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Susceptibility of *Neisseria gonorrhoeae* to Protegrins

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We developed a sensitive and quantitative radial diffusion method to ascertain the susceptibility of six strains of *Neisseria gonorrhoeae* to antimicrobial peptides derived from mammalian leukocytes. The test organisms included the well-characterized serum-resistant FA19 and serum-sensitive F62 strains plus four antibiotic-resistant clinical isolates. Although each *N. gonorrhoeae* strain was resistant to human neutrophil defensins, all six were exquisitely sensitive to protegrins, a family of small β-sheet antimicrobial peptides recently identified in porcine leukocytes. Protegrin-treated *N. gonorrhoeae* became vacuolated and had striking membrane changes when viewed by transmission and scanning electron microscopy. Because low concentrations of protegrins can also inactivate *Chlamydia trachomatis* and human immunodeficiency virus, they show promise for development as topical agents to avert sexually transmitted diseases.

The use of topical microbicides by women has been advanced as a new health initiative to prevent the spread of sexually transmitted diseases (STDs) (11). Ideally, such topical microbicides should be active against all of the major human STD pathogens. During the past decade, the existence of many endogenous, broad-spectrum antimicrobial peptides has been demonstrated in the blood leukocytes, epithelial cells, and mucosal tissues of humans and other mammals. These include classical and β-defensins and protegrins. Defensins are peptides of 3.5 to 5 kDa whose largely β-sheet structures are stabilized by three intramolecular cysteine disulfide bonds. Protegrins are potently antimicrobial peptides (8) that contain 16 to 18 amino acid residues and have a β-sheet structure which is stabilized by two intramolecular disulfide bonds. Protegrins were recently reported to inactivate both *Chlamydia trachomatis* elementary bodies (17) and human immunodeficiency virus type 1 (HIV-1) virions (16).

Neisseria gonorrhoeae causes more than 400,000 infections annually in the United States. Although most of these infections respond to appropriate antibiotics, the emergence of strains resistant to benzylpenicillin and/or tetracycline requires more costly treatments that are especially problematic in underdeveloped countries and economically disadvantaged communities. Because patients with chlamydial disease are often coinfected with gonococci, we examined the ability of protegrins to inactivate *N. gonorrhoeae*. To facilitate these studies, we devised a novel radial diffusion assay that allowed rapid, reproducible, and quantitative assessment of antimicrobial activity and confirmed our findings by classical colony-counting procedures. Both assays revealed that under identical assay conditions, gonococci are resistant to human neutrophil defensins but highly susceptible to protegrins.

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MATERIALS AND METHODS

Peptides. Native porcine protegrins PG-1, PG-2, and PG-3, human defensins HNP-1, HNP-2, and HNP-3, and rabbit defensin NP-2 were purified from leukocytes as previously described (5, 12). Only the synthetic form of PG-5 was tested. All synthetic protegrins were C-terminally amidated, prepared with F-moc chemistry (SynPep, Dublin, Calif.), and purified in our laboratory. The synthetic and native forms of PG-1 were identical in mass, net charge, behavior in sodium dodecyl sulfate (SDS) and acid-urea (AU) polyacrylamide gel electrophoresis (PAGE), and antimicrobial activity against a panel of gram-positive and gram-negative bacteria (data not shown). The 20-mer CG117–136 antibacterial peptide was synthesized and purified by J. Pohl of the Emory University Microchemical Facility, as described previously (15).

Bacteria. *N. gonorrhoeae* strains FA19 (serum resistant) and F62 (serum sensitive) have been described previously (1). Four clinical strains of *N. gonorrhoeae* were provided by Elizabeth Wagar of the UCLA Department of Clinical Microbiology. Three of these produced penicillinase, whereas the other, which was resistant to penicillins and tetracyclines, did not.

Growth conditions. Bacteria were streaked on *N. gonorrhoeae* test medium (NGTM) plates, supplied by Clinical Standard Laboratories (Rancho Dominguez, Calif.). NGTM plates contained 36 g of GC agar base and 10 ml of VIT-X enrichment per liter and conformed to National Committee for Clinical Laboratory Standards M2-A5 guidelines. The cultures were incubated at 37°C in 5% CO_2–room air overnight and passaged daily. GC broth (pH 7.2 to 7.3) contained, per liter, 15 g of proteose peptone (Difco Laboratories, Detroit, Mich.), 4 g of K_2HPO_4, 1 g of KH_2PO_4, 5 g of NaCl, 10 ml of supplement I (40% D-glucose, 1% L-glutamine, and 0.002% cocarboxylase), 1 ml of supplement II [0.5% $Fe(NO_3)_3$], and 10 ml of 4.3% (wt/vol) $NaHCO_3$. Bacteria isolated from NGTM plates were resuspended in 25 ml of the above GC broth and incubated in a 37°C waterbath with shaking for 3 h. By spectrophotometry, an optical density at 620 nm of 0.1 corresponded to 10^8 CFU/ml.

Preparation of radial diffusion plates. Underlay gels contained, per liter, 1.5 g of proteose peptone, 10 g of Sigma A-6013 agarose, 4 g of K_2HPO_4, 1 g of KH_2PO_4, 5 g of NaCl, and 1 g of soluble starch (Difco). Overlay gels contained 15 g of proteose peptone, 4 g of K_2HPO_4, 1 g of KH_2PO_4, 5 g of NaCl, 1 g of soluble starch, and 10 g of Bacto agar (Difco, catalog no. 0140-01) per liter, with 10 ml of supplement I and 1 ml of supplement II per liter (see above). The Bacto-agar was prepared separately and mixed with the other ingredients immediately before pouring, to maintain the overlay gel's optical clarity.

Radial diffusion assays. Ten milliliters of molten (43°C) underlay gel was mixed with 4×10^6 CFU of *N. gonorrhoeae* and poured into a square Lab Tek petri dish (9 by 9 cm). When the gel set, 25 evenly spaced wells were punched, and the plugs were removed with a Pasteur pipette. The wells had a diameter of 3 mm and could hold approximately 7.5 μl of liquid. Peptides were dissolved and serially diluted in 0.01% acetic acid, and 5 μl of each dilution was added to a well. The plates were incubated in a CO_2 incubator at 37°C for 3 h to allow the peptides to diffuse into the underlay gels. After pouring the overlay gel (10 ml) and incubating the plates overnight, the total diameters of the clear (colony-free)

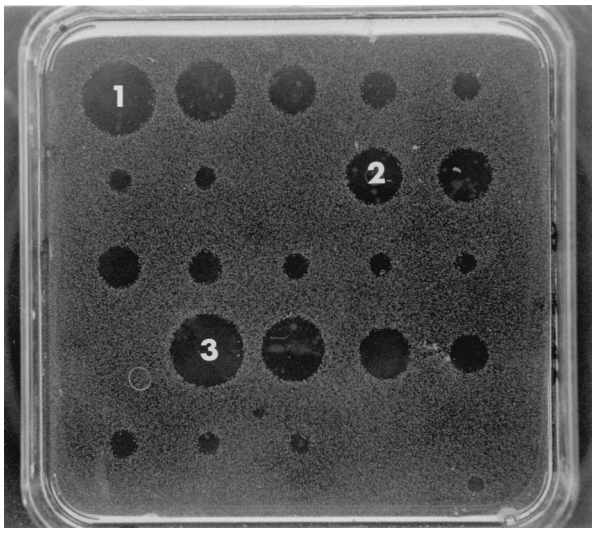

FIG. 1. Radial diffusion assay plate: a "half-log" dilution series for three protegrins tested against *N. gonorrhoeae* FA19. Wells received 5-μl aliquots of the following concentrations of each peptide: 500, 158, 50, 15.8, 5, 1.58, and 0.5 μg/ml. The highest protegrin concentration in each series is indicated by a numeral (1, PG-1; 2, PG-2; and 3, PG-3). The plate was stained with Coomassie brilliant blue to enhance colony contrast and photographed on Ilford XP400 film with oblique, quasi-dark-field transillumination.

zones surrounding the wells were measured, and antimicrobial activities were expressed in units as previously described (9).

Colony count assays. Nonpiliated, transparent variants were used to reduce the likelihood of aggregation involving *opa* or pilus proteins. In preliminary experiments (data not shown), we established that gonococcal susceptibility to protegrins was independent of piliation and of variation in colony opacity.

Synthetic PG-1 was mixed with $\approx 10^6$ mid-log-phase bacteria per ml in a final volume of 100 μl, which contained 9 parts GC broth and 1 part 0.01% acetic acid (final pH, 7.27). Mixtures were incubated in a 37°C shaking water bath, and 10-μl aliquots were removed at intervals and diluted with 990 μl of GC broth. Aliquots (40 μl) were transferred to NGTM plates with a Spiral Plater (Spiral Systems, Cincinnati, Ohio). After overnight incubation, the surviving colonies were counted.

Electron microscopy. Mid-logarithmic-phase *N. gonorrhoeae* strain FA19 (3.5×10^8 CFU/ml) was incubated for 60 min in GC broth with or without PG-1 (100 μg/ml). Samples (1 ml) were removed after 15 and 60 min. The samples were washed twice with 0.1 M sodium cacodylate buffer containing 0.2% $CaCl_2$, dehydrated in graded ethanol solutions, and embedded in LR-White. Thin (<1 μm) sections were stained with saturated uranyl acetate for 1 h at 60°C and examined with a Jeol 100 CX electron microscope at 80 kV.

For scanning electron microscopy, mid-log-phase *N. gonorrhoeae* FA-19 (4×10^8/ml) was incubated at room temperature in NGTM with or without PG-1 (50 μg/ml). Samples were removed after 15 min, suspended in 1 ml of 80 mM sodium cacodylate buffer with 0.2% $CaCl_2$ (pH 7.34), and filtered through 0.1-μm-pore-size Millipore filters (Millipore, Bedford, Mass.). The filters were fixed immediately in 2% cacodylate-buffered glutaraldehyde, washed in buffer, dehydrated in graded ethanols and hexamethyldisilazane, and gold coated with an EMS-76 Mini-coater (Ernest Fulham, Latham, N.Y.). Scanning electron microscopy was performed with a Cambridge 360 instrument (Leica Inc., Deerfield, Ill.).

RESULTS

Development of the radial diffusion assay. In order to establish a radial diffusion assay for *N. gonorrhoeae*, it was necessary to identify an optically clear medium that supported excellent growth and viability of an organism so prone to autolysis. During our preliminary experiments, we determined that (i) the simple buffer and agar formulation that we had used previously for other organisms (9) was unsatisfactory for *N. gonorrhoeae*, (ii) the organisms grew poorly in low-salt media, (iii) the presence of starch enhanced their survival, (iv) 1.5 g of proteose peptone per liter in the underlays was sufficient to ensure viability and (limited) growth during the first 3 h of incubation, (v) low-electroendosmosis (EEO) agarose was preferable to standard Bacto-agar in the underlay, presumably because the agarose was substantially free of polyanionic agar moieties that adsorbed positively charged peptides and prevented their migration into the gel, (vi) a standard agar medium could be used for the overlay, and (vii) to avoid precipitation, molten Bacto-agar should be prepared separately and combined with the other components immediately before the overlay gels were poured. The final procedures, as described in Materials and Methods, proved to be highly satisfactory. Figure 1 is a photograph of a radial diffusion assay plate.

Activity of defensins and protegrins. We compared the antimicrobial activities of four protegrins and three human defensins against several strains of *N. gonorrhoeae*. In pilot studies with classical colony-counting assays, we determined that colony variants of either strain F62 or FA19 that differed in piliation (Pil^+ or Pil^-) or colony opacity (Opa^+ or Opa^-) were equally susceptible (or resistant, in the case of defensins) to the antimicrobial peptides described herein (data not shown). Radial diffusion assays (Fig. 2) revealed that all four protegrins were highly effective against both the serum-resistant FA19 strain and the serum-sensitive F62 strain. The estimated minimal bactericidal concentration, which corresponds to the *x* intercept of the plots shown in Fig. 2, was 1 to 2 μg/ml for each of the peptides. PG-1, PG-3, and PG-5 had almost identical activity throughout the range of concentrations tested. PG-2 concentrations of >10 μg/ml gave somewhat smaller zones, reflecting either a slightly lower intrinsic potency or less efficient diffusion into the underlay. In contrast to the efficacy of protegrins, each of the human neutrophil defensins was completely inactive against both organisms.

These findings were confirmed by classical colony-counting experiments (Fig. 3), wherein PG-1 (5 μg/ml) was found to sterilize $>10^6$ CFU of *N. gonorrhoeae* per ml within 3 h. In another experiment (data not shown), we determined the 50% effective dose (ED_{50}) of PG-1 against *N. gonorrhoeae* strain F62, using a 45-min incubation time. The apparent ED_{50} of ≈3 μg/ml is consistent with the data shown in Fig. 3.

In other experiments involving gonococci and protegrins (data not shown), we observed that the native and synthetic forms of PG-1, PG-2, and PG-3 all showed virtually identical

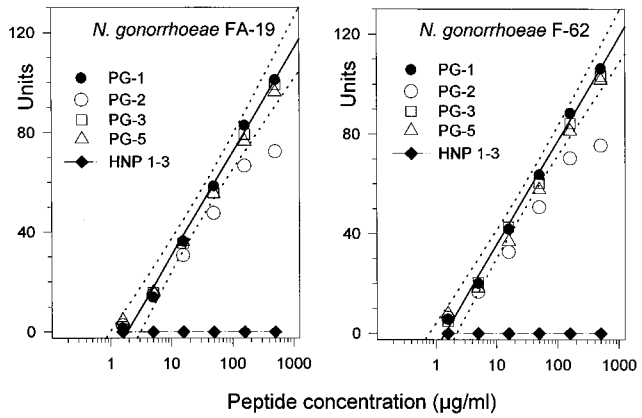

FIG. 2. Radial diffusion assays. Protegrins and defensins were tested against serum-resistant (FA19) and serum-sensitive (F62) strains of *N. gonorrhoeae*. Each symbol represents a mean from five separate experiments. HNP-1, -2, and -3, which showed no activity, are represented by the solid diamond. The line through the protegrin points is a least-mean-squares fit of the PG-1 datum points. The dotted lines show 99% confidence limits for the PG-1 data. When radial diffusion assays are performed and graphed in this manner, the *x* intercepts correspond to minimal bactericidal concentrations. Zone diameters are shown in units (1 unit = 0.1 mm).

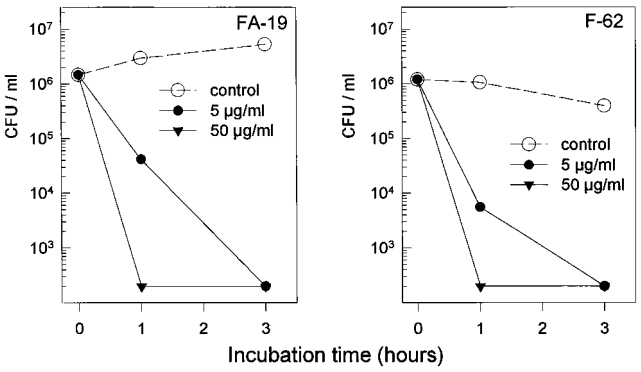

FIG. 3. Colony count assays. Approximately 10^6 CFU of the serum-resistant (FA19) and serum-sensitive (F62) strains of *N. gonorrhoeae* per ml were incubated for 1 or 3 h without (controls) or with protegrin PG-1.

activity. As judged by the $\log_{10}$ decrease in CFU 45 min after exposure to 100 μg of peptide per ml, *N. gonorrhoeae* F62 was considerably more susceptible to PG-1 (5.2 $\log_{10}$ decrease) than to rabbit defensin NP-1 (−0.09 $\log_{10}$ decrease), human defensin HNP-1 (−0.019 $\log_{10}$ decrease), or an antibacterial peptide (CG117–136) derived from human cathepsin G (−0.15 $\log_{10}$ decrease). In contrast to the resistance of gonococci, *Pseudomonas aeruginosa* was killed effectively by both NP-1 and CG117–136 (data not shown), demonstrating that the defensin preparations were active.

Antibiotic-resistant strains. As expected, protegrins were just as active against three penicillinase-producing strains of *N. gonorrhoeae* as they were against FA19 and F62 (Fig. 4). One of these strains showed small clear zones around wells that contained either 500 μg of human defensin HNP-1 (0.6 mm) or HNP-2 (2.0 mm) per ml but no activity around wells that contained 500 μg of HNP-3 per ml or the next lower dilution (158 μg/ml) of all three human defensins. The other two penicillinase-producing strains were resistant even to the 500-μg/ml concentration of human defensins. The strain resistant to penicillin and tetracyclines was also fully susceptible to protegrins and resistant to defensins (Fig. 4).

Morphological changes. Protegrin-treated *N. gonorrhoeae* FA19 manifested prominent changes evident by both phase and electron microscopy. Whereas control (or defensin treated) gonococci were uniformly phase dense, protegrin-treated organisms had phase-lucent centers. By transmission electron microscopy, the protegrin-treated gonococci (Fig. 5b) contained large central multiloculated vacuoles that were not found in the controls (Fig. 5a). The outer membrane of protegrin-treated gonococci also appeared frayed and was studded with multiple, beadlike structures that were approximately 100 nm in diameter (Fig. 5b, arrows).

Figure 6 shows control and protegrin-treated gonococci observed by scanning electron microscopy. In contrast to the bland surface topography of control gonococci (Fig. 6a), the surfaces of protegrin-treated cells resembled an actively volcanic terrain in that they displayed numerous cratered structures. These were often evenly spaced (at approximately 75-nm intervals) and formed ridgelike formations that were especially prominent along cleavage planes separating daughter cocci (Fig. 6b, arrows). The individual surface structures seen in scanning electron micrographs were approximately 100 nm in diameter and corresponded to the surface-adherent beadlets shown in Fig. 5b.

DISCUSSION

The protegrin (PG) family consists of five homologous peptides of porcine origin. Protegrins PG-1, -2, and -3 were isolated from leukocytes (8), whereas PG-4 and PG-5 were discovered by subsequent cDNA (19) or gene (18) cloning experiments. Native protegrins contain 16 to 18 amino acid residues, including four invariant cysteines which form intramolecular disulfide bonds (6) and an amidated C-terminal arginine (Fig. 7). Protegrins show substantial primary sequence homology to certain defensins (8). However, in size and conformation, they more closely resemble tachyplesins, a family of antimicrobial peptides found in horseshoe crab hemocytes (7).

Although we first demonstrated the potent activity of protegrins against *N. gonorrhoeae* by classical colony-counting techniques, such assays were laborious and slow. Accordingly, we devised a two-step radial diffusion assay for *N. gonorrhoeae* adapted from a previously described procedure (9). Unlike CFU assays, which are performed in suspension culture, the radial diffusion assay is not affected by aggregation, because the gonococci are immobilized in a thin agarose gel. Because of the small wells, 5-μl peptide samples, and thin underlay gels, a complete half-log dilution series from 500 down to 0.16 μg/ml could be performed with less than 4 μg of protegrin. Because the diameter of the clearing zone, expressed in units, was a linear function of the $\log_{10}$ peptide concentration added to the well, the *x* intercept of the plot corresponded to the lowest peptide concentration that caused clearing and provided an estimate of the minimal bactericidal concentration.

Although the finding of intracellular gram-negative diplococci in urethral exudates has long been a valuable tool for diagnosing gonorrhea, the viability of such intracellular gonococci has been controversial (4). It is currently believed that only a small percentage of gonococci remain viable within phagolysosomes after their ingestion by neutrophils (14). Since anaerobically maintained normal neutrophils and neutrophils obtained from patients with chronic granulomatous disease that are unable to generate oxidants can kill engulfed gonococci as well as normal neutrophils (2, 10), oxygen-independent mechanisms likely play an important role in equipping neutrophils to kill gonococci. Although defensins are remarkably abundant in human neutrophils and may contribute significantly to oxygen-independent killing of other bacteria, the present data suggest that gonococci are resistant to defensin-

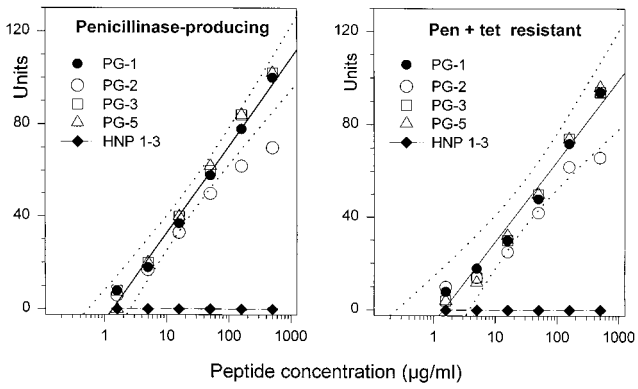

FIG. 4. Antibiotic-resistant *N. gonorrhoeae* strains. As radial diffusion assays performed with protegrins gave virtually identical results for the three penicillinase-producing strains, the left panel shows only the results obtained with one of the strains. The right panel shows the results obtained with the penicillin- and tetracycline-resistant (Pen + tet) clinical isolate. Zone diameters are shown in units (1 unit = 0.1 mm).

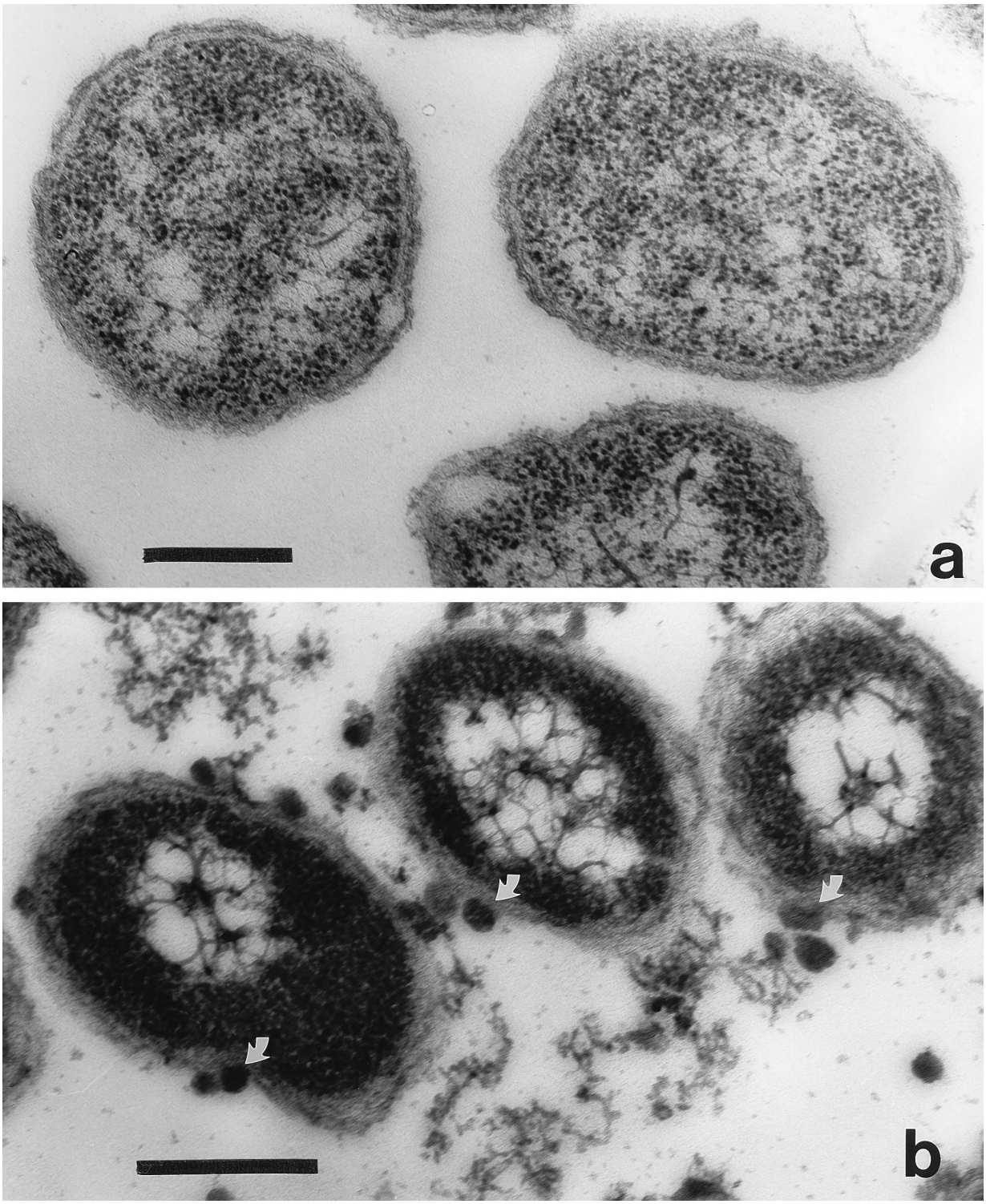

FIG. 5. Transmission electron micrographs of *N. gonorrhoeae*. (a) Control bacteria; (b) organisms 60 min after treatment with protegrin PG-1 (50 μg/ml). Note the central vacuolation of the protegrin-treated bacteria. The arrows in panel b point to some of the membrane-associated, electron-dense structures described in the text. Bars, 0.5 μm.

mediated microbicidal activity, in contrast to their reported susceptibility to other neutrophil proteins (3, 13). Presumably, the defensin-resistant phenotype of *N. gonorrhoeae* arose as a consequence of its repeated exposure to human neutrophils over the millennia and contributes to its pathogenic potential for humans.

This paper describes the potent activity of PG-1 against several clinical isolates of *N. gonorrhoeae*, including strains that

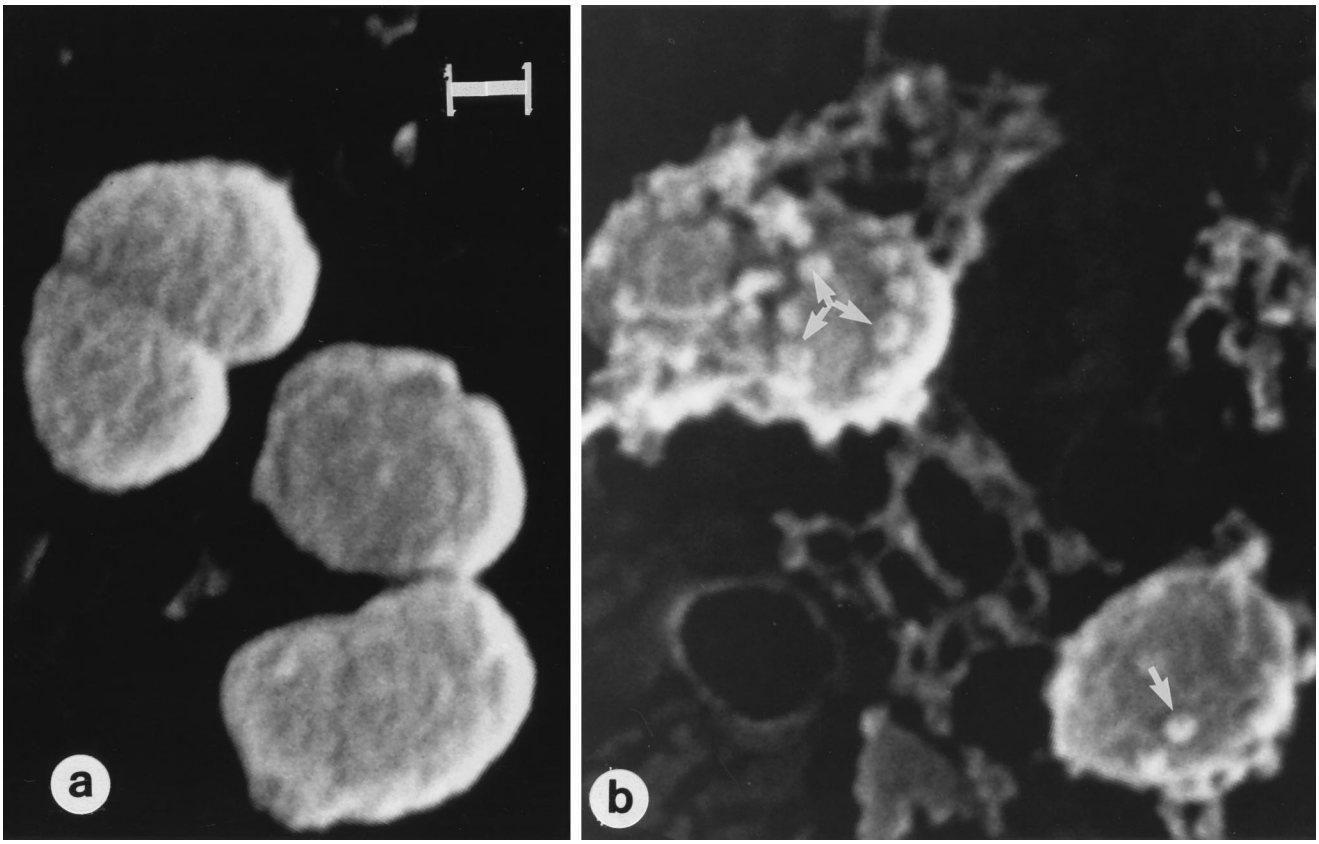

FIG. 6. Scanning electron micrographs of *N. gonorrhoeae*. (a) Control bacteria; (b) organisms 60 min after treatment with protegrin PG-1 (50 μg/ml). The arrows in panel b indicate several of the umbilicated, ≈100-nm-diameter surface structures described in the text. Bar, 0.2 μm.

differed in susceptibility to normal human serum and to antibiotics. Penicillin-resistant strains (one of which also expressed tetracycline resistance) were as susceptible as wild-type organisms, whether their penicillin resistance arose from plasmid-mediated beta-lactamase production or chromosomal determinants. PG-1 is also very active against *C. trachomatis* (17), an organism that also causes severe gynecologic sequelae, including pelvic inflammatory disease and endometritis. Furthermore, it has recently been shown that protegrins can inactivate HIV-1 (16). This remarkable constellation of attributes makes protegrins attractive prototypes for developing topical agents that can be used to prevent STDs. Tests to ascertain the ability of protegrins to inactivate other STD pathogens, such as *Treponema pallidum*, *Trichomonas vaginalis*, and herpes simplex virus, would be of interest.

PG-1	RGG	R	LCYCR	RRF	C	V	CV	GR*
PG-2	RGG	R	LCYCR	RRF	C	**I**	CV	*
PG-3	RGG	**G**	LCYCR	RRF	C	V	CV	GR*
PG-5	RGG	R	LCYCR	**P**RF	C	V	CV	GR*

FIG. 7. Primary sequences of protegrins. Residues that are invariant in all currently reported porcine are boxed and shaded, and the disulfide connectivity is shown. Residues that differ from those in PG-1 are displayed in boldface characters. The asterisk denotes C-terminal amidation.

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