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Susceptibility of *Chlamydia trachomatis* to Protegrins and Defensins

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We compared the susceptibilities of *Chlamydia trachomatis* elementary bodies (EBs) to human defensin HNP-2 and porcine protegrin PG-1, cysteine-rich beta-sheet antimicrobial peptides produced by mammalian leukocytes. Although both peptides protected McCoy cell monolayers from infection by chlamydial EBs, protegrins were especially potent. Protegrin-mediated inactivation of chlamydiae occurred rapidly, was relatively independent of the presence of serum, and was effective against serovars L2, D, and H. Protegrin-treated EBs showed striking morphological changes, with obvious damage to their limiting membranes and loss of their cytoplasmic contents and nucleoid. Their effectiveness against chlamydial EBs and other sexually transmitted pathogens combined with their relative lack of cytotoxicity suggests that protegrins and related molecules could serve as prototypes for topical agents to prevent sexually transmitted chlamydial infection.

Chlamydia trachomatis, a gram-negative eubacterial obligate intracellular pathogen, causes a number of human diseases, including trachoma and genitourinary tract infections that often result in sterility (21). Chlamydiae have a unique dimorphic growth cycle that allows their survival in two discontinuous habitats. Within eukaryotic host cells, Chlamydiae form inclusions and reorganize to a noninfectious reticulate body. These reticulate bodies eventually undergo nucleoid condensation and other changes to form releasable elementary bodies (EBs) that can survive the extracellular environment and infect other cells. Although their inner and outer membranes define a periplasmic space, Chlamydiae differ from other gram-negative bacteria by lacking peptidoglycan (2). The outer membrane of chlamydiae contains several cysteine-rich proteins, including a major outer membrane protein (3, 11) that is probably linked by disulfide bonds to maintain EB membrane structural integrity (32). Register et al. reported that human polymorphonuclear leukocyte granule fractions had antimicrobial activity against *C. trachomatis* and *Chlamydia psittaci* (28).

Defensins are antimicrobial and cytotoxic peptides that contain 29 to 35 amino acid residues, including six invariant cysteines whose intramolecular disulfide bond formation stabilizes a triple-stranded beta-sheet conformation (12, 18). Defensins are abundant in human, rat, and rabbit polymorphonuclear leukocytes but are absent in mouse and horse neutrophils. More recent studies show that purified antimicrobial peptides, defensins, are effective against both gram-negative and gram-positive bacteria as well as fungi (20), mycobacteria, spirochetes, and some viruses. Their mechanism of action appears to involve initial electrostatic interactions with negatively charged target cell surface molecules, followed by insertion into the cell membranes which they permeabilize by forming voltage-regulated channels (17, 18).

Protegrins, a recently identified family of small peptides (16 to 18 amino acid residues), were found in porcine leukocytes and also exert antimicrobial activity against bacteria, fungi, and some enveloped viruses (15). Protegrins resemble tachyplesins, a family of host defense peptides found in the hemocytes of horseshoe crabs, in many respects, including size, the presence of two intramolecular cystine disulfide bonds, and the presence of an amidated C-terminal residue (22). Although protegrins are much smaller than defensins, they show primary sequence homology to certain defensins (15). Recent studies suggest that protegrins can bind to lipopolysaccharide, a property that may help them to insert into the membranes of gram-negative bacteria and permeabilize them (19).

Although protegrins and defensins have been shown to exert potent microbicidal activity against *Escherichia coli*, *Listeria monocytogenes*, and *Candida albicans* (15, 18), their effects on Chlamydiae have not been described. We addressed this question by evaluating the ability of defensins and protegrins to reduce the numbers of inclusion-forming units (IFU) in a well-documented chlamydial culture system.

MATERIALS AND METHODS

Peptides. Human defensins HNP-1 and HNP-2 (29) and rabbit defensin NP-1 were purified from leukocytes as described previously (9). Synthetic protegrins were prepared by solid-phase synthesis, purified after reduction by reverse-phase high-performance liquid chromatography (RP-HPLC), folded, and repurified to homogeneity by RP-HPLC. Synthetic and native protegrins were identical in mass (by fast atom bombardment-mass spectrometry), electrophoretic mobility (by acid-urea-polyacrylamide gel electrophoresis), retention time (by RP-HPLC), and antimicrobial activity against *E. coli* and *L. monocytogenes*. All peptides were stored as lyophilized powders. Peptide stock solutions were prepared from lyophilized powders in sterile acidified water (0.01% glacial acetic acid) at 0.25 mg/ml or 1 mg/ml and subsequently stored at −20°C.

Shell vial assay. We examined the ability of antibiotic peptides to prevent chlamydial infection in a standard shell vial chlamydial assay (4). Briefly, we used coverslipped monolayers of McCoy cells in vials with a final volume of 1 ml of Eagle's minimal essential medium (EMEM) that contained 1 mg of cycloheximide per ml and 10% fetal bovine serum (Bartels Diagnostics, Deerfield, Ill.). Prior to preincubation with peptides, serial 10-fold dilutions of EB seed were made in SPG medium, a mixture of 0.2 M sucrose, 0.004 M KH_2PO_4, 0.009 M Na_2HPO_4, and 0.004 M glutamic acid. A 10^{-2} dilution of the seed yielded 9.2×10^6 IFU/ml. One hundred microliters of this 10^{-2} dilution (9.2×10^5 IFU) was preincubated with different concentrations of peptides in a total volume that was adjusted to 300 μl by adding SPG medium. Just prior to chlamydial inoculation, the maintenance medium from McCoy cells was aspirated without disturbing the cell layer and replaced with 300 μl of the chlamydia-peptide mixture in SPG medium. The vials were centrifuged at 1,500 × *g* for 1 h at 20°C, and thereafter, 1 ml of cycloheximide-containing EMEM (BioWhittaker, Walkersville, Md.) was added per shell vial. The vials were capped and incubated at 37°C for 48 h. After the medium was aspirated, the coverslips were rinsed twice with phosphate-buffered saline (PBS), fixed with ethanol for 10 min, and allowed to dry. For staining, a drop of PBS plus 30 μl of Microtrak fluorescein isothiocyanate-linked monoclonal antibody was added to each shell vial per the manufacturer's instructions (Syva Company, San Jose, Calif.), and after incubating the vials at 35°C for 30 min and washing the cells twice with distilled water, the coverslips

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TABLE 1. Efficacy of antimicrobial peptides against *C. trachomatis*[a]

Peptide	Supplement	IFU count[b] after preincubation of:		
		1 h	2 h	4 h
None	None	222	292	460
PG-1	None	0	0	0
	PG-1	0	0	0
HNP-1	None	35	43	12
	HNP-1	41	19	
NP-1	None	42	20	11
	NP-1	32	7	5

[a] *C. trachomatis* serovar L2 was preincubated for 1 to 4 h with 50 μg of the indicated peptide or an equivalent volume of acidified water and then transferred to shell vials that contained McCoy cells in EMEM. Some of these vials also received supplemental peptide (≈75 μg/ml), as noted.

[b] The IFU values are means of duplicate coverslips.

were mounted on slides and the number of IFU per coverslip was ascertained by fluorescence microscopy. Each IFU can be considered equivalent to a colony of conventional free-living bacteria on a standard culture plate.

Chlamydia inoculation and culture. *C. trachomatis* serovar L2 (L2/434/Bu) was prepared in the laboratory of E. Wagar as described previously (16) and used in all experiments with this serovar. *C. trachomatis* serovars D and H1 were a gift from J. Ito (City of Hope, Duarte, Calif.) and were grown and purified as described previously (serovar D [14] and serovar H1 [13]). Since all seed cultures were prepared from sonicated tissue cultures, i.e., serovar L2 in L929 mouse fibroblasts (ATCC CCL 1) and serovars D and H1 in McCoy cells (ATCC CRL 1696), some host protein was present in seed aliquots even after their concentration by centrifugation. Since the titer of each seed batch was determined at the time of preparation (prior to conducting experiments with antibiotic peptides) and adjusted to produce countable numbers, this approach also diluted background eukaryotic protein carried over during seed preparation. Experiments were repeated two or three times with two to three coverslips per experiment unless otherwise indicated.

Preincubation with antibiotic peptides. One hundred microliters of the diluted chlamydial seed (serovar L2) containing 9.2×10^5 IFU was incubated with the indicated concentrations of antibiotic peptide (or acidified water) in a final volume of 300 μl in 1.5-ml Eppendorf tubes for 1, 2, or 4 h at room temperature (RT). Ten minutes before the samples were inoculated, the maintenance medium (1 ml) was aspirated from McCoy shell vials to allow the peptide-treated and control inocula to be added and centrifuged for 1 h at 1,500 × *g*. Without removing the inocula, the vials received 1 ml of additional EMEM-cycloheximide and were cultured at 35°C for 48 h. In some experiments, additional defensin or protegrin was added to the medium to supplement the final concentrations by ~75 μg/ml. At the completion of culture, the coverslips were immunostained and IFU counts were performed.

Concentration dependence. To determine the concentration of antimicrobial peptides required to kill *C. trachomatis*, we conducted three experiments, wherein serovars L2, D, and H1 were exposed for 2 h at RT to protegrin PG-1 (3.3 to 167 μg/ml) or human defensin HNP-2 (41.6 to 334 μg/ml). With the exception of the protegrin or defensin present in the initial inoculum, no supplemental protegrin or defensin was added to the medium during the 48-h culture period.

Effect of serum. Fetal calf serum is a traditional component of chlamydial culture systems. Because some host serum is also likely to be present at cervical sites in vivo, we examined the effects of 10% fetal bovine serum on the ability of protegrin or defensin to inactivate *C. trachomatis* serovar L2 in a 2-h RT preincubation with 50 μg (167 μg/ml) of peptide, with acidified water as the control.

Delayed addition of peptides. In some experiments, *C. trachomatis* serovar L2 was first exposed to the peptides (38 μg/ml) 1 h after the organisms had been added to McCoy cells, centrifuged, and incubated at 35°C for 1 h. Thereafter, the vials were cultured for 48 h and processed as described above.

Preincubation of monolayer with peptides. To determine whether the antibiotic peptides acted on the target cells as well as the Chlamydiae, we added 25 μg of protegrin or defensin (or an equivalent volume of acidified water) to the McCoy cell monolayers and incubated the cultures for 2 h at RT. Thereafter, this medium was removed, and *C. trachomatis* serovar L2 was inoculated. One milliliter of peptide-free EMEM containing cycloheximide was then added per shell vial, and culture was performed as described above.

Removal of peptide by ultrafiltration. To determine whether prolonged chlamydia-peptide contact was required for inactivation, we added 83.5 μg of protegrin or defensin per ml or an equivalent volume of acidified water to 9.2×10^5 or 1.84×10^6 IFU of *C. trachomatis* serovar L2 in 300 μl of SPG medium and incubated the mixture for 2 h at RT. Thereafter, the mixtures were placed into Centricon 10 ultrafiltration tubes (Amicon, Beverly, Mass.) and centrifuged at 4,451 × *g* for 30 min at 4°C to separate the unbound antibiotic peptide. The retained Chlamydiae were recovered and added to the shell vials as described previously.

Cytotoxicity testing. We assessed the cytotoxicity of defensins and protegrins for McCoy cells by determining their effects on membrane permeability and on growth kinetics (protegrin PG-1 only). Membrane permeability was studied by using the Live/Dead EukoLight Viability/Cytotoxicity Kit (Molecular Probes, Eugene, Oreg.) as described in the manufacturer's suggestions. The effect of defensins and protegrins on McCoy cell growth was measured by culturing subconfluent monolayers for 48 h in RPMI 1640 with 10% fetal calf serum plus 0, 5, or 10 μg of PG-1 per ml and then performing cell counts at 2, 24, and 48 h by removing the cells from the coverslips with 0.25% trypsin in PBS. Experiments were performed twice with duplicate coverslips. In addition, to make certain that protegrin treatment did not render McCoy cells incapable of supporting chlamydial growth, we pretreated the monolayers for 6 h with 300 μl of SPG medium that contained 0, 5, or 10 μg of PG-1. The protegrin-containing solutions were aspirated and replaced with 1 ml of EMEM with 10% fetal calf serum and cycloheximide (the customary medium) and cultured for 18 h at 35°C before infecting with chlamydial EBs by the usual procedure described elsewhere in this report. The IFU were counted after 48 h.

Electron microscopy. Untreated and protegrin-treated *C. trachomatis* serovar L2 organisms were fixed with 2% glutaraldehyde in PBS and postfixed in 1% osmium tetroxide (OsO_4) in PBS. After dehydration with ethanol, they were embedded in Spurr (Polysciences, Warrington, Pa.), a low-viscosity embedding medium (31). Thin sections, approximately 80- to 100-nm thick, were stained with uranyl acetate and lead citrate and examined with a JEOL (Tokyo, Japan) model 100CX electron microscope.

RESULTS

Efficacy against *C. trachomatis*. Our first experiment, described in Table 1, indicated that both protegrins and defensins inhibited *C. trachomatis*. Although protegrin PG-1 completely prevented productive infection, rabbit defensin NP-1 and human defensin HNP-1 allowed residual inclusion formation, albeit at significantly reduced levels. In these and subsequent experiments, McCoy cell monolayers treated and cultured with protegrins or defensins appeared intact, with no detectable change in cell density compared with that of untreated McCoy monolayers.

Dosage and antibiotic effects. The inactivation of *C. trachomatis* by protegrins and defensins was confirmed by additional experiments shown in Fig. 1. Note that protegrin PG-1 was approximately 20-fold more potent than human defensin

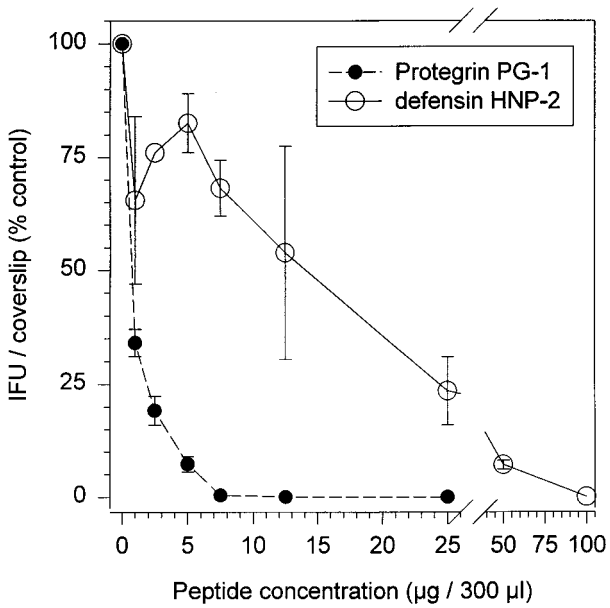

FIG. 1. Effect of synthetic protegrin PG-1 and human defensin HNP-2 on *C. trachomatis* serovar L2. *C. trachomatis* was incubated with various concentrations of antibiotic peptides for 2 h at RT, and infectivity was determined by IFU counts. The IFU values shown are means from three experiments.

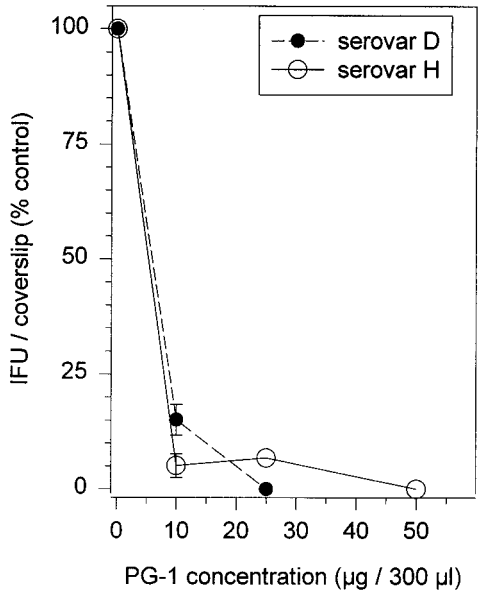

FIG. 2. Effect of synthetic protegrin PG-1 on *C. trachomatis* serovars D and H. The serovars were incubated with PG-1 for 2 h at RT, and infectivity was determined by IFU counts. The IFU values shown are means from three experiments.

HNP-2 on a weight basis (approximately 10-fold more potent on a molar basis). As shown in Fig. 2, PG-1 also inactivated *C. trachomatis* serovars D and H. Although HNP-2 was also active against these serovars, higher concentrations were required for an equivalent effect (data not shown).

Effect of serum. As shown in Table 2, the presence of fetal calf serum (1 or 10%) did not reduce the activity of protegrin against Chlamydiae, whereas the activity of HNP-2 was significantly reduced. The data of Table 2 indicate that the inactivation of chlamydiae occurred during the 2-h incubation period rather than during the ensuing 48 h of tissue culture.

Site of antibiotic peptide effect. When antibiotic peptides were added to chlamydiae 1 h after the centrifugation of *C. trachomatis* EBs onto the cell monolayers, protegrin PG-1 still reduced the number of IFU by 67%, whereas defensin HNP-2 exerted no detectable inhibitory effect on the chlamydiae under these conditions (Table 3). These findings suggest that the antibiotic effect of both peptides is largely exerted extracellularly and that intracellular *C. trachomatis* organisms are not as susceptible to protegrins as extracellular EBs.

TABLE 2. Effect of serum on inactivation of *C. trachomatis* serovar L2

Peptide	% Serum	IFU count after:	
		2-h preincubation[a]	No preincubation[b]
None	0	115	115
PG-1	0	0	92
	1	0	86
	10	0	80
HNP-2	0	1	69
	1	ND[c]	69
	10	61	112

[a] Fifty micrograms of peptide was added to *C. trachomatis* in the presence or absence of fetal bovine serum (serum). The mixture was preincubated for 2 h and then centrifuged onto the McCoy cell monolayers for 60 min and cultured for 48 h. The peptide-free control was preincubated with SPG medium and acidified water and then handled as described above.

[b] *C. trachomatis* with or without fetal bovine serum (serum) was centrifuged onto McCoy cell monolayers for 60 min, and then 50 μg of peptide was added and the incubation was continued for 48 h.

[c] ND, not determined.

TABLE 3. Effect of delayed addition of protegrin or defensin on *C. trachomatis* serovar L2

Treatment[a]	Mean IFU/coverslip[b]		
	Delayed addition[c]	Pretreated monolayers[d]	Centricon experiment[e]
Negative control	0	0	0
Positive control	115	130	41
Protegrin PG-1	49	0	0
Defensin HNP-2	139	137	55

[a] The negative controls received no inoculum, and the positive controls received no peptide treatment.

[b] All data show mean IFU per coverslip after 48 h of incubation and were derived from duplicate samples.

[c] Peptide (50 μg) was added to the shell vials 1 h after the *Chlamydia* inoculum was introduced and centrifuged onto the McCoy cells.

[d] McCoy cell monolayers were preincubated with 25 μg of peptide for 2 h at room temperature, washed, and infected with the standard inoculum of *C. trachomatis.*

[e] Chlamydiae that had been incubated with 25 μg of peptide for 2 h at RT were washed by ultrafiltration in Centricon 10 units, reconstituted to the original volume with SPG medium, and inoculated onto McCoy cell monolayers.

When McCoy cell monolayers were preincubated with protegrin for 2 h, washed to remove the peptides, and then immediately inoculated with chlamydiae and cultured in peptide-free EMEM, complete inhibition of IFU was observed. In contrast, similar treatment with human defensin, HNP-2, caused no reduction in IFU (Table 3). This suggests that protegrins may associate with the host cell, either by uptake or binding to its membrane, and that such cell-associated protegrin molecules retain their ability to inactivate chlamydiae.

Even after protegrin-treated *C. trachomatis* organisms were subjected to centrifugal ultrafiltration to remove unbound peptide, they failed to form IFU, whereas after they were similarly exposed to defensin HNP-2, they retained their ability to infect McCoy cells (Table 3). This finding suggests that protegrins (but not defensins) induce permanent and irreversible alterations in the chlamydial EBs that preclude later productive infection.

Cytotoxicity testing. As judged by the EukoLight procedure, the effects of defensins and protegrins on the permeability of McCoy cell membranes was slight. Monolayers incubated with 10 μg of PG-1 per ml or 60 μg of HNP-2 per ml for 48 h showed <1% nonviable cells in the centers of the coverslips. At the edges, where cell densities were considerably lower, approximately 5% of control cells and 10 to 15% of defensin- or protegrin-treated cells were nonviable. Although PG-1 was not cytocidal for McCoy cells, it was cytostatic, as indicated by the following experiment. The starting number of McCoy cells per shell vial was $(0.34 \pm 0.03) \times 10^6$ (mean ± standard error of the mean). The following cell counts were obtained after culture in the presence or absence of 10 μg of PG-1 per ml. After 24 h, control (protegrin-free) shell vials contained $(1.04 \pm 0.11) \times 10^6$ cells, and protegrin-treated vials contained $(0.78 \pm 0.04) \times 10^6$ cells. After 48 h, control vials contained $(1.97 \pm 0.22) \times 10^6$ cells, and protegrin-treated vials contained $(0.85 \pm 0.12) \times 10^6$ cells. In other experiments (data not shown), we determined that McCoy cells which had been exposed to protegrin PG-1 (5 or 10 μg) or defensin HNP-2 (10 or 60 μg) for 6 h and then grown in peptide-free medium for 18 h supported chlamydial growth to the same extent as control cells that had never been exposed to

either peptide. From the observations described above, we concluded that the antichlamydial effects of protegrins and defensins were not attributable to host cell cytotoxicity.

Electron microscopy. Electron microscopy of *C. trachomatis* seed stocks revealed intact bodies measuring about 0.3 μm in diameter, with an intact double membrane and condensed nucleoid, consistent with the size and ultrastructure of chlamydial EBs. Protegrin-treated inocula lacked intact EBs but contained membrane-bound shells comparable in size to EBs (Fig. 3). These had a single membrane with amorphous material attached both inside and outside. The amorphous cell-derived debris in the seed EB cultures resulted from the sonication procedure used to obtain infectious chlamydiae.

DISCUSSION

Gram-negative bacteria are sensitive to a wide variety of cationic peptides (39), including histones (8), protamines (8, 36–38), polymyxin-like antibiotics (34), and cationic polypeptides from polymorphonuclear leukocytes (5, 6, 30). Among the antimicrobial peptides of mammalian phagocytes are defensins (18) and protegrins (10, 15, 40).

We found that chlamydial EBs show marked sensitivity to synthetic protegrins, host defense peptides whose natural counterparts were originally purified from porcine leukocytes (15). Human and rabbit defensins had similar effects but were considerably less potent. The finding that both protegrins and defensins could protect otherwise permissive target cells from chlamydial infection is noteworthy given several biological features of chlamydiae. The metabolically inert but infectious chlamydial EB is surrounded by a multiply cross-linked outer membrane, producing a stable spore-like structure that can persist in the extracellular environment. These cross-linkages are formed by cysteine-rich proteins present in the outer membrane complex (7, 23). Protegrin is a small polypeptide whose four cysteine residues are cross-linked to stabilize a beta-sheet domain (10, 15). Given the construction of the chlamydial outer membrane and the spore-like properties of the chlamydial EB, whether protegrins or defensins would exert significant antimicrobial effects in this system could not be predicted.

Our dose-response experiments showed that relatively low concentrations of protegrin were effective against several serovars of *C. trachomatis* (Fig. 1 and 2). The ability of protegrins (but not defensins) to inactivate chlamydiae in the presence of 10% fetal bovine serum is especially encouraging in that the use of antimicrobial peptides to protect cervical surfaces from chlamydial infection will require agents that maintain activity in the presence of serum. It has been noted that defensins interact with serum (24–26) and that the presence of serum reduces their antibiotic effect (18). In contrast, no inhibitory effect of serum on protegrin activity against *C. trachomatis* was noted (this study), and similar findings have been obtained in studies with other gram-negative bacteria (19).

The lessened efficacy of protegrin after chlamydiae had entered the intracellular milieu suggest that its principal effects are exerted prior to or shortly after intracellular uptake perhaps by directly inactivating EBs or by preventing an early post-cell entry event (Table 3). The mechanism of protegrin-mediated activity against *C. trachomatis* remains to be ascertained. Also, protegrin may prevent uptake of EB by host cells, since we found a strong effect when monolayers were pretreated with protegrin. However the results of the Centricon 10 experiments (Table 3) and the striking morphological changes seen in electron micrographs of protegrin-treated *C. trachomatis* organisms indicate a direct effect on the pathogen itself.

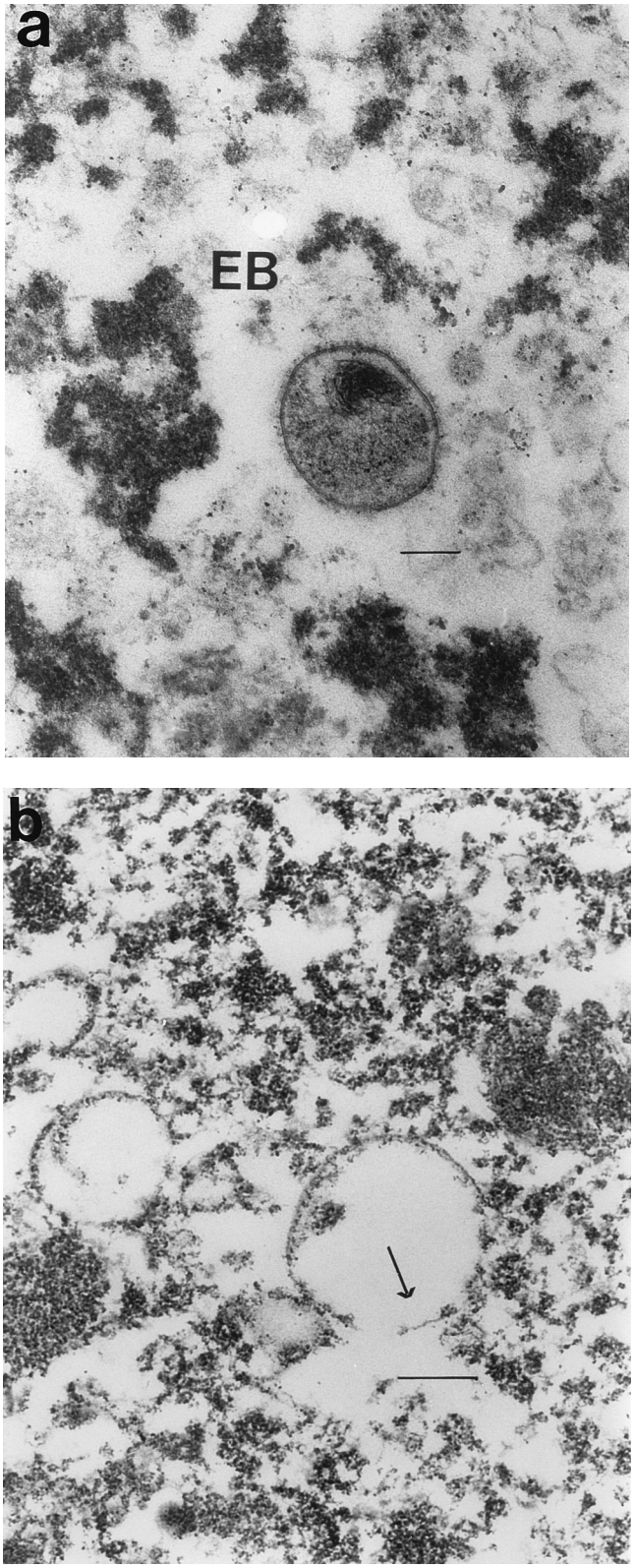

FIG. 3. Electron micrograph. (a) *C. trachomatis* serovar L2 showing a representative, intact chlamydial EB particle bounded by a double membrane. (b) Synthetic PG-1-treated *C. trachomatis* serovar L2, showing membrane shells, apparently with a single membrane, to which amorphous material is attached. Bar, 0.1 μm.

Perhaps protegrins will prove to alter both the intrinsic resistance of the host cell and the integrity of the microorganism.

To date, the major attempts to prevent chlamydial infection have included vaccine development and antibiotic therapy. The early vaccine trials with formalinized whole EBs were not protective, and more recent subunit constructs, including several epitopes of major outer membrane protein, have yet to meet with success (1, 32, 33). Effective antibiotics include tetracyclines, erythromycin, and azithromycin, all of which inhibit chlamydial protein synthesis. However, the prophylactic use of antibiotics is compromised by the tendency of chlamydial infections to go unrecognized. Clearly, new strategies for treatment of such infections are needed.

These experiments clearly establish that protegrins, endogenous antimicrobial peptides derived from porcine leukocytes, impair the ability of *C. trachomatis* EBs to infect cells productively. Because protegrins can also inactivate human immunodeficiency virus type 1 (35) and have exceptionally potent activity against *Neisseria gonorrhoeae* (27), this class of peptide antibiotics would appear to hold considerable promise for use as topical agents to prevent sexually transmitted diseases.

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