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In Vitro Activity of the Antimicrobial Peptides Human and Rabbit Defensins and Porcine Leukocyte Protegrin against *Mycobacterium tuberculosis*

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Three independent assay methods were used to investigate the activities of antimicrobial peptides (human and rabbit defensins and protegrin from porcine leukocytes) against *Mycobacterium tuberculosis* in vitro. *M. tuberculosis* H37Ra was cultured in the presence of human neutrophil peptide 1, synthetic rabbit neutrophil peptide 1, or porcine protegrin 1 at 37°C for 6 to 48 h, and antimycobacterial activity was measured by CFU assay. These peptides at a concentration of 50 μg/ml showed significant antibacterial effects on *M. tuberculosis* after 24 and 48 h of incubation (85.9 to 97.5% at 24 h and 91.6 to 99.4% at 48 h). A radiometric method and a radial diffusion assay confirmed these observations. Antibacterial activity against *M. tuberculosis* was independent of calcium (1.0 mM) or magnesium (1.0 mM) and not inhibited by sodium chloride (100 mM). The optimal pH for antibacterial activity against *M. tuberculosis* was greater than 4.0. Three clinical isolates of *M. tuberculosis* were also studied, and these peptides showed 86.3 to 99.0% reduction in CFU of these organisms. Morphological studies using scanning electron microscopy showed that defensins caused lesions on the surface of H37Ra. These observations suggest that antimicrobial peptides such as defensins and protegrins may represent an important component of the host defense mechanism against *M. tuberculosis* and offer a potential new approach to therapy.

Mycobacterium tuberculosis is still a leading cause of worldwide disease morbidity and is responsible for more deaths each year than any other single pathogen (32). In addition, the AIDS epidemic has exacerbated the problem. Interest in tuberculosis has been rekindled by the recent resurgence of cases both in the United States and worldwide. The increasing number of multidrug-resistant *M. tuberculosis* isolates that can be exceedingly difficult and expensive to treat is of particular concern (17). The combination of the increased frequency of infection due to *M. tuberculosis* and the increase in multidrug-resistant *M. tuberculosis* isolates in patients with AIDS has raised a great deal of concern across the country.

Defensins are endogenous antimicrobial peptides (AMPs) that contain 29 to 35 amino acid residues (27). These peptides were first recognized in rabbit and guinea pig neutrophils and in rabbit alveolar macrophages as "lysosomal cationic proteins" with antimicrobial properties (42, 43). Much earlier, calf thymus peptide (7) and lysozyme (34) were shown to inhibit the growth of pathogenic mycobacteria. More than 15 mammalian defensins derived from five species have been purified and sequenced, and human neutrophils were found to contain four defensin peptides (15). Defensins have been shown to possess antifungal (10), antibacterial (13, 16, 37), and antiviral (4) activities in vitro. We have previously demonstrated that the defensins human neutrophil peptide 1 (HNP-1), HNP-2, and HNP-3 have activity against *Mycobacterium avium-Mycobacterium intracellulare* (33). More recently, we have synthesized rabbit neutrophil peptide 1 (NP-1), referred to as synthetic NP-1 (sNP-1). Its biological activity against several pathogenic fungi is identical to that of natural NP-1 (35).

Protegrins (PGs) are a new family of small (M_r = 2,000) cysteine-rich AMPs discovered in porcine leukocytes. These proteins have recently been purified, and their primary structures and antibiotic properties have been characterized (23). Porcine leukocytes contain three homologous peptides, PG-1, PG-2, and PG-3, that have manifested potent bactericidal activity against *Escherichia coli*, *Listeria monocytogenes*, and *Candida albicans* in vitro.

This study was designed to assess the effects of AMPs (defensins and protegrin) on *M. tuberculosis*. We found that AMPs have potent activity in killing *M. tuberculosis*, including clinical isolates. We also describe the morphological changes of *M. tuberculosis* induced by defensins.

MATERIALS AND METHODS

Peptides. HNP-1, HNP-2, and HNP-3 were prepared from normal human peripheral blood leukocytes (16), and NP-2 was prepared from rabbit peripheral blood leukocytes, as previously described (37a). The synthesis of sNP-1 was accomplished by single coupling of each amino acid with 98.5% coupling efficiency at each step, using the FastMoc chemistry (35). PG-1 was purified from porcine blood by using a recently described method (23). All peptides were dissolved in 0.01% acetic acid and stored as a stock solution of 1 mg/ml at −20°C.

Strains of *M. tuberculosis* and culture conditions. Most studies were performed with *M. tuberculosis* H37Ra obtained from the American Type Culture Collection (Rockville, Md.). Recent clinical isolates of *M. tuberculosis*, 7632G, 9034G,

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and 11170G, were provided by Sharon Reed, Microbiology Laboratory at UCSD Medical Center, San Diego, Calif. All cultures were grown in Middlebrook 7H11 broth (Difco Laboratories, Detroit, Mich.) with oleic acid-bovine serum albumin-dextrose-catalase (OADC) complex (Difco) at 37°C, with vigorous agitation once a day. Rapidly growing organisms were harvested from 7- to 10-day-old cultures by centrifugation at 1,000 × *g* for 20 min and washed once in Middlebrook 7H11 broth with OADC complex, diluted 1:100 in 10 mM sodium phosphate buffer (Ca^{2+} and Mg^{2+} free, pH 7.4), termed 1:100 Middlebrook medium, and resuspended at the appropriate optical density (OD) in 1:100 Middlebrook medium. The OD was used to estimate the concentration of mycobacteria for setting up experiments. *M. tuberculosis* with an OD at 600 nm of 0.2 yielded about 4.5×10^7 CFU/ml. The 1:100-diluted sample of *M. tuberculosis* with an OD of 0.2 was found to have a concentration of 5.1×10^5 CFU/ml determined by the CFU assay.

Standard colony count assay for antimycobacterial activity. Standard colony count assays were performed to assess the bactericidal activity against *M. avium-M. intracellulare* as previously described (13). In these assays, *M. tuberculosis* was adjusted to 4.5×10^7 or 5.1×10^5 CFU/ml in 1:100 Middlebrook medium. Ninety-microliter aliquots of *M. tuberculosis* suspension were added to wells in a 96-well culture plate (96 Well Cell Cluster; Costar Corporation, Pleasanton, Calif.). In experimental wells, 10 μl of an appropriate concentration of the AMP in 0.01% acetic acid was added, while 0.01% acetic acid only was added to the control wells. The plate was incubated at 37°C for 6 to 48 h as described for individual experiments in Results. At the end of incubation, surviving *M. tuberculosis* bacilli were enumerated by CFU. Serial 10-fold dilutions were performed in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8 mM phosphate [Ca^{2+} and Mg^{2+} free, pH 7.4]) in a 96-well culture plate. Ten microliters of each dilution was plated on Middlebrook 7H10 agar plates. Plates were incubated at 37°C for 14 to 21 days in 5% CO_2. Colonies were counted at intervals until no new colonies appeared (6). The activity of AMP was estimated by the following formula: % killing = [(CFU in control well − CFU in AMP-treated well)/CFU in control well] × 100 (33).

To examine the effect of pH on the activity of AMP, 1:100 Middlebrook medium was adjusted to various pH levels between 2.0 and 8.0 by using HCl or NaOH. pH was measured at the beginning and the end of incubation. To examine the effects of calcium, magnesium, and sodium chloride, the medium was adjusted to final concentrations of calcium (1.0 mM), magnesium (1.0 mM), and sodium chloride (100 mM).

All experiments were performed in duplicate or triplicate and were repeated two or more times. Significance of killing differences between AMP-treated and control wells at identical time points was tested by Student's *t* test.

Enumeration of *M. tuberculosis* clumping. If a condition being tested causes bacterial clumping, CFU will be decreased. Therefore, it was necessary to determine whether reduction in CFU was caused by clumping or by killing. To calculate the decrease in CFU caused by clumping, *M. tuberculosis* H37Ra organisms in control and AMP-treated wells were fixed with 5% paraformaldehyde in Hanks' balanced salt solution. A total of 100 bacteria, including singles, doubles, and triples, etc., were counted under a microscope with the help of a hemocytometer at a magnification of ×400. Each single, double, triple, or large clump of bacteria was expected to give rise to only a single colony on agar. For example, if one started with 100 individual organisms, one would find, after 14 days, 100 colonies. However, if these organisms clumped and all formed doubles, one would observe only 50 colonies, even if all 100 organisms were alive. These direct microscopic counts, therefore, provide the basis for calculating the corrected colony count and the decrease in bacterial growth caused by the bacterial effect of defensins versus the decrease in CFU caused by clumping. The expected colony count (reduction due to clumping alone) was calculated as follows: expected colony count = (clumping in AMP-treated well by microscopy/clumping in control well by microscopy) × CFU in control well. Percent killing due to AMP was calculated as follows: % killing corrected for clumping = [(expected colony count − observed colony count in the AMP-treated well)/expected colony count] × 100 (33).

Radiometric assay compared with CFU assay. *M. tuberculosis* H37Ra suspended at 5.0×10^5 CFU/ml in 1:100 Middlebrook medium was cultured in the presence of PG-1 (final concentrations of 5 and 50 μg/ml) or its vehicle (0.01% acetic acid) alone for 48 h at 37°C. After this incubation, *M. tuberculosis* suspensions were evaluated for clumping and CFU. These suspensions were further diluted 100 times with 1:100 Middlebrook medium, and 5-μl aliquots of these suspensions were mixed with 195 μl of PBS and inoculated to BACTEC 12B vials (Becton Dickinson Diagnostic Instruments Systems, Pittsburg, Pa.). The vials contained no AMP. The growth index (GI) was monitored every day until it reached 100. To assess the quantitative activity of PG-1 in this radiometric assay, a control mycobacterial suspension which had been incubated for 48 h was diluted 1:2, 1:4, and 1:10 with PBS and then inoculated into BACTEC vials in the same manner to obtain GI curves of various initial mycobacterial counts. For example, a volume of 1:2-diluted suspension of the control well was considered equivalent to 50% of the bacteria killed (50% killing). Therefore, for the same volume, 1:4 and 1:10 dilutions were taken to be equivalent to 75 and 90% killing, respectively. The effect of PG-1 was estimated by comparing GI curves of various initial mycobacterial counts with those of PG-1-treated cultures.

Radial diffusion assay. A radial diffusion assay was performed to assess the activities of AMP against *M. tuberculosis* growing in the agarose gel. An H37Ra suspension at 4.5×10^6 CFU was incorporated into 3-mm-thick agarose gels that contained nine evenly spaced wells, 3 mm in diameter. The underlayer agar (pH 7.4) consisted of 10 mM sodium phosphate buffer, 1% (wt/vol) agarose (Agarose A-0169; Sigma Chemical Co., St. Louis, Mo.), and 0.05% (wt/vol) Middlebrook 7H9 broth (Difco). After 24 h of growth in the agarose, mycobactericidal activity was tested by placing 5-μl samples of HNP-1 and sNP-1 at concentrations of 10, 25, 50, 100, 250, and 500 μg/ml in each well, incubating them for 24 h to permit the peptides to diffuse into the agarose, and pouring a nutrient-rich overlay that allowed the surviving mycobacteria to grow and form colonies. The overlay consisted of distilled water, 1% (wt/vol) agarose, 0.5% (wt/vol) Middlebrook 7H9 broth, and 10% (vol/vol) OADC complex. The plates were incubated for 2 to 3 weeks at 37°C. The diameter of the clear zone was measured and expressed in arbitrary units (0.1 mm = 1 U) after subtraction of the diameter of the well (3 mm) (28).

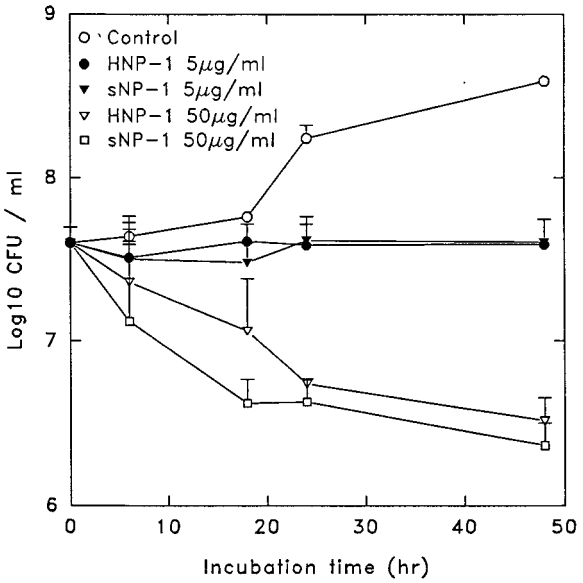

FIG. 1. Effects of HNP-1 and sNP-1 against *M. tuberculosis* H37Ra. *M. tuberculosis* in 1:100 Middlebrook medium at 4.5×10^7 CFU/ml was cultured in the presence of HNP-1 and sNP-1 (5 and 50 μg/ml) at 37°C for 6, 18, 24, and 48 h in 5% CO_2. After incubation, CFU were enumerated. Data indicate the means ± standard deviations of assays performed twice in duplicate. (Error bars do not appear when smaller than symbols.)

Scanning EM. The effects of HNP-1 and sNP-1 on the morphology of *M. tuberculosis* were examined by scanning electron microscopy (EM). H37Ra was exposed to 0.01% acetic acid and HNP-1 or sNP-1 at 50 μg/ml for 20 min, 60 min, 6 h, and 24 h in Terasaki plates (Miles Laboratories, Inc., Naperville, Ill.). The bacteria were then fixed with 2.5% paraformaldehyde (all EM chemicals were from Ted Pella Inc., Redding, Calif.) and 2.5% glutaraldehyde solution in cacodylate buffer (0.1 M + 0.1 M sucrose [pH 7.2]) (11) for 15 min, washed twice with 0.1 M cacodylate buffer, and fixed overnight at 4°C with 2.5% glutaraldehyde in 0.1 M cacodylate buffer. This procedure was followed by postfixation in 1% osmium tetroxide in 0.1 M cacodylate buffer and then dehydration in a graded (50 to 100%) ethanol series. The Terasaki plate was cut into small pieces with a jeweler's saw, critical point dried with a CO_2 apparatus (model 1200; Ted Pella Inc.), sputter-coated with gold in a Technics Hummer apparatus (Technics, Alexandria, Va.), and examined on a Cambridge 360 Stereoscan electron microscope (Cambridge Instruments Co. Ltd., London, England) at a magnification of ×17,000.

RESULTS

Mycobactericidal activities of AMPs. The effects of AMPs against *M. tuberculosis* were examined by placing *M. tuberculosis* suspended at 4.5×10^7 and 5.1×10^5 CFU/ml in the presence of AMPs at concentrations of 5 and 50 μg/ml for 6 to 48 h at 37°C on 1:100 Middlebrook medium. After incubation, CFU were determined as described in Materials and Methods.

HNP-1 and sNP-1 decreased CFU of *M. tuberculosis* in a concentration- and time-dependent manner (Fig. 1). At 50

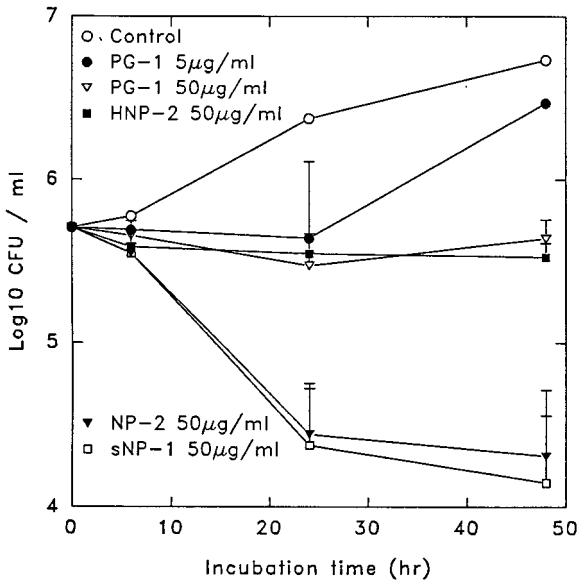

FIG. 2. Effect of PG-1 compared with those of defensins against *M. tuberculosis* H37Ra. H37Ra in 1:100 Middlebrook medium at 5.1×10^5 CFU/ml was cultured in the presence of PG-1 (5 and 50 μg/ml) and of NP-2, sNP-1, and HNP-2 (50 μg/ml) at 37°C for 6, 24, and 48 h in 5% CO_2. After incubation, CFU were enumerated. Data indicate the means ± standard deviations of assays performed twice in duplicate. (Error bars do not appear when smaller than symbols.)

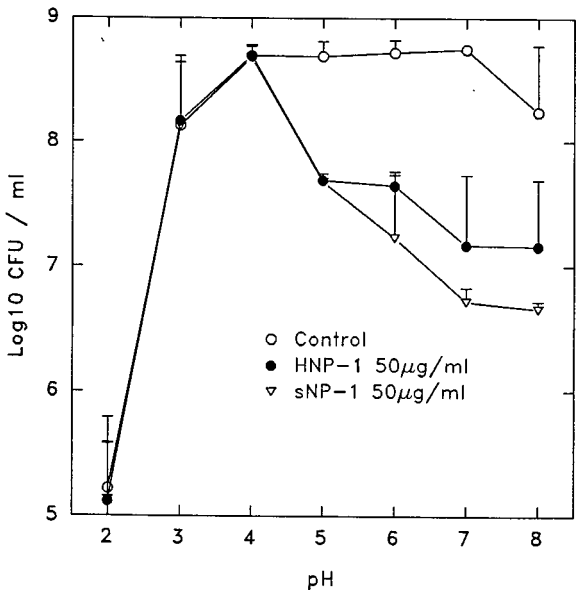

FIG. 3. Effects of pH on HNP-1 and sNP-1 against *M. tuberculosis*. In this assay, 1:100 Middlebrook medium was adjusted to various pH levels between 2.0 and 8.0. Strain H37Ra at 4.5×10^7 CFU/ml placed in pH-adjusted medium was cultured in the presence of HNP-1 or sNP-1 at a final concentration of 50 μg/ml. Incubation was at 37°C for 48 h in 5% CO_2. Data indicate the means ± standard deviations of assays performed twice in duplicate. (Error bars do not appear when smaller than symbols.)

μg/ml, HNP-1 decreased CFU at 48 h by 99% ($P < 0.01$). At 50 μg/ml, sNP-1 decreased CFU at 48 h by 99% ($P < 0.01$).

The activity of PG-1 was also concentration and time dependent (Fig. 2). PG-1 at 50 μg/ml inhibited 91.6% of *M. tuberculosis* growth after 48 h of incubation ($P < 0.01$).

Clumping of *M. tuberculosis* after treatment with AMP. Some clumping was observed in control wells, and more was evident in AMP-treated wells. The expected colony count reduction as a result of clumping caused by AMP was calculated according to the formula presented in Materials and Methods. Killing of *M. tuberculosis*, corrected for clumping, was estimated as actual killing of *M. tuberculosis* by AMP (Table 1). Treatment with AMP resulted in a reduction of 90.2 to 99.0% of surviving *M. tuberculosis* after correction for clumping. AMP reduced CFU both by clumping and by killing of organisms, which is expressed as percent killing in Table 1. However, AMP killing of organisms was the dominant factor in the reduction of CFU.

Effect of pH on AMP activity. The effect of pH on AMP activity against *M. tuberculosis* was examined by incubating organisms with AMP in 1:100 Middlebrook medium adjusted to various pH levels. The pH was stable throughout the experiment in all wells, as determined by checking the pH at the beginning and the end of the incubation. Growth of *M. tuberculosis* was not inhibited by HNP-1 or sNP-1 at or below pH 4.0. At higher pH, growth was significant in control wells, but it was strongly inhibited by defensins (Fig. 3). No significant activity was noted at or below pH 4.0 after treatment with PG-1. At pH 5 and pH 6, values for percent killing were 86 and 92, respectively.

Effect of calcium, magnesium, or sodium chloride on activ-

TABLE 1. *M. tuberculosis* clumping after treating with HNP-1, sNP-1, and PG-1[a]

AMP	Control[b] (CFU/ml) (A)	Results after treatment with AMP[b]			
		Expected colony count corrected for clumping (CFU/ml) (B)	Observed colony count (CFU/ml) (C)	% Killing (D)	Corrected for clumping (%) (E)
HNP-1					
Expt 1	$(3.9 \pm 0.1) \times 10^8$	$(2.1 \pm 0.1) \times 10^8$	$(4.2 \pm 1.3) \times 10^6$	98.9 ± 0.3	98.0 ± 0.6
Expt 2	$(4.1 \pm 0.4) \times 10^8$	$(3.1 \pm 0.3) \times 10^8$	$(5.9 \pm 0.1) \times 10^6$	98.6 ± 0.1	98.1 ± 0.1
sNP-1					
Expt 1	$(3.9 \pm 0.1) \times 10^8$	$(2.4 \pm 0.1) \times 10^8$	$(3.0 \pm 0.6) \times 10^6$	99.2 ± 0.1	98.7 ± 0.2
Expt 2	$(5.1 \pm 0.5) \times 10^8$	$(3.0 \pm 0.3) \times 10^8$	$(3.0 \pm 0.1) \times 10^6$	99.4 ± 0.1	99.0 ± 0.1
PG-1					
Expt 1	$(5.7 \pm 0.4) \times 10^6$	$(5.2 \pm 0.3) \times 10^6$	$(3.6 \pm 0.4) \times 10^5$	93.6 ± 0.6	92.9 ± 0.7
Expt 2	$(5.5 \pm 0.5) \times 10^6$	$(3.8 \pm 0.3) \times 10^6$	$(3.7 \pm 0.8) \times 10^5$	93.3 ± 1.5	90.2 ± 2.2

[a] *M. tuberculosis* H37Ra was suspended at 4.5×10^7 or 5.1×10^5 CFU/ml and incubated with 50 μg of HNP-1, sNP-1, or PG-1 per ml at 37°C for 48 h.
[b] Results are means ± standard deviations of assays performed in duplicate or triplicate. D = [(A − C)/A] × 100. E = [(B − C)/B] × 100.

TABLE 2. Activities of HNP-1, sNP-1, and PG-1 with clinical isolates of *M. tuberculosis*[a]

Isolate	% Killing[b]					
	HNP-1		sNP-1		PG-1	
	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2
7632G	86.3 ± 1.8	89.8 ± 0.1	98.3 ± 0.3	95.5 ± 0.7	98.9 ± 0.1	98.0 ± 0.2
9034G	86.8 ± 3.3	88.3 ± 0.3	99.0 ± 0.1	95.0 ± 0.8	99.0 ± 0.3 9	8.2 ± 0.1
11170G	88.4 ± 2.6	91.1 ± 0.6	98.9 ± 0.1	93.2 ± 0.8	98.5 ± 0.2	98.8 ± 0.1

[a] Each isolate was incubated with 50 μg of HNP-1, sNP-1, and PG-1 per ml at 37°C for 48 h.
[b] Results are the means ± standard deviations of assays performed in duplicate.

ities of AMPs. The effect of calcium, magnesium, or sodium chloride on AMP activity against *M. tuberculosis* was examined by placing organisms with 50 μg of AMP per ml for 48 h at 37°C in Middlebrook medium adjusted to a final concentration of calcium (1.0 mM), magnesium (1.0 mM), or sodium chloride (100 mM). PG-1 was active in all of these solutions (88.2 to 98.2% killing). HNP-1 and sNP-1 were also active in the presence of calcium, magnesium, or sodium chloride (89.9 to 99.2% killing by HNP-1 and 89.9 to 99.9% killing by sNP-1).

Activities of AMPs on clinical isolates of *M. tuberculosis*. Three different clinical isolates of *M. tuberculosis* were tested by the CFU assay. Mycobacteria with or without AMP were cultured. All isolates of *M. tuberculosis* were susceptible to killing by HNP-1, sNP-1, and PG-1, with the average percent killing ranging from 86.3 to 99.0 (Table 2).

Activities of HNP-2 and HNP-3 against *M. tuberculosis* compared with that of HNP-1. HNP-2 and HNP-3 were incubated with *M. tuberculosis* H37Ra, and CFU counts were compared with those in samples incubated with HNP-1. HNP-1, HNP-2, and HNP-3 were equally effective in killing *M. tuberculosis*. At 50 μg/ml, HNP-2 decreased CFU of *M. tuberculosis* at 48 h by 99% ($P < 0.01$). At 50 μg/ml, HNP-3 decreased CFU of *M. tuberculosis* at 48 h by 98% ($P < 0.01$) (Fig. 4).

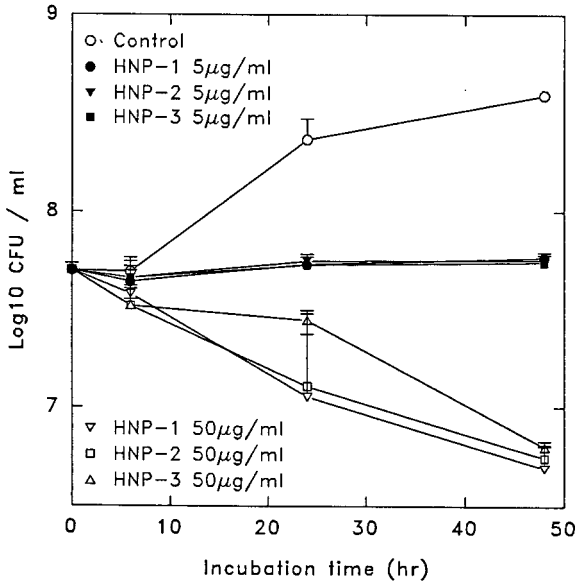

FIG. 4. Effects of HNP-2 and HNP-3 compared with that of HNP-1 against *M. tuberculosis*. *M. tuberculosis* at 4.5×10^7 CFU/ml in 1:100 Middlebrook medium was incubated in the presence of defensins (5 and 50 μg/ml) at 37°C for 6, 24, and 48 h. Data indicate the means ± standard deviations of assays performed twice in duplicate. (Error bars do not appear when smaller than symbols.)

Radiometric method. GI curves of various initial *M. tuberculosis* counts and PG-1-treated suspensions are shown in Fig. 5. Note that the GI curve of the PG-1 (5 μg/ml)-treated suspension is very close to that of 75% killing. The PG-1 (50 μg/ml)-treated suspension showed over 90% killing. By comparison, when the viability of bacteria in those suspensions was enumerated by CFU assay, treatment with PG-1 resulted in reductions of 62.5 and 94.7%, respectively, after correction for clumping (Table 3).

Radial diffusion assay. The activities of HNP-1 and sNP-1 against *M. tuberculosis* H37Ra were assessed in agarose gels. A representative view of a gel in this assay is shown in Fig. 6. In Fig. 7, the activities of HNP-1 and sNP-1 are expressed in units as described in Materials and Methods. Both AMPs were active against *M. tuberculosis*. The minimal concentration of both peptides that resulted in inhibition of mycobacterial growth was 25 μg/ml. The control, 0.01% acetic acid, gave no clear zone.

Morphological changes induced by defensins. The morphological changes in *M. tuberculosis* induced by defensins HNP-1

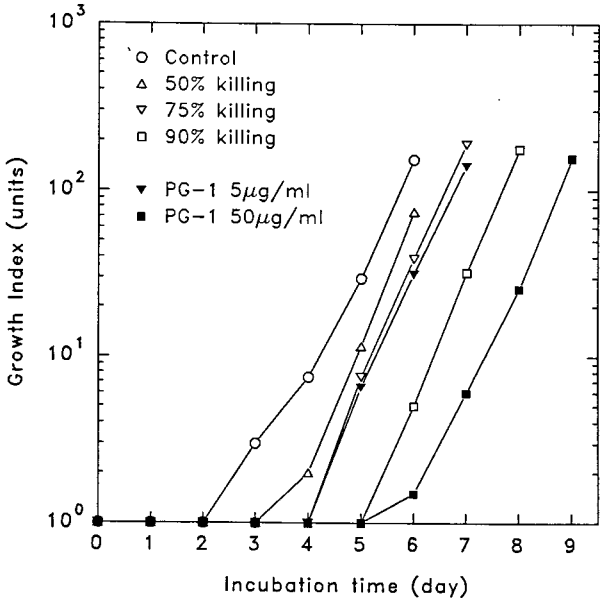

FIG. 5. Radiometric methods. BACTEC vials containing 12B medium were inoculated with *M. tuberculosis* H37Ra at various initial viable counts by diluting a control suspension to generate a standard curve, against which the GI of a PG-1-treated suspension was compared. The GI was monitored every day until it reached 100. A GI equal to the GI of the 1:2 dilution indicates that 50% of the bacteria in the experimental well were killed (50% killing). GI values represent means of assays performed in duplicate. (Experiments were performed three times.)

TABLE 3. Effect of PG-1 against *M. tuberculosis* by CFU assay[a]

Sample	CFU/ml[b]	% Killing corrected for clumping
Control	$(5.9 \pm 0.6) \times 10^4$	
PG-1		
5 μg/ml	$(1.9 \pm 0.8) \times 10^4$	62.5
50 μg/ml	$(2.5 \pm 0.7) \times 10^3$	94.7

[a] *M. tuberculosis* was suspended at 5.0×10^3 CFU/ml and incubated with 5 and 50 μg of PG-1 per ml at 37°C for 48 h.
[b] Results are means ± standard deviations of assays performed in duplicate. (Experiments were performed three times.)

and sNP-1 were examined by scanning EM. Representative views of selected experiments are shown in Fig. 8. A control containing 0.01% acetic acid had no effect on the morphology of H37Ra (Fig. 8A). On HNP-1- or sNP-1-treated mycobacteria, 70- to 100-nm "warts" could be seen within 20 min (Fig. 8B). These lesions became more numerous with time (Fig. 8C and D). The effects of sNP-1 and HNP-1 on the morphology of *M. tuberculosis* were identical.

DISCUSSION

In this study, we have demonstrated that human neutrophil defensins, synthetic rabbit defensin, and porcine protegrin have the ability to kill *M. tuberculosis*, including clinical isolates, in vitro.

As mycobacteria tend to clump, it has been suggested that clumping of mycobacteria during the killing assay could be misinterpreted as bacterial killing (5). If defensins indeed cause additional clumping, this effect will result in the reduction of CFU and could mimic killing (33). Therefore, the clumping of *M. tuberculosis* before and after treatment with AMP was determined by microscopic evaluations and killing of *M. tuberculosis* corrected for clumping was estimated as actual killing of *M. tuberculosis* by AMP. Actually, AMP reduced CFU both by clumping and by killing organisms. However, our data for percent killing corrected for clumping along with percent killing indicated that the effect of AMP on *M. tuberculosis* was predominantly killing.

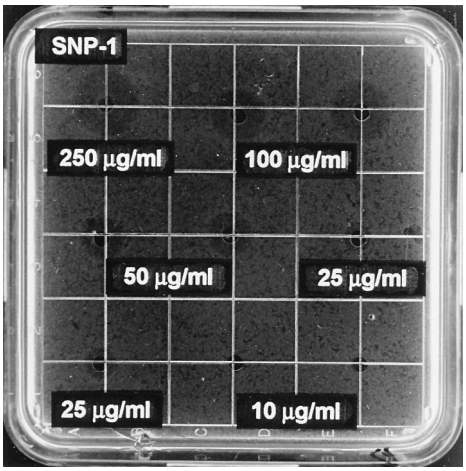

FIG. 6. Radial diffusion assay. *M. tuberculosis* H37Ra at 4.5×10^6 CFU was incorporated into an agarose gel that contained nine evenly spaced wells, 3 mm in diameter. Mycobactericidal activity was tested by placing 5-μl AMP samples (in 0.01% acetic acid) in each well, incubating them for 24 h to permit the AMP to diffuse into the agarose, and pouring a nutrient-rich overlay that allowed the surviving *M. tuberculosis* to grow and form colonies. The control, 0.01% acetic acid, gave no clear zone.

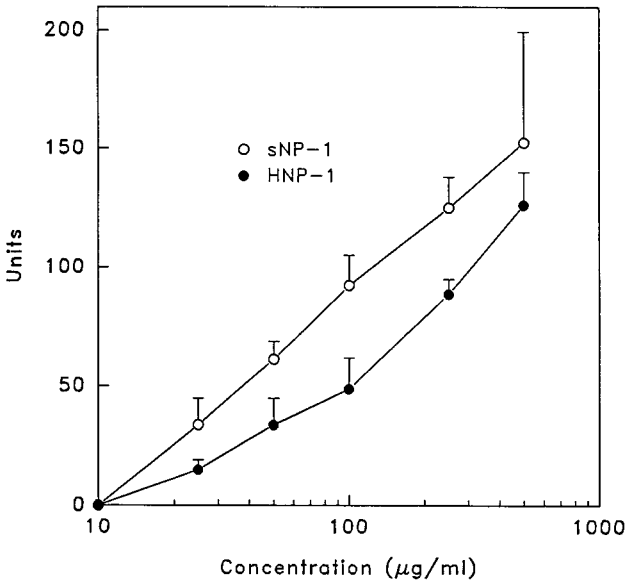

FIG. 7. Activities of HNP-1 and sNP-1 against *M. tuberculosis* H37Ra in a radial diffusion assay. The diameters of the clear zones were measured and expressed in arbitrary units (0.1 mm = 1 U) after subtraction of the diameter of the well (3 mm). Data indicate the means ± standard deviations of assays performed twice in duplicate.

The effect of PG-1 was confirmed by a quantitative radiometric method to circumvent potential bias in colony-counting results as a result of the characteristic clumpy growth of *M. tuberculosis*. We have shown here that radiometric results correlate well with percent killing that has been corrected for clumping determined by the CFU assay. The radiometric method detected growth of mycobacteria by measurement of $^{14}CO_2$ released as a consequence of the catabolism of [^{14}C] palmitic acid included as a substrate in the broth medium (21). On the basis of release of $^{14}CO_2$, one cannot directly distinguish between inhibition of cell division and inhibition of metabolic activity. However, it has been reported that inhibition of metabolism is equivalent to inhibition of growth, and radiometric results correlate very well with CFU-per-milliliter measurements (18, 19).

Further confirmation of our results was obtained by a radial diffusion assay with *M. tuberculosis*. This assay was designed to achieve maximal sensitivity with minimal consumption of reagents (9, 20, 28). Since *M. tuberculosis* bacilli were entrapped in an agarose gel, clumping was precluded. Thus, the results confirm that the decreased CFU noted in our colony count experiments resulted primarily from the mycobactericidal or mycobacteriostatic activity of the AMP.

Macrophages are known to actively maintain a low pH (36). However, a recent experiment reported that living *M. tuberculosis* organisms are located in human macrophages in vesicles which are not acidic (3). The activity of AMP against *M. tuberculosis* was relatively independent of H^+ from pH 5.0 to 8.0. In addition, killing of *M. tuberculosis* was calcium, magnesium, and sodium chloride independent. These data are very similar to the effects of HNP-1 on *M. avium-M. intracellulare* (33) and differ from the effect of HNP-1 on *C. albicans* (26). Human defensin-mediated candidacidal activity was inhibited by both magnesium and calcium. These data imply that both HNP-1

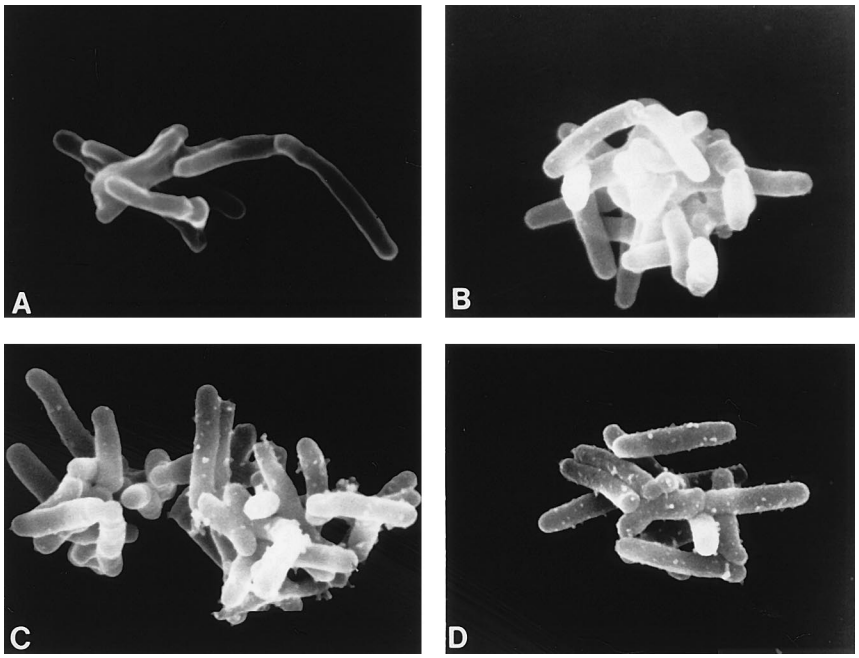

FIG. 8. Scanning electron micrographs of morphological changes in defensin-treated *M. tuberculosis*. The organisms were examined on a Cambridge 360 Stereoscan electron microscope (Cambridge Instruments). All photographs were taken at a magnification of ×17,000. (A) Control *M. tuberculosis* exposed to 0.01% acetic acid for 24 h. (B) *M. tuberculosis* exposed to 50 μg of sNP-1 per ml for 20 min, showing warts forming on the surface of one of the organisms. (C) *M. tuberculosis* exposed to 50 μg of sNP-1 per ml for 24 h, showing wart formation on most of the organisms. (D) *M. tuberculosis* exposed to 50 μg of HNP-1 per ml for 24 h, which caused the same changes as did sNP-1.

and sNP-1 may act on *M. tuberculosis* by using a mechanism that differs from that involved in their candidacidal activity.

The wart formation detected by scanning EM suggests that the defensins have their site of action on the surface of *M. tuberculosis* and result in the loss of viability and reduction of CFU. It is not yet certain whether membrane disruption is in itself sufficient to kill the bacterium or whether additional intracellular events are required for killing. EM of *E. coli* that had been killed by defensins revealed the presence of striking electron-dense deposits in the periplasmic space and affixed to the outer membrane (24). In a recent study (38), transmission EM of defensin-treated *S. aureus* confirmed that the cytoplasmic membranes are the targets of defensins. Biochemical studies demonstrated a change in outer membrane and inner membrane permeability. Since *M. tuberculosis* does not have separate outer and inner membranes, it is necessary to investigate the components and permeability of the cell membrane, in studies including transmission EM, so as to clarify the mechanism of killing mycobacteria.

Our observations raise fundamental questions about the role of AMPs in the host defense against mycobacteria. The AMPs of phagocytes are believed to play a biologic role in the nonoxidative defense mechanisms of these cells (15). On the basis of structural features, leukocyte AMPs have been grouped into several families (12). Macrophages from humans have been shown to contain lysozyme (25), whereas rabbit alveolar macrophages synthesize and contain defensins. To date, no defensin has been detected in human macrophages. On the other hand, human neutrophils contain a large number of cytoplasmic granules which have been shown to contain several AMPs, including defensins, which have been extensively studied (27). The role of polymorphonuclear leukocytes (PMN) in mycobacteriosis is controversial. In an exhaustive review of the literature, Edwards and Kirkpatrick concluded that the role of PMN in the host response to mycobacteria is transient and inconsequential (8). PMN have been reported as unable to kill pathogenic mycobacteria (30, 40). On the other hand, Brown et al. reported that PMN are capable of killing *M. tuberculosis* in vitro (2). They also showed that killing by normal PMN was effective in the absence of serum and independent of the oxygen metabolic burst (22).

From the point of view of PMN-macrophage cooperation, the report that in vitro antimycobacterial activity of macrophages is enhanced by internalizing granulocyte material from PMN (39) led us to the idea that macrophages may incorporate some materials derived from PMN to kill mycobacteria. The authors of the study propose that PMN participate in the host response to mycobacterial infection not as phagocytes but as accessory cells providing the molecules lacking in the mature macrophages. The recruitment of PMN in the early lesions of tuberculosis has been observed (31). The macrophage migration that follows the PMN influx is the effect of a chemotactic factor released by the PMN in response to the mycobacteria (1). HNP-1 and HNP-2 are chemotactic for human monocytes in vitro, and it is suggested that the release of defensins from neutrophils during infection may attract monocytes to the involved sites (14). When PMN are stimulated by phagocytosis, defensins are released into the extracellular fluids (41). The released defensins may contribute to extracellular antimicrobial activity. It remains to be determined whether the uptake of the AMP by macrophages can equip them to inhibit or kill internalized *M. tuberculosis*. Since mycobacteria are intracellular parasites of macrophages, they can either provide the organisms sanctuary and sustenance or restrict their growth and eventually eliminate them (29). Under these circumstances, our data support the possibility that defensins play an important role in host defense mechanisms against *M. tuberculosis*.

AMPs kill *M. tuberculosis*. The role of this mechanism in

normal host defense against tuberculosis needs to be further investigated. It is exciting to consider ways to exploit this activity as a potential for the treatment of tuberculosis.

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