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Periathamby A. Raj
Marquette University

K. J. Antonyraj
Marquette University

T. Karunakaran
Marquette University

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Large-scale synthesis and functional elements for the antimicrobial activity of defensins

Periathamby Antony Raj
Division of Periodontics, School of Dentistry, Marquette University
Milwaukee, WI

Kavitha J. Antonyraj
Department of Oral Biology and
Periodontal Disease Research Center, State University of New York at Buffalo
Buffalo, NY

Thonthi Karunakaran
Department of Oral Biology and
Periodontal Disease Research Center, State University of New York at Buffalo
Buffalo, NY

Human neutrophil defensins, and their analogues incorporating anionic, hydrophobic or cationic residues at the N- and Ctermini, were synthesized by solid-phase procedures. The synthetic defensins were examined for their microbicidal activity against *Candida albicans*, two Gram-negative bacteria (*Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*) and two Gram-positive bacteria (*Streptococcus gordonii* and *Streptococcus mutans*). The human neutrophil peptide 1 (HNP1) and HNP2 were found to be potent candidacidal agents. HNP3, which differs by one amino acid at the N-

Biochemical Journal, Vol. 347, No. 3 (May 2000): pg. 633-641. DOI.

terminus of its sequence, was totally inactive. The Gram-negative bacteria *A*. *actinomycetemcomitans* and *P*. *gingivalis* and the Grampositive bacteria *S*. *gordonii* and *S*. *mutans* were insensitive to human defensins. However, the insertion of two basic residues, such as arginine, at both the N-terminus and the C-terminus of HNP2 significantly enhanced antifungal and antibacterial activity. The addition of anionic residues, such as aspartic acid, at the N- and C-termini rendered the molecule totally inactive. The presence of two hydrophobic amino acids, such as valine, at the N-terminus of HNP2 and of two basic arginine residues at its C-terminus resulted in molecules that were optimally active against these oral pathogens. The results suggest that the N- and C-terminal residues in defensin peptides are the crucial functional elements that determine their microbicidal potency. The three-dimensional structure of all defensins constitutes the same amphiphilic β-sheet structure, with the polar face formed by the N- and C-terminal residues playing an important role in defining microbicidal potency and the antimicrobial spectrum. The enhance microbicidal activity observed for defensin peptides with two basic residues at both the N- and C-termini could be due to optimization of the amphiphilicity of the structure, which could facilitate specific interactions with the microbial membranes.

Key words: antimicrobial agents, antiparallel β-sheet peptides, cysteine-rich cationic polypeptides, defensin analogues, peptide disulphides.

Introduction

The defensins comprise a group of cysteine-rich cationic antimicrobial polypeptides that have been identified and characterized in animals (including humans), plants and insects. They are composed of 29-42 amino acids and contain three disulphide bonds, formed by three pairs of cysteine residues [1-4]. Defensins have been isolated from various tissue sources, such as mammalian trachea, intestine, tongue and skin, and human plasma, oral mucosa, salivary glands, saliva and ocular surface [5-13]. Defensins are involved in the oxygen-independent mechanism of the host immune response [14]. The `classic' or α-defensins were the first group of defensins to be isolated from human and animal phagocytes, and they comprise a large subfamily of polypeptides containing 29-36 residues [3,15]. The more

recently isolated β-defensins are more basic and longer (34-42 residues), and are expressed in various mucosa and epithelial cells [5,16]. The tri-disulphide motifs in α- and β-defensins differ [16,17].

Defensins have been reported to exhibit powerful antibacterial, antifungal and antiviral activity against a wide variety of microorganisms [17-19]. The antibiotic activity of defensins has generally been attributed to their effects on microbial membranes [19,20]. They have been reported to interact with lipopolysaccharides in Gram-negative bacteria, polysaccharides (teichoic acid) in Gram-positive bacteria, and phospholipids [17]. The three-dimensional structures of a few defensin peptides have been reported in the crystalline state [21] and in solution [22-26]. These studies have shown that defensins exist as triple-stranded amphiphilic β-sheet structures in aqueous solution, and that the amphiphilicity of the molecule might facilitate their spontaneous insertion into microbial membranes, thereby disrupting membrane permeability and integrity [24,25]. Recent studies have shown that the mechanism of antibiotic activity of defensins could involve both cell membrane and intracellular targets [27].

Variations exist in the sequences of α-defensins isolated from different biological sources in humans and animals, particularly at the N-and C-termini and in the middle portion, as shown in Figure 1. The sequence identity, the structure reported for a few α-defensins and the conserved disulphide constraints suggest that the overall three-dimensional structure of α-defensins is maintained, despite variations in individual sequences. However, the differences in α-defensin sequences have been found to be reflected in their specificity for microbes and their spectrum of antimicrobial activity. For instance, human neutrophil defensins HNP1 (human neutrophil peptide 1) and HNP2 have been reported to be potent candidacidal agents. However, HNP3, which differs only in one amino acid at the N-terminus, has been found to be least active against *C. albicans* [28]. HNP1 and HNP2 have been found to be equipotent in their ability to kill *Capnocytophaga* species, and their activity has been reported to be significantly greater than that of HNP3 [29]. Even though human defensins (HNP1, HNP2 and HNP3) are not active against *Actinobacillus actinomycetemcomitans*, rabbit defensing NP1, which shows variations

Biochemical Journal, Vol. 347, No. 3 (May 2000): pg. 633-641. DOI.

at the N- and C-termini and in the middle portion of the sequence, has been reported to exert pronounced activity against *A. actinomycetemcomitans* strains [29]. Such wide functional diversity has also been observed for rat defensins of similar structure [30]. The three-dimensional structure of defensins has been reported to primarily assume a `basket-like' amphiphilic structure. The open end of the basket constituted by the N- and C-terminal residues forms the polar face, whereas the lower and bottom portion of the basket assumes the apolar face of the amphiphilic structure [21,25]. The wide functional diversity observed in parallel with variations in the sequence at the N- and C-termini seems to indicate that the residues at the polar face could be the crucial determinants of microbicidal potency and the antimicrobial spectrum. In this paper we describe a new method for the large-scale synthesis of α-defensins, and investigate the influence of their N- and C-terminal residues on their antimicrobial spectrum and microbicidal potency.

Experimental

General materials and methods

Chemicals and solvents were of the highest purity available and were used without further purification. Amino acid derivatives and *p*-benzyloxybenzyl alcohol (Wang) resins were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and Bachem (Torrance, CA, U.S.A.). The peptide sequence was assembled using a manual peptide synthesizer. HPLC was carried out on a Rainin-HPXL system interfaced to a Macintosh SE/30 computer using a Rainin Dynamax C18 column (10 mm x 250 mm) coupled to a guard column (10 mm x 50 mm) and employing acetonitrile/water [each containing 0.1% trifluoroacetic acid (TFA)] linear gradient elution with a flow rate of 2.0 ml·min^{-1}. The dried peptide was hydrolysed in sealed tubes for 24 h at 110°C using 6 M HCl in the presence of 1% phenol vapour, and amino acid analysis was performed on a Perkin—Elmer 420A derivatizer and analyser system. The amino acid sequence of the peptide (~1 nmol) was checked by sequence analysis using a Perkin—Elmer Model 477A protein sequencer. Fast-atom bombardment (FAB) mass analysis was carried out on an M-Scan VG Analytical ZAB 2-SE high-field mass spectrometer. A caesium ion gun was used to generate ions for the

Biochemical Journal, Vol. 347, No. 3 (May 2000): pg. 633-641. DOI.

acquired spectra, which were recorded using a PDP 11-250J data system.

Peptide synthesis and purification

The linear defensin sequences (HNP1, HNP2, HNP3, HNPa, HNPb, HNPc, HNPd and HNPe) shown in Figure 1 were assembled by standard solid-phase procedures [31] using Wang resins linked to the C-terminal Fmoc (9-fluorenylmethyloxycarbonyl) amino acid (0.2-0.3 mmol/g). The general scheme employed for the synthesis of defensin peptides is shown in Scheme 1. The side-chain protecting groups were the following: Glu, Thr and Tyr, O-tButyl; Trp, tBoc (t-butyloxycarbonyl); Arg, *N*-2,2,5,7,8-pentamethylchroman-6-sulphonyl; N- and C-terminal Cys, Trityl ; Cys-4 and Cys-19, S-tButyl; Cys-9 and Cys-29, acetamidomethyl (Acm). The coupling reactions were carried out with a 3-fold excess of Fmoc amino acid in a mixture (50%, v/v) of *N*,*N*-dimethylformamide and dichloromethane using dicyclohexylcarbodi-imide as the coupling reagent in the presence of 1-hydroxybenzotriazole. Completion of the coupling reaction was monitored by Kaiser's test [32]. The Fmoc group on the N-terminus was deprotected in 10 min with a 25% solution of piperidine in dimethylformamide. The protected peptidyl resin was then treated with 90% (v/v) TFA in dichloromethane containing thioanisole (2.5%) and dimethyl sulphide (2.5%). When the peptide was cleaved from the resin, the crude linear peptide (93-95%) was obtained. The N- and C-terminal Cys residues with the S-Trityl protecting group were deprotected at the same time when the peptide was cleaved from the resin by treatment with TFA, whereas the Acm and S-tButyl protecting groups on Cys-4/Cys-19 and Cys-9/Cys-29 respectively were intact. The crude linear peptides were purified by reverse-phase HPLC on a Rainin Dynamax-60A C18 column (10 mm x 250 mm) coupled to a guard column (10mm x 50 mm) employing acetonitrile/water (each containing 0.1% TFA) linear gradient elution (10-50% acetonitrile in water over a period of 45-50 min) with a flow rate of 2.0 ml·min^{-1} (detection at 230 nm and 280 nm). The fractions of each resolved peak were pooled, lyophilized and subjected to amino acid and sequence analyses, which identified and verified the authentic peptide sequence.

Biochemical Journal, Vol. 347, No. 3 (May 2000): pg. 633-641. DOI.

Disulphide-bond formation between the N- and C-terminal cysteine residues

The HPLC-purified linear peptide (0.45 g) with free thiol groups on the N- and C-terminal cysteine residues was suspended in 2.5 litres of water to bring the concentration of the peptide to ~0.05 mM, and the pH was brought to 6.8 by the addition of 0.1M ammonium acetate solution. A 0.2 M solution of potassium ferricyanide was added in minute drops over a period of 12 h while maintaining the pH at 6.8-7.0, until the yellow colour persisted. A 5 ml excess of potassium ferricyanide was then added to ensure oxidation. The pH of the solution was maintained at 6.8-7.0 by using 0.1 M ammonium acetate and 20% acetic acid. The solution was concentrated to 50 ml at 40-45°C and then passed through an activated thiol-Sepharose 4B resin (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) column (10 mm x 75 mm) to remove any peptide containing free thiol groups. The peptide from the column was eluted with 0.1 M ammonium acetate buffer (pH 6.8). The aqueous peptide solution was pooled and lyophilized. The dried material was purified by reverse-phase HPLC using the semi-preparative Rainin Dynamax-60A C18 column, as described above. The fractions of the major well resolved peak were pooled, lyophilized and subjected to amino acid and sequence analyses. The amino acid composition, sequence and mass-spectral analysis of the purified material verified the correct sequence. The yield of the purified product was found to be 66-68%.

Disulphide-bond formation between Cys-4 and Cys-19

The purified material obtained as described above was dissolved in trifluoroethanol (300 ml) and reduced with 12.3 ml of tributylphosphine at room temperature for 3 h to deprotect the S-tButyl groups on Cys residues at positions 4 and 19 (Scheme 1). The reaction mixture was then concentrated to a small volume (2 ml) and the product was precipitated by the addition of diethyl ether. The separated solid was filtered and washed with ether (3 x 25 ml) and dried. The dried material (~0.35 g) was suspended in 2.0 litres of water to bring the peptide concentration to 0.05 mM and then subjected to oxidation using a 0.2 M solution of potassium ferricyanide at pH 6.8, and the peptide was isolated as described in the previous

section. The dried material was subjected to reverse-phase HPLC and the fractions of the major resolved peak were pooled and analysed. The amino acid composition, sequence and mass-spectral analyses identified the peptide. The yield of the purified product was found to be 56-58%.

Disulphide-bond formation between Cys-9 and Cys-29

The total material obtained after the formation of the second disulphide bond (~0.3 g) was dissolved in a mixture of water/methanol (6 :1, v/v) so that the peptide concentration was 0.05 mM. The peptide solution was stirred while adding a 1 mM solution of iodine (125 ml) in methanol dropwise during 1 h. The red solution was cooled to 0 °C and treated with 1 M sodium thiosulphate until the red colour disappeared. The methanol was removed by rotary evaporation at the water aspirator and then the material was lyophilized. The crude material obtained was dissolved in 50 ml of water and passed through an activated thiol-Sepharose B column (5 mm x 75 mm); the column was then washed with (3 x 25 ml) 0.1 M ammonium acetate buffer at pH 6.8. The aqueous solution was lyophilized. The dried material was purified by reverse-phase HPLC as described above. All fractions of the well-resolved peaks were pooled and lyophilized. The sequence, mass-spectral and amino acid analyses of the major component identified it as the synthetic defensin sequence. The identity of the synthetic peptide was assessed by comparing its HPLC retention time, CD spectrum and mass spectrum with those of the native defensin peptides isolated from human neutrophils. The yield of the product after HPLC purification was found to be 45-48%.

Organisms and growth conditions

Candida albicans strain ATCC 28366 obtained from the American Type Culture Collection (Rockville, MD, U.S.A.) and a clinical isolate from a patient with denture-induced stomatitis (DIS) were used. The identity of the clinical isolate was verified by the Yeast System (Flow Laboratories, McLean, VA, U.S.A.). Organisms were streaked on to Sabouraud Dextrose Agar plates (Difco Laboratories, Detroit, MI, U.S.A.) and maintained at 4°C. One colony of *C. albicans* from this plate was inoculated into 10 ml of yeast synthetic growth medium

Biochemical Journal, Vol. 347, No. 3 (May 2000): pg. 633-641. DOI.

containing sucrose, salts and biotin [33], and incubated for 48 h at 25°C in a shaker rotating at 200 rev./min. After this period, the population of yeast cells was in late-exponential-phase growth. Cell morphology was determined by phase-contrast microscopy and found to be uniformly blastospores. Subcultures were done every 3 days by inoculating 100 μl of 3-day-old culture into 10 ml of fresh synthetic medium.

Measurement of candidacidal activity

The antifungal activity of the synthetic human neutrophil defensins and their analogues was assessed *in vitro* against *Candida albicans*. The loss of cell viability is expressed as a function of peptide concentration. The assay was performed as described for the candidacidal activity of salivary histatin 5 and its fragments [34]. We refer to the rapid candidacidal action of peptides as a loss of viability of cells, since inability of the yeast to replicate following removal of peptides is considered to represent a non-viable cell. Thus the assay used in the present study measured only fungicidal activity. Cells from 48 h cultures were harvested, washed and resuspended to a concentration of 5×10^7 cells/ml in 0.01 M sodium phosphate buffer (pH 7.4). Stock solutions of peptides (range 0.05-200 μM) were made in 0.01 M sodium phosphate buffer. Peptide concentrations were determined by amino acid analysis. A microassay system was utilized in which 100 μl of the stock solution of test peptide was incubated with 100 μl (5×10^6 cells) of yeast suspension in sterile glass test tubes at 37°C for 1.5 h. Control suspensions consisting of 100 μl of cells and 100 μl of sodium phosphate buffer were incubated simultaneously. Each tube of cell suspension was shaken for 10 s every 15 min during this time. A 100 μl aliquot of cells was removed from the tube, diluted to 5×10^2 cells/ml in sodium phosphate buffer and then vortex-mixed. Aliquots of 0.5 ml of each suspension were spread on to plates (15 mm x 100 mm) of Sabouraud Dextrose Agar in duplicate. *C. albicans* was grown overnight at 37°C, and the candidacidal activity of the test peptide was assessed as the ratio of colonies per test plate to the number of colonies on control (no peptide) plates, and expressed as a percentage.

Biochemical Journal, Vol. 347, No. 3 (May 2000): pg. 633-641. DOI.

Bacterial strains and cultivation

The cultivation of bacterial strains was carried out as described in our previous publications [35,36]. Briefly, *Porphyromonas gingivalis* strains W50 and 381 were maintained on blood agar plates containing tryptic soy agar (Difco Laboratories) supplemented with hemin (1%), menadione (0.02%) and de-fibrinated sheep blood (5%). *Actinobacillus actinomycetemcomitans* strains Y4 and 67 were maintained in tryptic soy agar plates containing yeast extract (0.5%). *Streptococcus mutans* GS5 and *Streptococcus gordonii* DL1 were grown on Todd-Hewitt agar plates. For liquid culture, these bacteria were grown in their respective broth. All experiments were carried out in an anaerobic chamber (85% N_2, 10% H_2 and 5% CO_2) (Coy Laboratory Products), and bacteria were confirmed routinely by standard microbiological methods.

Bactericidal assay

The bactericidal assays were performed as described previously [35,36]. The antimicrobial activity of defensins was tested against *P. gingi.alis* (W50 and 381), *A. actinomycetemcomitans* (Y4 and 67), *S. gordonii* (GS5) and *S. mutans* (DL1). Bacterial strains were grown in freshly prepared broth to 10^9 cells and subsequently diluted to 10^6 cells/ml in 10 mM sodium phosphate buffer (pH 7.4). Cells were harvested and washed twice in sodium phosphate buffer. Washed cells were incubated with various concentrations of the peptide in a total volume of 200 μl at 37°C in an anaerobic chamber for 1.5 h as described previously [36]. The cells were diluted to 10^3 cells/ml and plated on the respective agar plates. The plates were incubated anaerobically at 37°C for 6 days, and the number of viable *P. gingivalis* colonies was calculated in comparison with the control. In the case of *A. actinomycetemcomitans*, *S. gordonii* and *S. mutans*, the colonies were counted after 3 days and compared with the control plates. The bactericidal activity of the peptide was expressed as [1—(cell survival after peptide incubation)/(cell survival in buffer alone)] x 100, which represents the percentage killing of the bacterial cells. The loss of cell viability is expressed as a function of peptide concentration. The peptide concentration that induced 90% of the maximal loss of cell viability (EI_{90}) was determined from the concentration-effect curves.

Biochemical Journal, Vol. 347, No. 3 (May 2000): pg. 633-641. DOI.

Purification of native human neutrophil defensins

In order to determine the identity of the synthetic molecules and to verify the formation of correct disulphide bonds between the respective cysteine residues, we purified a few native human defensins. Human neutrophils were isolated from whole blood collected from healthy human volunteers, as described in our previous publication [37]. Cell purity (90-95% neutrophils) was assessed by Wright-Giemsa staining. Leucocytes were homogenized, centrifuged and chromatographed using the method reported previously [38]. The mixture of HNP1, HNP2 and HNP3 obtained after initial chromatographic separation was subjected to reverse-phase HPLC on a Rainin Dynamax-60A semi-preparative C18 column (10 mm x 250 mm) coupled to a guard column (10 mm x 50 mm) employing acetonitrile/water (each containing 0.1% TFA) linear gradient elution (flow rate 2.0 ml·min^{-1}). Processing of 2.8 x 10^9 cells yielded ~4.5, 4.0 and 4.2 mg of HNP1, HNP2 and HNP3 respectively. The homogeneity of peptides was examined by reverse-phase HPLC on a Rainin Dynamax-60A analytical C18 column (4.6 mm x 250 mm) coupled to a guard column (4.6 mm x 15 mm) employing acetonitrile/water (each containing 0.1% TFA) linear gradient elution (flow rate 0.8 ml·min^{-1}) with detection at 230 nm and 280 nm. The native molecules were further verified by SDS/PAGE, cationic PAGE, amino acid analysis and FAB-MS analysis.

CD spectroscopy

CD measurements were carried out on an Aviv 62DS spectro-polarimeter equipped with a thermostatic temperature controller and interfaced to an IBM microcomputer. CD spectra were recorded in a quartz cell of 0.05 mm pathlength at 30°C between 180 and 250 nm, using a 1.0 nm bandwidth and a scanning rate of 10 nm/min with a wavelength step of 0.2 nm and a time constant of 2 s. The region from 250 to 400 nm was recorded using a 1 mm pathlength cell. Peptides were dissolved in 10 mM sodium phosphate buffer at pH 7.2 to a concentration of 0.1-0.3 mM. CD band intensities were determined as molar ellipticity ($[\theta]_M$), in deg·cm^{2}·dmol^{-1}.

Biochemical Journal, Vol. 347, No. 3 (May 2000): pg. 633-641. DOI.

RESULTS AND DISCUSSION

Synthesis of defensin peptides

The method described for the synthesis of defensin peptides yielded nearly 17-20% of final purified product. The identity of the synthetic molecules was verified by comparing the SDS/PAGE and cationic PAGE profiles and the CD and FABMS spectra with those of the native peptides. The FAB mass spectra of synthetic and native HNP1 showed the molecular ion cluster centered at *m*/*z* 3442, which is consistent with the molecular mass. The intensity of the disulphide n-σ* CD bands that generally occur between 250 and 320 nm is highly sensitive to the dihedral angle, flexibility and chirality of the disulphide chromophore [39,40]. Hence we examined the CD bands in this region for both synthetic peptides and native molecules. The CD spectra of synthetic and native HNP1 in the region 250-400 nm, which reflects the contribution of disulphide n-σ* and of aromatic residues, were comparable. The CD spectra of the synthetic and native HNP1 in the region between 180 and 250 nm, which represents the peptide secondary structure, were almost identical. Analysis on SDS/12.5%-PAGE indicated that the native and synthetic molecules migrated to the same extent. In addition, the microbicidal activity of synthetic and native HNP1 (described in subsequent sections) were also comparable. Collectively, the data suggest that the synthetic molecules are identical with the native peptides.

The formation of the three distinct disulphide bonds between the respective cysteine residues is the major task in the synthesis of defensins. Simultaneous oxidation of the six thiol groups might lead to the formation of undesired disulphide bridges and polymerized side-products, leading to a very poor yield of defensins. We found a high percentage of polymerized sideproducts when all six thiol groups were oxidized simultaneously, even at very low peptide concentration. The formation of undesired polymerized side-products during the simultaneous oxidation of all six or four cysteine residues has been reported [41], although it might be possible to purify trace amounts of the correct peptide from these side-products [42]. Therefore we rationally selected three different protecting groups, namely Trityl (for N- and C-terminal cysteines), S-tButyl (for Cys-4 and Cys-19) and

Biochemical Journal, Vol. 347, No. 3 (May 2000): pg. 633-641. DOI.

Acm (for Cys-9 and Cys-29), for the three pairs of cysteine residues that form the disulphide bonds. The three thiol protecting groups were deprotected under different conditions in three different stages and oxidized to form the respective disulphide bridges, as described in Scheme 1. Reverse-phase HPLC traces of the crude peptide with one disulphide bond between Cys-1 and Cys-30, of that with two disulphides between Cys-1 and Cys-30 and between Cys-4 and Cys-19, of the crude HNP1 and of the purified HNP1 are provided in Figure 2.

The scheme employed for the synthesis of defensins eliminates the formation of any undesired disulphide bonds.Wefirst cyclized the peptide by forming the disulphide bridge between the N- and C-terminal cysteines, thereby allowing the other cysteine residues to achieve a favourable orientation in space to ease the formation of disulphide bonds between the respective cysteine residues. The three-dimensional structure of defensins clearly suggests that the folding of the C-terminus towards the N-terminus brings the other two pairs of cysteine residues into close proximity, favouring the formation of the disulphide bonds [21,25]. Hence we chose to form the first disulphide bridge between the N- and C-terminal Cys residues. The trace amounts of any free thiolcontaining sequences at each stage of formation of disulphide bonds were removed by treatment with a column of activated thiol-Sepharose 4B resin (Amersham Pharmacia Biotech), which selectively adsorbs even trace amounts of thiol-containing sequences. The use of activated thiol-Sepharose 4B resin at each stage of formation of disulphide bonds eliminates the accumulation of any thiol-containing products. The oxidation of cysteine residues in all three different stages was carried out in solution at a peptide concentration of 0.05 mM, with vigorous stirring to minimize polymerization. Chemical synthesis of a few defensing peptides using only two different protecting groups, and formation of disulphide bonds in two stages, has been reported previously, with a final yield of only 7% [41]. The simultaneous removal of the protecting groups on four cysteine residues and oxidation of the thiol groups might have led to the formation of undesired side-products, and accordingly lowered the percentage yield of the products. The method described in this paper, using three distinct thiol protecting groups and

Biochemical Journal, Vol. 347, No. 3 (May 2000): pg. 633-641. DOI.

forming disulphide bonds in three different stages, is very efficient, giving a high yield (17-20%) of pure defensin peptides.

Candidacidal activity

The efficacy of defensin peptides in inducing loss of viability of *Candida albicans* was measured at various peptide concentrations (Figure 3). The data provided in Figure 3 are for the DIS strain; *C. albicans* strain 28366 has been found to be 10-20% more susceptible to these peptides. The percentage loss of viability of *C. albicans* induced by synthetic HNP1 at various concentrations (Figure 3) is comparable with that observed for the native molecule, suggesting identity of the molecules. The EI_{90} value for HNP1 was ~14 μM. The candidacidal activity of HNP2 was nearly 5-fold lower than that of HNP1. The EI_{90} for HNP2, which lacks the N-terminal Ala residue, was ~65 μM, indicating the importance of the residues preceding cysteine at the N-terminus. The *Candida albicans* strains were insensitive to HNP3, which has a negatively charged Asp residue at this position. The results are consistent with the previously reported candidacidal activity of human defensins [28], although the *C. albicans* strains and the assay procedures used were different.

HNPa, which has two Asp residues at both the N- and the C-terminus, was totally inactive against *C. albicans*, suggesting that negatively charged side chains at the termini render the molecule inactive. Compared with HNP1, a 5-fold increase in candidacidal activity was observed when two polar amino acids, such as threonine, were present at the N- and C-termini, as in the case of HNPc (EI_{90} ~ 3 μM). HNPb, which has two hydrophobic valine residues at the N-terminus and two cationic arginine residues at the C-terminus, had 2-fold greater candidacidal activity (EI_{90} ~ 6 μM). Compared with HNPb, a 2-fold increase in candidacidal activity was observed for HNPd (EI_{90} ~ 1.5 μM), which has two threonine residues in place of valines at the N-terminus. The presence of two arginine residues at both the N- and the C-terminus enhanced candidacidal activity significantly : HNPe (EI_{90} ~ 0.5 μM) was 10-fold more potent than HNPb, and markedly (30-fold) more active than HNP1. Collectively, the candidacidal activities of human defensins and their analogues suggest that polar and charged side chains at the N- and C-termini, respectively

Biochemical Journal, Vol. 347, No. 3 (May 2000): pg. 633-641. DOI.

preceding and succeeding the cysteine residues, appear to be functionally very important.

Bactericidal activity

The microbicidal activities of human neutrophil defensins and their analogues against the Gram-negative *P. gingivalis* and *A. actinomycetemcomitans* strains are summarized in Figures 4 and 5 respectively. *P. gingivalis* (strains W50 and 381) and *A. actinomycetemcomitans* (strains Y4 and 67) were insensitive to human neutrophil defensins HNP1, HNP2 and HNP3 up to a peptide concentration of 200 μM. The strains were also insensitive to the analogue HNPa, which has two Asp residues at both the N- and C-termini. Both *P. gingivalis* and *A. actinomyctemcomitans* strains were highly sensitive to the analogues HNPb, HNPc, HNPd and HNPe, which have two additional residues at the N- and C-termini of human defensing peptides (Figures 4 and 5). HNPb, which has two hydrophobic valines preceding the cysteine at the N-terminus and two arginines following the cysteine at the C-terminus, was microbicidal against both *P. gingivalis* and *A. actinomycetemcomitans* (Figures 4 and 5). The EI_{90} for this peptide was ~ 25 μM. This HNPb peptide is similar to rabbit defensin peptide NP1, which has two valine and two arginine residues at the N- and C-termini respectively. However, NP1 has variations in the middle portion of the sequence as compared with human defensins HNP1, HNP2 and HNP3 (Figure 1). Therefore the comparable cidal activities of HNPb and NP1 seem to indicate that the residues at the N- and C-termini of defensin peptides may be functionally very important for microbicidal activity and specificity. The microbicidal activity of NP1 against *A. actinomycetemcomitans* reported previously is comparable with our present results with HNPb [29].

The presence of two polar side chains, such as threonine residues, at the N- and C-termini of human defensins also elicited similar antimicrobial activity against these two Gram-negative bacteria (Figures 4 and 5). However, the presence of threonines appeared to increase the cidal potency slightly. The EI_{90} for HNPc was ~ 14 μM, suggesting an approx. 2-fold increase in microbicidal activity as compared with HNPb. The presence of two threonine residues at the N-

Biochemical Journal, Vol. 347, No. 3 (May 2000): pg. 633-641. DOI.

terminus and two arginine residues at the C-terminus, as in the case of HNPd (EI_{90} ~ 5 μM), enhanced the activity 5-fold as compared with HNPb (Figures 4 and 5). The addition of two polar and charged arginine residues at the N- and C-termini, as in the case of HNPe (EI_{90} ~ 1 μM), enhanced microbicidal activity 25-fold as compared with HNPb (Figures 4 and 5).

Thus, even though HNP1 and HNP2 are potent antifungal agents against *C*. *albicans*, they are minimally active against the Gram-negative bacteria *P*. *gingivalis* and *A*. *actinomycetemcomitans*. The insertion of two valine side chains at the N-terminus and two arginine residues at the C-terminus of HNP2 elicits antibacterial activity against these organisms. This suggests that the additional N- and C-terminal side chains are indeed necessary to elicit optimal antibacterial activity against these organisms. Comparison of the activities of HNPb, HNPc, HNPd and HNPe suggests that the antibacterial activity tends to increase with the increase in polarity caused by the additional residues at the N- and C-termini. The data suggest that the presence of two additional polar and charged side chains preceding and following the cysteine residues at the N- and C-termini respectively of human defensins is required for optimal antibacterial activity.

Streptococcus gordonii (strain GL1) and *Streptococcus mutans* (strain GS5) were also insensitive to the human neutrophil defensins HNP1, HNP2 and HNP3 and the analogue HNPa. However, both strains were sensitive to HNPb, HNPc, HNPd and HNPe (Figure 6), at slightly higher concentrations than were required for activity against the Gram-negative bacteria *P*. *gingi*.*alis* and *A*. *actinomycetemcomitans*. At a concentration of 55 μM, 90% killing was observed for HNPb. HNPc (EI_{90} ~ 27 μM), which has two polar threonine residues at both the N- and C-termini, was slightly (2-fold) more active than HNPb. HNPd, which has two threonines and two arginines at the N- and C-termini respectively, was nearly 5-fold more active (EI_{90} ~ 15 μM) than HNPb (Figure 6). The presence of two arginines at both the N- and C-termini caused HNPe to be significantly more active (EI_{90} ~ 6 μM) than HNPb.

The trend for the increase in microbicidal activity with sequence alterations at their N- and C-termini appears to be the same for Gram-positive and Gram-negative organisms (Figure 6). In other words, the

Biochemical Journal, Vol. 347, No. 3 (May 2000): pg. 633-641. DOI.

antibacterial activity of defensin peptides increases with an increase in the cationic charge and polarity of the residues preceding and following cysteine residues at the N- and C-termini respectively. The slight differences in the cidal potencies of the defensin analogues observed for Gram-negative as compared with Gram-positive organisms could be due to differences in membrane structure and composition of lipids. Defensin-like peptides have been reported to have better antimicrobial activity against Gram-negative bacteria, and their activity has been related to their ability to permeabilize the outer membrane [43,44].

Molecular structure and antimicrobial activity

α-Defensins isolated from various biological sources in humans and animals show conservation of the three disulphide bridges and retain nearly 50% sequence identity. There are variations in the sequences at the N- and C-termini and in the middle portion of these peptides. These differences have been found to be reflected in their antimicrobial spectrum and specificity of organisms for which they are cidal [14,28]. This functional diversity observed for similarly structured molecules raises interest in identifying the crucial residues responsible for their specificity and antimicrobial potency. Hence we selected the human neutrophil defensins HNP1, HNP2 and HNP3, which have identical sequences from the N-terminal cysteine to the Cterminal cysteine, and examined analogues with minor variations only at the N- and C-termini, to assess unambiguously the influence of terminal residues on their antimicrobial spectrum and antimicrobial potency.

HNP2, which does not have any additional residues preceding the N-terminal cysteine or following the C-terminal cysteine (Figure 1), is an optimally active candidacidal agent. The presence of an Ala residue at the N-terminus enhances the candidacidal activity, as observed in the case of HNP1 (Figure 3). On the other hand, the presence of a negatively charged Asp residue at this position (HNP3) renders the molecule totally inactive against *C*. *albicans*. HNP1, HNP2 and HNP3 do not exhibit any microbicidal activity against the Gram-negative bacteria *P*. *gingivalis* and *A*. *actinomycetemcomitans* or the Gram-positive bacteria *S*. *gordonii* and *S*. *mutans*. The same effect is

Biochemical Journal, Vol. 347, No. 3 (May 2000): pg. 633-641. DOI.

observed for HNPa, which has two Asp residues at both the N- and C-termini, indicating that negative charge at these positions renders defensins inactive. However, the presence of two valines at the N-terminus and two arginines at the C-terminus renders human defensins active against the Gram-negative and Gram-positive organisms examined in this investigation. Antibacterial potency is also enhanced by the presence of polar residues, such as threonine, and significantly enhanced by the presence of positively charged arginine residues, as shown by the relative antibacterial and candidacidal activities of HNPb, HNPc, HNPd and HNPe (Figures 3-6). An arginine residue has a hydrophobic hydrocarbon chain attached to a polar and charged guanidine function. The enhanced microbicidal activity observed for HNPe, and comparison of its activity with that of the other analogues, seems to suggest that the interactions of defensins with microbes could involve hydrophobic, hydrogen-bonding and electrostatic interactions. It is also possible that an increase in the cationic charge preceding the N-terminal cysteine and following the C-terminal cysteine could be an important determinant of the antimicrobial spectrum and microbicidal potency of the defensins. However, residues with polar side chains, such as threonine, in these positions also elicit high cidal potency against the organisms tested, as shown by the microbicidal activities of HNPc and HNPd.

The three-dimensional structure of defensins established by X-ray diffraction and NMR studies contains an amphiphilic three-stranded antiparallel β-sheet structure [21,24,25]. The molecules assume a basket-like structure. The open end of the basket constituted by the N- and C-terminal residues forms the polar face, whereas the lower and bottom portion of the basket assumes the apolar face of the amphiphilic structure. The presence of two residues both preceding and following the cysteines at the N- and C-termini respectively might not alter the overall three-dimensional structure of the molecules. The enhanced activity of defensin analogues with an additional two residues at both the N- and C-termini suggests that the projecting side chains at the polar face of the amphiphilic structure could, indeed, be crucial requirements for their specificity and microbicidal activity. The increase in microbicidal activity observed for analogues with polar and charged residues at the N- and C-termini correlates with an increase in polarity of the polar face of the amphiphilic structure. Similar effects

Biochemical Journal, Vol. 347, No. 3 (May 2000): pg. 633-641. DOI.

may be observed by altering the hydrophobicity of the apolar face of the amphiphilic molecule. However, additional data are required to support this argument. Nevertheless, the present data provide support for the proposal that the N- and C-terminal residues preceding and following the cysteine residues respectively are important functional elements for the antimicrobial spectrum and microbicidal potency of defensins. This could be due to an alteration in the amphiphilicity of the molecule that might have a profound influence on microbial membranes, which differ in their structure and lipid composition depending on the micro-organisms involved.

In summary, the synthetic scheme described in this paper represents a convenient procedure for the large-scale synthesis of defensins, which have potential applications in the pharmaceutical industry. The present studies suggest that the two residues at the N- and C-termini that respectively precede and follow cysteine residues could be the determinants of the microbicidal potency and the antimicrobial spectrum of defensins. Attempts to compromise the amphiphilicity of the defensin structure by altering the polarity of the polar face appear to significantly modify specificity and microbicidal potency. Hence, by rationally choosing these residues, it should be posssible to obtain potent antimicrobial agents to target selected infectious pathogens.

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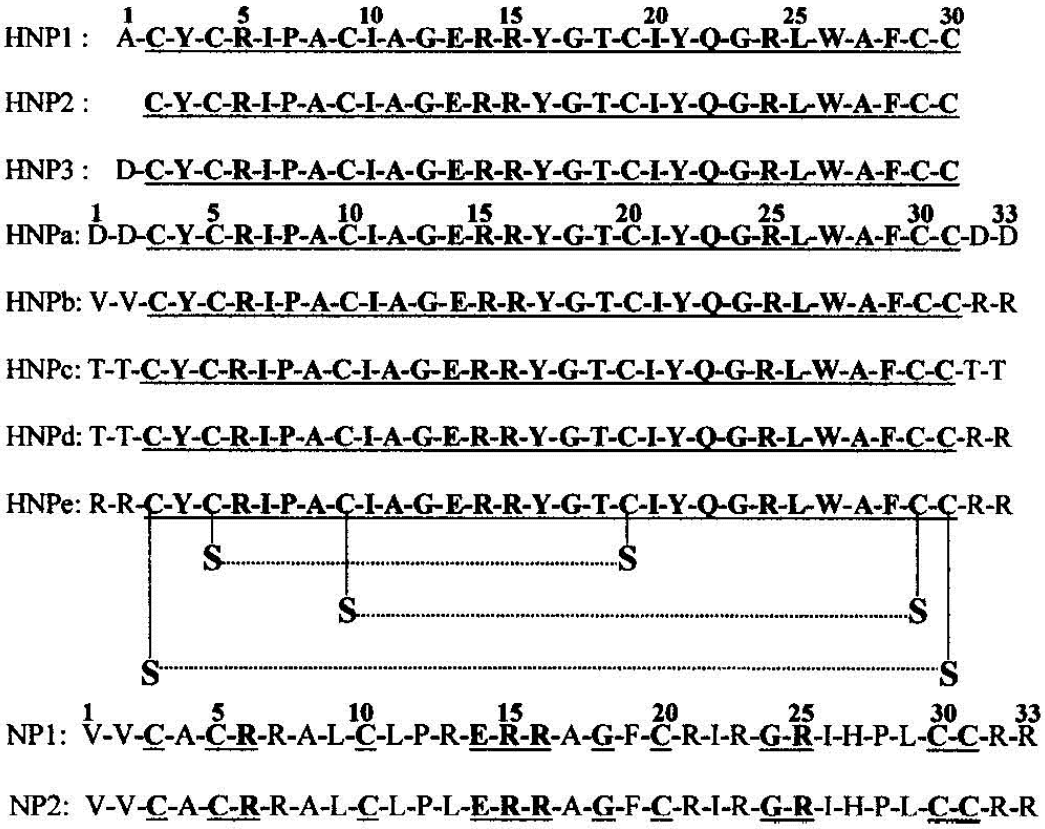
Figure 1 Amino acid sequences of human (HNP) and rabbit (NP) defensins and synthetic analogues

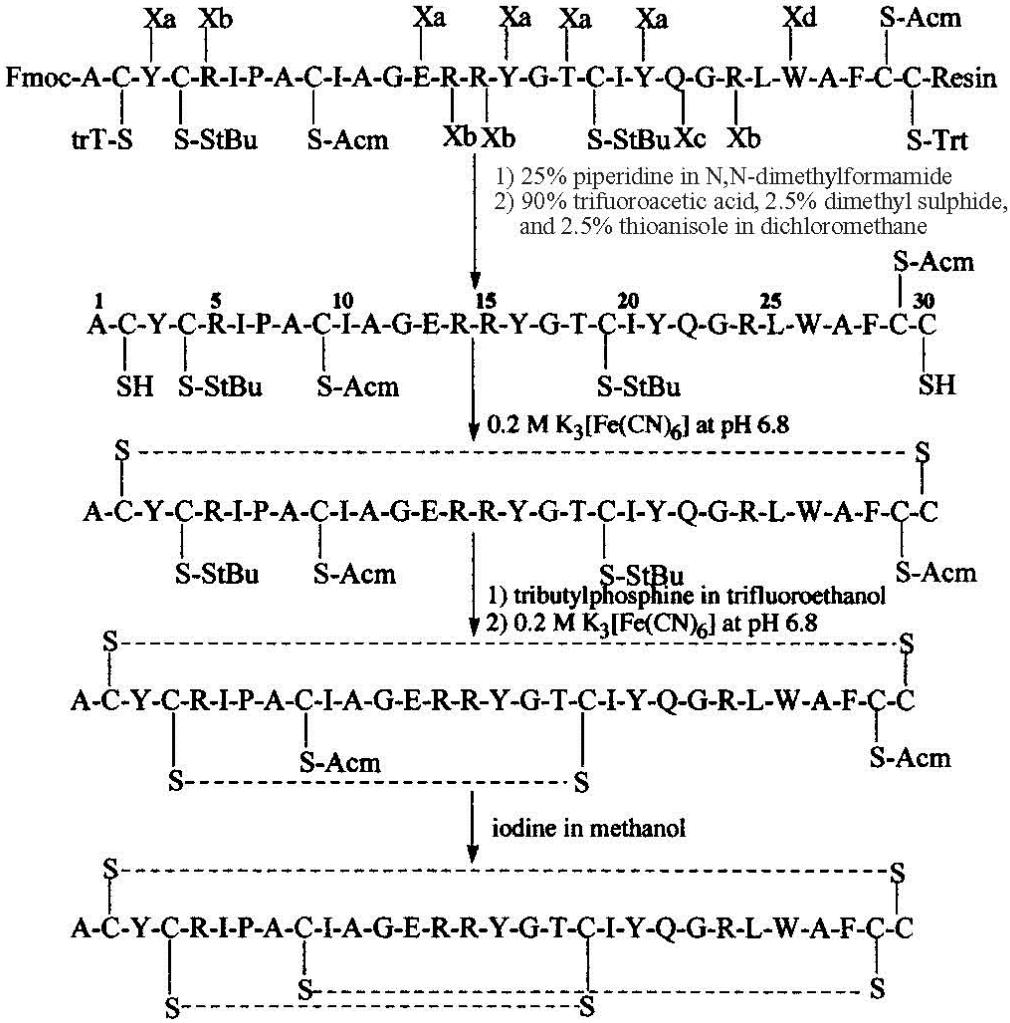
The one-letter symbols are used for amino acids in the sequences [45]. Conserved amino acid residues are in bold and underlined. The broken lines indicate the disulphide bridges between the respective cysteine residues. HNP1, HNP2 and HNP3 are native peptides; HNPa, HNPb, HNPc, HNPd and HNPe are synthetic analogues.



Biochemical Journal, Vol. 347, No. 3 (May 2000): pg. 633-641. DOI.

Scheme 1 Scheme and protecting groups employed for the synthesis of human neutrophil defensins and their analogues

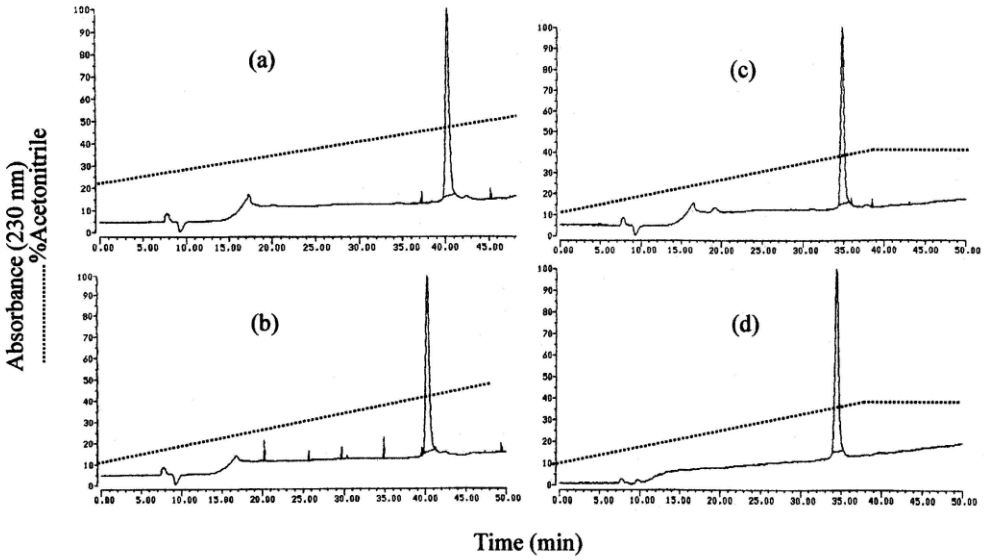
The sequence shown in the scheme is that of HNP1. tBu, tertiarybutyl ; Trt, Trityl ; Xa, O-tButyl ; Xb, *N*-2,2,5,7,8-pentamethylchroman-6-sulphonyl ; Xc, N-Trityl ; Xd, tBoc.



Biochemical Journal, Vol. 347, No. 3 (May 2000): pg. 633-641. DOI.

Figure 2 Reverse-phase HPLC of crude peptide intermediates and purified human defensin HNP1

The solvent system employed was acetonitrile/water (both containing 0.1% TFA). (*a*) Crude peptide with one disulphide bond between Cys-1 and Cys-30; (*b*) crude peptide with two disulphide bonds, between Cys-1 and Cys-30 and between Cys-4 and Cys-19 ; (*c*) crude HNP1; (*d*) purified HNP1. The gradient used is indicated by the broken line (% acetonitrile). Peptide samples (C150 lg) were loaded into an injection loop of 250 μl capacity and detected at 230 nm.



Biochemical Journal, Vol. 347, No. 3 (May 2000): pg. 633-641. DOI.

Figure 3 Concentration-dependence of loss of viability of Candida albicans (DIS) induced by human neutrophil defensins and their analogues

Left panel: a, native HNP1; b, synthetic HNP1; c, synthetic HNP2; d, synthetic HNP3. Right panel: a, HNPb; b, HNPc; c, HNPd; d, HNPe; e, HNPa. *C. albicans* cells (DIS) were incubated for 1.5 h at 37°C with defensin peptides (0.05-200 μM) as described in the Experimental section. Loss of cell viability is expressed as [1— (cell survival after peptide incubation)/(cell survival in buffer alone)] x 100. Each data point is the mean (±S.D.) of duplicate determinations from three independent experiments

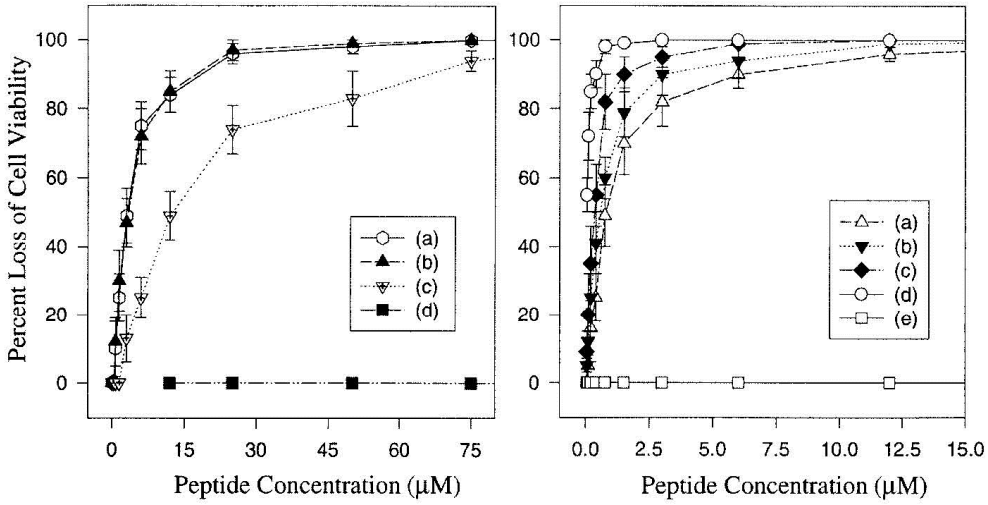
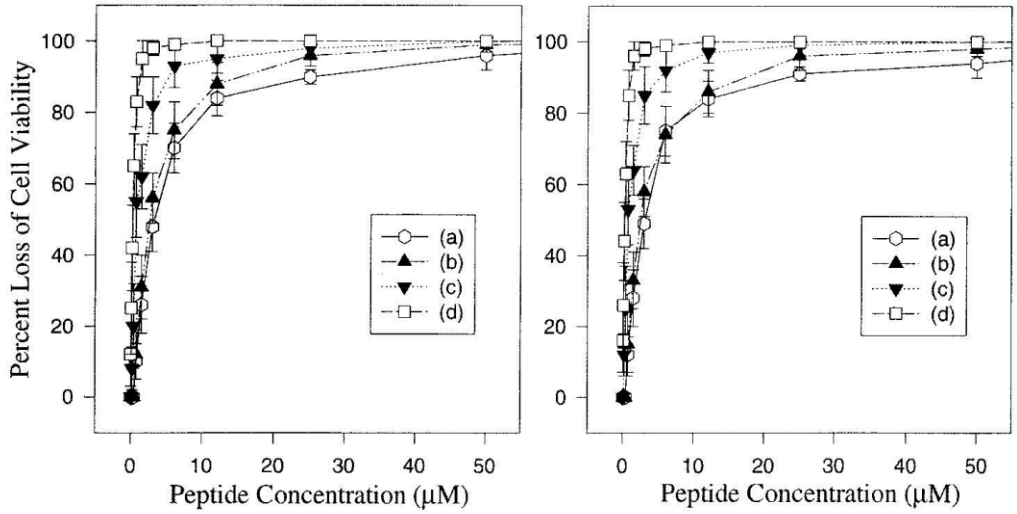


Figure 4 Concentration-dependence of loss of viability of Porphyromonas gingivalis strains W50 (left) and 380 (right) induced by human neutrophil defensing analogues HNPb (a), HNPc (b), HNPd (c) and HNPe (d)

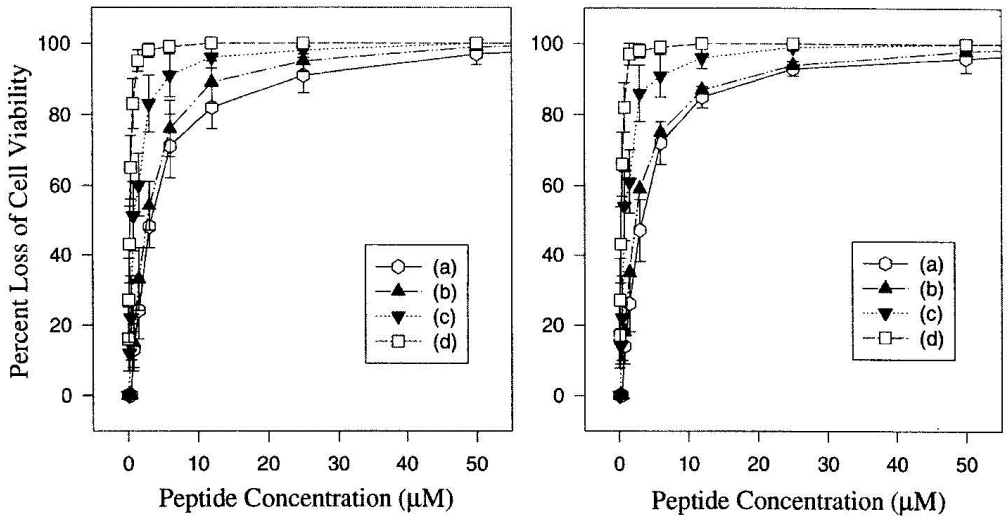
P. gingivalis cells were incubated for 1.5 h at 37°C with defensin peptides (0.05-200 μM) as described in the Experimental section. Loss of cell viability is expressed as [1 — (cell survival after peptide incubation)/(cell survival in buffer alone)] x 100. Each data point is the mean (±S.D.) of duplicate determinations from three independent experiments.



Biochemical Journal, Vol. 347, No. 3 (May 2000): pg. 633-641. DOI.

Figure 5 Concentration-dependence of loss of viability of Actinobacillus actinomycetemcomitans strains Y4 (left) and 67 (right) induced by human neutrophil defensin analogues HNPb (a), HNPc (b), HNPd (c) and HNPe (d)

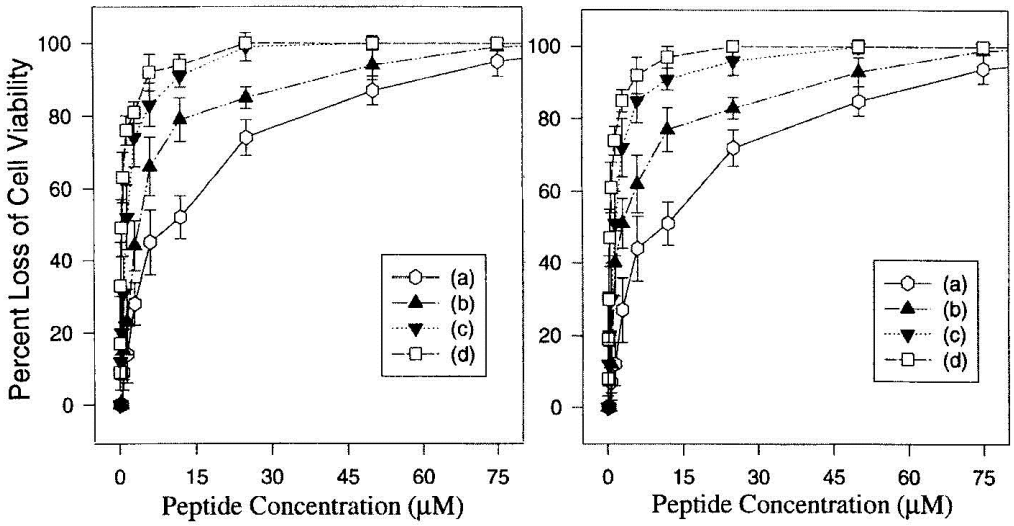
A. actinomycetemcomitans cells were incubated for 1.5 h at 37°C with defensin peptides (0.05-200 μM) as described in the Experimental section. Loss of cell viability is expressed as [1 — (cell survival after peptide incubation)/(cell survival in buffer alone)] x 100. Each data point is the mean (±S.D.) of duplicate determinations from three independent experiments.



Biochemical Journal, Vol. 347, No. 3 (May 2000): pg. 633-641. DOI.

Figure 6 Concentration-dependence of loss of viability of Streptococcus gordonii DL1 (left) and Streptococcus mutans GS5 (right) induced by human neutrophil defensin analogues HNPb (a), HNPc (b), HNPd (c) and HNPe (d)

S. gordonii and *S. mutans* cells were incubated for 1.5 h at 37°C with defensin peptides (0.05-200 μM) as described in the Experimental section. Loss of cell viability is expressed as [1 — (cell survival after peptide incubation)/(cell survival in buffer alone)] x 100. Each data point is the mean (±S.D.) of duplicate determinations from three independent experiments



Biochemical Journal, Vol. 347, No. 3 (May 2000): pg. 633-641. DOI.