Antibacterial Activity of Human Neutrophil Defensin HNP-1 Analogs without Cysteines

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The antibacterial activity of human neutrophil defensin HNP-1 analogs without cysteines has been investigated. A peptide corresponding to the HNP-1 sequence without the six cysteines (HNP-1ΔC) exhibited antibacterial activity toward gram-negative and gram-positive bacteria. Truncated analogs wherein the nine N-terminal residues of HNP-1 and the remaining three cysteines were deleted (HNP-1ΔC18) or the G was replaced with A (HNP-1ΔC18A) also exhibited antibacterial activity. Substantial activity was observed for HNP-1ΔC and HNP-1ΔC18 in the presence of 100 mM NaCl, except in the case of Pseudomonas aeruginosa. The linear peptides were active in the presence of carbonyl cyanide m-chlorophenylhydrazone (CCCP), indicating that proton motive force was not essential for killing of bacteria by the peptides. In fact, in the presence of CCCP, the peptides were active against P. aeruginosa even in the presence of 100 mM NaCl. The antibacterial activity of HNP-1ΔC, but not that of the shorter, 18-residue peptides, was attenuated in the presence of serum. The generation of defensins without cysteines would be easier than that of disulfide-linked defensins. Hence, linear defensins could have potential as therapeutic agents.

Mammalian defensins are a family of cationic antimicrobial peptides characterized by three disulfide bridges (13, 22, 35). They are expressed in granulocytes, monocytes, macrophages, and epithelial cells of the skin, intestines, and other tissues (2, 13, 39). Based on the pattern of disulfide bonding, mammalian defensins have been classified into α and β families. In another class named the θ-defensins, in addition to three disulfide bridges, the amino- and carboxyl-terminal ends are linked via an amide bond (13). In humans, θ-defensins are produced in neutrophils and Paneth cells of the small intestine, while β-defensins are expressed by epithelial cells at various sites (13, 22). The θ-defensins have been identified only in primates (13, 19). The expression of defensins is constitutive as well as induced by infection and inflammation (13, 22, 23). Defensin biosynthesis and release have been observed to be regulated by microbial signals, developmental signals, and cytokines (13, 14).

Apart from their role as microbicidal agents, defensins have also been implicated as effective immune modulators in adaptive immunity (11, 54). They have potency to enhance phagocytosis, induce the activation and degranulation of mast cells, and indirectly regulate innate antimicrobial immunity by affecting the production of chemokines such as interleukin-8, interleukin-10, and tumor necrosis factor from different cells (3, 24, 53). Studies have also revealed the role of defensins in adaptive immunity, as reflected by their chemotactic effect on monocytes, T cells, and immature dendritic cells (5, 32, 35, 43, 48, 54).

Human neutrophil defensins (HNP-1 to HNP-4) are stored in azurophilic granules of the neutrophil. They are transferred to phagolysosomes upon phagocytosis to kill the ingested microbes (14, 15). The crystal structure of HNP-3 reveals a dimeric structure, with each monomer containing a triple-stranded β-sheet region (16). Nuclear magnetic resonance studies indicate that HNP-1 also forms dimers or higher-order aggregates in solution. The three β-strands observed in HNP-3 are also present (34, 57). In an effort to determine how essential the disulfide connections are for antibacterial activity and structure, we have investigated the antibacterial activity of a peptide spanning residues A1 to F28 of HNP-1 which does not have the six cysteines present in HNP-1 (Table 1). We have also examined the effect of deleting the amino acids AYRIPA from the N-terminal end of this peptide and the effect of replacing G with A in the truncated analog (Table 1) on antibacterial activity and structure.

**MATERIALS AND METHODS**

9-Fluorenylmethoxy carbonyl (Fmoc) amino acids were obtained from Novabiochem AG (Switzerland) and Advanced Chemtech (Louisville, KY). The Fmoc amide handle crown was from Mimotopes (Australia). 1-N-Phenyl naphthalamine (NPN) and carbonyl cyanide m-chlorophenylhydrazone (CCCP) were obtained from Sigma. All other chemicals used were of the highest grade available.

**Peptide synthesis.** Peptides were synthesized using an Fmoc amide handle crown employing F-moc chemistry (1). The synthesized peptides were cleaved and deprotected by using a mixture containing 82.5% trifluoroacetic acid (TFA), 5% phenol, 5% H2O, 5% thioanisole, and 2.5% ethanedithiol for 12 to 15 h at room temperature. HNP-1 was synthesized on 4-hydroxymethylphenoxymethyl resin (Applied Biosystems; 0.9 mmol/g) (28) with the cysteines protected by acetalidomethyl (46). The removal of acetalidomethyl was effected by Hg(II) acetate and β-mercaptoethanol treatment (28, 46) followed by gel filtration on a Biogel P-2 column. HNP-1 was then oxidized in 20% dimethyl sulfoxide-water (42) at a peptide concentration of 0.2 to 0.4 mM. This was followed by gel filtration with 50% acetonitrile-water containing 0.01% TFA. The peptides were purified on a Hewlett-Packard 1100 series high-performance liquid chromatography instrument with a reversed-phase C4 or C18 Bio-Rad column. The solvents consisted of 0.1% TFA in H2O as mobile phase A and 0.1% TFA in CH3CN as mobile phase B. The purified peptides were then characterized by matrix-assisted laser desorption ionization–time-of-flight mass spectrometry on a Voyager DE STR mass spectrometer (Applied Biosystems) at the Proteomics Facility of the Centre for Cellular and Molecular Biology. Stock solutions of HNP-1 analogs were prepared by dissolving the peptides in water. A stock solution of HNP-1 was

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preparred in 0.01% acetic acid. The concentrations were determined by monitoring the absorbance of tryptophan at 280 nm.

**Antibacterial activity.** The bacterial strains used were *Escherichia coli* W 160-37 (47), *Staphylococcus aureus* (NCTC 8530), and *Pseudomonas aeruginosa* (NCTC 6750). Bactericidal activity was determined as follows. Mid-log-phase bacteria were obtained by growing bacteria in nutrient broth (Bacto; Difco), washing them twice with 10 mM sodium phosphate buffer (pH 7.4), and then diluting them in the same buffer to give approximately 10^6 CFU/mL. A final volume of 100 μL was used in sterile 96-well plates to examine the antibacterial activity of the peptides. After incubation of the bacteria with different concentrations of peptides for 2 h at 37°C, 20 μL was plated on nutrient agar plates. The plates were incubated for 18 h at 37°C, and the colonies formed were counted. The lowest concentration of peptide at which there was complete killing was taken as the lethal concentration (LC). In control experiments, cells were incubated with only 0.01% acetic acid (the solvent used to dissolve HNP-1).

**Kinetics of bacterial killing.** The kinetics of bacterial killing were determined against gram-negative and gram-positive bacteria. Mid-log-phase bacteria (10^5 CFU/ml) were incubated with LC50s of peptides in 10 mM sodium phosphate buffer (pH 7.4) in a final volume of 100 μL. Aliquots of 20 μL were removed at fixed intervals and plated on nutrient agar plates. The CFU were counted after an incubation of 16 to 18 h at 37°C. The values mentioned in the results are the averages of two independent experiments.

**Salt sensitivity.** Salts (NaCl 100 mM; MgCl₂ and CaCl₂, 1 mM and 2 mM) were added to the incubation buffer to examine the effect of salt on the antibacterial activity of the peptides. The effect of salts was selected at the lethal concentrations of the peptides. *E. coli* MG1655 (10^5 CFU/ml) was used to examine inhibition by MgCl₂ and CaCl₂. The values determined were the averages of three independent experiments. The variation was within ±5%.

**Outer membrane permeabilization assay.** The ability of peptides to permeabilize the outer membranes of *E. coli* MG 1655 cells was investigated using an NPN uptake assay (25, 38). Briefly, cells at an optical density at 600 nm (OD₆₀₀) of 0.1 were suspended in 5 mM HEPES (pH 7.4) with 10 μM NPN. After 15 min of incubation, peptides were added, and the fluorescence of NPN was monitored. The excitation wavelength used was 350 nm, and the emission wavelength was 420 nm. The experiment was carried out at 25°C.

**Inner membrane permeabilization assay.** The ability of peptides to permeabilize the inner membranes of bacteria was studied using *E. coli* GJ 2455 (laci lacZ+ strain derived from *E. coli* MG1655 at Centre for Cellular and Molecular Biology), which constitutively expresses β-galactosidase in its cytoplasm. α-Nitrophenyl-β-D-galactopyranoside (ONPG) was used as the substrate for β-galactosidase. Late-logarithmic-phase (OD₆₀₀ = 0.5 to 0.6) cells were washed and diluted to an OD₆₀₀ of 0.03 in 10 mM sodium phosphate buffer (pH 7.4), and 0.55 mM ONPG was added. After the addition of different peptide concentrations, OD measurements were made every 5 min at 550 nm and 420 nm, and absorbance calculations [4(550) – (1.75 × A₄₂₀)] were taken as a measure of the β-galactosidase activity. The production of o-nitrophenol was monitored at 420 nm. The value 1.75 × A₄₂₀ represents the light scattering by cell debris at 420 nm. Bacterial cells without any peptide were used as a control.

**Effect of dissipation of proton motive force on antibacterial activity.** The effect of a loss of proton motive force due to the action of CCCP was examined as follows. Bacteria were preincubated with 20 μM CCCP for 20 min, followed by the addition of peptides at the LC₅₀ with or without NaCl. The effect of incubation with 50 μM CCCP was also examined. CCCP at concentrations of 20 μM and 50 μM has been used for dissipation of the proton motive force in bacteria (10, 33, 40). The bactericidal activity was determined as described earlier. The values determined were the averages of three independent experiments. The variation was within ±5%.

**Hemolytic activity.** Human erythrocytes were used to evaluate the hemolytic activity of the peptides. Erythrocytes were obtained by centrifuging (800 × g) heparinized blood and then were washed thrice with 5 mM HEPES (pH 7.4) containing 150 mM NaCl. Aliquots containing 10^7 red blood cells/ml were incubated in the presence of different peptide concentrations in 0.5-ml Eppendorf tubes at a final volume of 100 μL for 30 min at 37°C with gentle mixing. The tubes were centrifuged, and the absorbance of the supernatants was measured at 540 nm. Erythrocyte lysis occurring in distilled water was taken as maximal lysis.

**Serum sensitivity assay.** To study the effect of serum components on the antimicrobial activity of the peptides, serum was isolated from human blood after the removal of erythrocytes by centrifugation. Total protein was estimated using the Folin-Ciocalteau-Lowry method (26). For both antimicrobial activity and hemolytic activity, serum at 1 mg/ml was included in the buffer and incubated for 15 min before the addition of peptides. Cells with only serum were used as a control.

**Circular dichroism (CD).** A JASCO J-715 spectropolarimeter was used to record the spectra of peptides in water, trifluoroethanol (TFE), and 10 mM sodium dodecyl sulfate (SDS) at 25°C. A quartz cell with a 1-mm path length was used. Spectra were recorded in the region from 195 to 250 nm. The concentrations of peptides were 25 μM for HNP-1ΔC, 30 μM for HNP-1ΔC18, and 20 μM for HNP-1ΔC18A.

**RESULTS**

**Antibacterial activity of peptides.** The ability of the linear peptides and HNP-1 to kill both gram-positive and gram-negative bacteria was investigated. The lethal concentrations for killing *E. coli*, *P. aeruginosa*, and *S. aureus* are shown in Table 2. The results indicate that HNP-1 analogs which lack cysteines, and hence the disulfide cross-links, can still display significant antibacterial activity. However, HNP-1 exhibited greater activity than the analogs. The deletion of AYRIPA from HNP-1ΔC resulted in the attenuation of antibacterial activity. The replacement of G by A in HNP-1ΔC18 marginally increased the potency against *E. coli*.

**Bacterial killing** (reduction in log₅₀ CFU/ml) as a function of time at the LC is shown in Fig. 1. HNP-1ΔC killed *E. coli*, *S. aureus*, and *P. aeruginosa* more rapidly than HNP-1. The shorter analogs also killed *E. coli* and *S. aureus* more rapidly than HNP-1. However, the rates of killing of *P. aeruginosa* by the 18-residue peptides and HNP-1 were comparable. Rapid killing of *S. aureus* by HNP-1ΔC18A compared to that by the other peptides was observed.

**Effect of salt and serum on antimicrobial activity.** The effects of NaCl on the activities of HNP-1 and its analogs are summarized in Table 3. The activity of HNP-1ΔC was marginally lower against *E. coli* and *S. aureus* but decreased considerably against *P. aeruginosa*. The shorter peptides exhibited relatively greater losses in activity against *E. coli*. When G was replaced by A in HNP-1ΔC18, the loss in activity against *P. aeruginosa* and *S. aureus* was relatively less. When the effects of divalent cations on HNP-1 and its analogs were examined, there was a complete loss of antibacterial activity in the presence of 1 mM MgCl₂ for the analogs, unlike

**TABLE 1. Primary structure of HNP-1 and its analogs**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>HNP-1</td>
<td>AYPVPAAGERRYGTYIQGRWF-LYS-CONH₂</td>
</tr>
<tr>
<td>HNP-1ΔC18</td>
<td>AYPVPAAGERRYGTYIQGRWF-CONH₂</td>
</tr>
<tr>
<td>HNP-1ΔC18A</td>
<td>IAERYRTAYGARWF-CONH₂</td>
</tr>
</tbody>
</table>

**TABLE 2. Antibacterial activity of peptides**

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>Lethal concentration (μM)³ of peptide</th>
<th>HNP-1ΔC18A</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> W 160-37</td>
<td>1.0 ± 0.2</td>
<td>14.0 ± 2.0</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> NCTC 6750</td>
<td>1.0 ± 0.2</td>
<td>14.0 ± 2.0</td>
</tr>
<tr>
<td><em>S. aureus</em> NCTC 8530</td>
<td>0.8 ± 0.2</td>
<td>7.0 ± 2.0</td>
</tr>
</tbody>
</table>

³ The values reported for the analogs are means ± maximum variations observed in three independent experiments.
HNP-1, which was active even in the presence of 2 mM MgCl$_2$ (data not shown). However, the presence of 2 mM CaCl$_2$ seemed to have only a marginal effect on the antibacterial activity of the three linear analogs toward E. coli MG1655. A complete loss in the activity of HNP-1 was observed in the presence of Ca$^{2+}$ (data not shown), as reported earlier (20). Both Mg$^{2+}$ and Ca$^{2+}$ bind to lipopolysaccharides and stabilize the outer membrane of E. coli (45). Our results suggest that there could be differences in affinity between the linear analogs and HNP-1 for lipopolysaccharides.

The antibacterial activities of the 18-residue peptides were not affected in the presence of serum (Table 3, values in parentheses). HNP-1 and HNP-1ΔC showed reduced activities against E. coli. While HNP-1 was rendered inactive against P. aeruginosa and S. aureus, HNP-1ΔC showed a considerably reduced activity (Table 3).

### Outer and inner membrane permeabilization assays
Defensins are known to act by sequentially permeabilizing the outer and inner membranes of E. coli (20). NPN, a neutral hydrophobic probe, has been used to examine the permeabilization of the outer membranes of gram-negative bacteria (38). All of the analogs permeabilized the outer membrane of E. coli in a concentration-dependent manner, as observed by an increase in NPN fluorescence (Fig. 2), despite differences in the inhibition of antibacterial activity by Mg$^{2+}$ and Ca$^{2+}$ between HNP-1 and the linear analogs.

When the ability of the peptides to permeabilize the inner membrane of E. coli was examined by the ONPG assay (21), no influx of ONPG was observed, suggesting that the peptides do not permeabilize the inner membrane (data not shown), unlike HNP-1, which was effective in permeabilizing the inner membrane.

### Effect of dissipation of proton motive force on antibacterial activity
The effect of depolarizing the bacterial inner membrane with CCCP on the antibacterial activity of the peptides was examined. HNP-1 and its analogs exhibited bactericidal activities against E. coli, P. aeruginosa, and S. aureus at their LCs in the absence of a proton motive force as a result of adding 20 μM CCCP. Similar results were observed when 50 μM CCCP was used (data not shown). The peptides were effective against P. aeruginosa in the absence of a proton motive force, even in the presence of 100 mM NaCl (data not shown).

### Lysis of human erythrocytes
The ability of the peptides to lyse human red blood cells is shown in Table 4. For comparable concentrations of peptides, maximum hemolysis was observed with HNP-1ΔC18A, which was, however, neutralized in the presence of human serum.

### Conformation of peptides
Circular dichroism (CD) spectroscopy was carried out for all three peptides in water, undiluted TFE, and 10 mM SDS (Fig. 3). The spectra of the peptides in water are characteristic of unordered conformations. The spectra of HNP-1ΔC in TFE and SDS suggest a population with a β-hairpin conformation (4, 6, 18) characterized by a negative ellipticity near 205 nm and a crossover at ~200 nm. In TFE, both the HNP-1ΔC18 and HNP-1ΔC18A spectra display minima at ~205 nm and ~223 nm, with a crossover at ~200 nm. The spectra indicate the presence of populations with both β-hairpin and helical conformations (4, 6, 18). In 10 mM SDS, HNP-1ΔC18A shows a minimum at

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**TABLE 3. Effect of NaCl and human serum on antibacterial activity of peptides**

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>% Bacterial killing in presence of NaCl</th>
<th>HNP-1</th>
<th>HNP-1ΔC</th>
<th>HNP-1ΔC18</th>
<th>HNP-1ΔC18A</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>(in presence of human serum$^a$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W 160-37</td>
<td>68 (50)</td>
<td>81 (47)</td>
<td>68 (100)</td>
<td>59 (100)</td>
<td></td>
</tr>
<tr>
<td>P. aeruginosa NCTC 6750</td>
<td>45 (0)</td>
<td>10 (13)</td>
<td>12 (87)</td>
<td>41 (98)</td>
<td></td>
</tr>
<tr>
<td>S. aureus NCTC 8530</td>
<td>33 (0)</td>
<td>92 (24)</td>
<td>63 (99)</td>
<td>84 (99)</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Mid-log-phase bacteria (10$^5$ CFU/ml) were incubated with the LCs of peptides in the presence of 100 mM NaCl. The values reported are the averages of three independent experiments. The variation was within ±5%.
~214 nm and a crossover at ~200 nm, which may indicate a population largely consisting of a β-structure.

**DISCUSSION**

In this study, we have examined the antibacterial activities of linear peptides derived from the HNP-1 sequence without cysteines. The linear analogs of HNP-1 do possess antibacterial activity, although their lethal concentrations are approximately 10- to 20-fold higher than that of HNP-1.

The antibacterial activities exhibited by HNP-1ΔC18 and HNP-1ΔC18A suggest that the six N-terminal amino acids AYRIPA present in HNP-1 are dispensable for activity, even in the context of the linear sequence. It was shown earlier that peptides derived from the HNP-1 sequence without AYRIPA, but constrained by one or two disulfide bridges, have antibacterial activity (28). The residue G does not appear to play a crucial structural role, as its replacement with A does not diminish the activity. CD data indicate that linear HNP-1ΔC does tend to adopt a β-structure, suggesting that there is an intrinsic propensity of the peptide sequence to adopt this conformation rather than its being due to constraints imposed by disulfide bridges. When G is replaced with A, the peptide favors a helical conformation. This observation suggests that the positioning of cationic and hydrophobic residues in β-strands in HNP-1 is not the only structural feature that is essential for the exhibition of antibacterial activity.

We have observed that the activity of HNP-1 is not completely abolished in the presence of 100 mM NaCl, although there have been reports that the antibacterial activity of HNP-1 is completely lost in the presence of high salt concentrations (31, 44). It is conceivable that a loss in activity in the presence of NaCl may depend on the assay employed. A loss of antibacterial activity in the presence of a high salt concentration has been observed for HNP-1 in the radial diffusion assay, which has been extensively used to determine the antibacterial activities of mammalian defensins (44, 56). Also, the inhibition of antibacterial activity by human neutrophil defensins appears to be strain dependent (12, 44). Like HNP-1, all of the linear peptides permeabilize the outer membrane of *E. coli*. Subsequently, the linear peptides do not permeabilize the inner membrane to cause an influx of ONPG, as observed with HNP-1 (20, 21). However, it is likely that the site of action is the inner membrane, considering the rapid rate of killing. The

**TABLE 4. Hemolytic activity of peptides**

<table>
<thead>
<tr>
<th>Concn of peptide (μM)</th>
<th>% Hemolysis by peptide†</th>
</tr>
</thead>
<tbody>
<tr>
<td>HNP-1</td>
<td>HNP-1ΔC</td>
</tr>
<tr>
<td>20</td>
<td>15</td>
</tr>
<tr>
<td>40</td>
<td>20</td>
</tr>
</tbody>
</table>

†Erythrocytes (10⁷ cells/ml) were incubated with peptides in 5 mM HEPES (pH 7.4) containing 150 mM NaCl. The values reported are the averages of three independent experiments. The variation was within ±5%.
linear analogs and HNP-1 are active even in the presence of CCCP. Since CCCP would discharge the pH gradient and destroy the proton motive force across the inner membrane, it appears that HNP-1 and the linear analogs can exert their antibacterial activity even in the absence of a proton motive force. It has been observed that the susceptibility of Neisseria gonorrhoeae to the human defensin HNP-2 is not abolished in the presence of CCCP (40). The bactericidal activity of HNP-1 against S. aureus is also not dependent on the transcytoplasmic membrane electrical potential (52). Also, the loss of activity against P. aeruginosa in the presence of 100 mM NaCl is not observed when CCCP is present. The concentration of cytoplasmic Na⁺ is maintained by a Na⁺-proton antiporter driven by the proton motive force (10). CCCP inhibits the antiporter function (10, 33). In the presence of a high NaCl concentration in the external medium, the membrane potential does not favor associations of the peptides with the membrane of P. aeruginosa. However, when the membrane potential is dissipated by the addition of CCCP, the high salt concentration is no longer inhibitory. The linear peptides appear to exert their antibacterial activity in the same way as polymyxin B (8). As in the case of polymyxin B (8), the antibacterial activity of the linear peptides is inhibited by Mg²⁺. Tam and coworkers have engineered a salt-insensitive rabbit α-defensin with end-to-end cyclization (56). They have speculated that the clustering of cationic charges as a result of cyclization may promote salt insensitivity. In the absence of disulfide constraints, it is unlikely that a similar clustering of charges occurs in HNP-1C18. Hence, the linearization of defensin may be a good strategy to generate salt-insensitive antibacterial peptides.

The hemolytic assay indicates a reduction in hemolysis of human erythrocytes with the analog HNP-1C18 and an increase with HNP-1C18A. This may be due to decreased hydrophobicity in the case of HNP-1C18 and a comparatively increased helicity as well as hydrophobicity for HNP-1C18A, as observed for linear host defense antibacterial peptides (30, 37, 41). Earlier studies have shown that the microbicidal activity of HNPs is affected by the presence of serum (7, 55). We have also observed that serum inhibits the antibacterial activity of HNP-1. The HNP-1 analogs, particularly the truncated ones, show bactericidal activity even in the presence of human serum.

In summary, our studies have provided new insights into the structure-function relationship of HNP-1. While it has been demonstrated that all three disulfides or the order of connectivity is not an essential feature for activity in defensins (27, 29, 49), our results indicate that linear peptides without cysteines can still possess antibacterial activity, although it is 10- to 20-fold lower than that of HNP-1. The antibacterial activity is only marginally inhibited by high concentrations of NaCl, especially against E. coli and S. aureus. The peptides are active even in the absence of a proton motive force. The generation of linear defensins would be easier than that of disulfide-linked defensins, although defensins have been generated by chemical synthesis and recombination methods (6, 9, 17, 27–29, 36, 49–51, 56). Hence, linear defensins could have potential for development as therapeutic agents.

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REFERENCES


