent on structural considerations (hydrogen bonding potential and hydrophilicity) imparted by their sequences can be inferred by the influence of specific alanine substitutions in their primary sequence, alterations and/or inversion of the configuration of histidine, and the addition of a large hydrophobic heptanoyl group to the N-terminal amino group. Recent analyses of the cDNA for these serine proteases (6) revealed the phylogenetic relationship between cat G, human granzyme B, and several mouse granzymes. Briefly, through such analyses it was postulated that cat G and granzymes B from the human and mouse were more closely related to each other than cat G and the other granzymes.

The close phylogenetic relationship between cat G and human granzyme B is also supported by our finding of the antibacterial capacity of the synthetic peptides derived from their internal sequences but not those of the other human and mouse granzymes. Due to the lack of purified human granzyme B, we have not examined its antibacterial capacity but our results suggest that it has antibacterial potential through peptide sequence HPAYNPK. If so, then human granzyme B may participate in cytotoxic action against transformed cells, in conjunction with other CTL proteins such as perforin (10), as well as antibacterial activity against microbial pathogens. We are currently evaluating the cytotoxic action of several of the peptides reported herein using both normal and transformed cells as well as the mechanism(s) whereby these peptides exert bactericidal action against gram-negative and -positive bacteria. Such information may also provide important insight into the still unknown function(s) (5, 6) of the granzymes in CTL-mediated killing of target cells.

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**Fig. 1.** Bactericidal action of cat G synthetic peptides (HPQYNQR, Ac-HPQYNQR) (see Table II), and human granzyme B-derived peptide HPAYNPK under different pH conditions (A) and ionic strength (B), and at different time points (C). The synthetic peptides (5 × 10−4 M) and purified, enzymatically inactive cat G (1.8 × 10−6 M) were tested against S. aureus for the anti-staphylococcal action of full-length cat G, the antibacterial action of several of the peptides reported herein using both normal and transformed cells as well as the mechanism(s) whereby these peptides exert bactericidal action against gram-negative and -positive bacteria. Such information may also provide important insight into the still unknown function(s) (5, 6) of the granzymes in CTL-mediated killing of target cells.
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