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Treponema denticola Is Resistant to Human β-Defensins

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Spirochetes, including *Treponema denticola*, are implicated in the pathogenesis of periodontal disease. Because *T. denticola* lacks lipopolysaccharides that serve as targets for human β-defensin (hβD) binding, we postulated that *T. denticola* would resist killing by hβD. We showed that *T. denticola* is resistant to hβD-1 and -2. Protease inhibitors did not enhance killing of *T. denticola* by hβD-2, suggesting that degradation of hβD-2 by treponemal proteases is not a major factor in *T. denticola* resistance.

Periodontal disease is a destructive inflammatory condition resulting in tooth loss; severe disease requires clinical intervention and affects 6 million Americans (17, 31). Periodontal disease is linked to cardiovascular disease, as well as delivery of preterm, low-birth-weight infants, increasing its recognition as a public health concern (19, 22, 24). Oral spirochetes, including *Treponema denticola*, are implicated in the pathogenesis of periodontal disease (2, 6, 15), and spirochetes comprise 40% of the microflora found in diseased sites (16).

Microorganisms induce a variety of responses from epithelial cells, including the expression of antimicrobial peptides, such as β-defensins. These small, cationic peptides interact with negatively charged cell wall components of bacteria and fungi, disrupting membrane integrity (9). Human β-defensin 1 (hβD-1) is expressed constitutively by gingival epithelial cells, while hβD-2 expression is induced in response to periodontal microorganisms such as *Fusobacterium nucleatum* (12, 13). Some antimicrobial peptides have significant in vitro bactericidal activity against periodontal pathogens, including *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans*, and *Eikenella corrodens* (20, 21); the role of antimicrobial peptides in controlling periodontal pathogens in vivo is unknown.

Defensins are known to interact strongly with lipopolysaccharide (LPS) due to its negative charge. *T. denticola* lacks a traditional LPS, as do many other spirochetes (25). Further, *T. denticola* is resistant to polymyxin B, a cationic peptide antibiotic that interacts with LPS (1), at more than 1,000 μg/ml. These facts prompted us to investigate whether *T. denticola* is resistant to human β-defensins.

T. denticola strains ATCC 35405, ATCC 35404, ATCC 33521, and GM-1 (30) were obtained from Pamela Braham (University of Washington) and maintained as previously described (4). *Escherichia coli* strain DH5α was obtained from Patricia Totten (University of Washington) and maintained in Luria-Bertani (LB) medium at 37°C. Four-day, late-logarithmic-phase cultures of *T. denticola* and overnight cultures of *E. coli* were centrifuged at 10,000 × *g* for 10 min at 20°C. Bacteria were washed once and resuspended in phosphate-buffered saline to yield a final concentration of 10^7 organisms/ml. Bacteria with or without hβD-1 or -2 (Peptides International, Louisville, Ky., and United Biochemical Research, Inc., Seattle, Wash.) were added to duplicate wells of a 96-well polypropylene plate (Costar) at physiologically relevant concentrations ranging from 0.01 to 100 μg/ml and incubated at 37°C for 4 h (18, 26). Motile treponemes were enumerated by dark-field microscopy. *E. coli* cells were diluted in distilled H_2O and plated on LB agar overnight to enumerate CFU. The percentage of killing was calculated as follows: 100 − [(number of viable treated organisms/number of viable untreated organisms) × 100]. All *T. denticola* strains tested were significantly less susceptible to killing by either defensin than was *E. coli* (Fig. 1). There was no statistical difference in the number of live treponemes after incubation with or without peptide ($P > 0.05$, Student's two-tailed *t* test assuming unequal variances). Similar results were seen with 3-day, mid-logarithmic-phase *T. denticola* ATCC 35404, and no increase in killing of *T. denticola* was seen even after 24 h of exposure to 100 μg of hβD per ml (data not shown). The different susceptibilities of various strains of *T. denticola* to hβD-1 may influence their relative abilities to colonize the oral cavity (13). No strain-to-strain differences in susceptibility were seen with hβD-2. These data demonstrate that, unlike *E. coli*, *T. denticola* is able to resist killing by human β-defensins.

Because loss of spirochetal motility does not always correlate with loss of viability, we tested metabolic activity of defensin-exposed *T. denticola* using Alamar Blue, a dye that is reduced in the presence of actively metabolizing organisms (28). This method has been used successfully to monitor antimicrobial activity against *Mycobacterium tuberculosis* (8). As there was little difference in hβD-2 sensitivity among strains, *T. denticola* ATCC 35404 was used for all experiments. Organisms (final concentration, 10^7/ml) were incubated with 10 μg of hβD-2 per ml or 0.1% sodium dodecyl sulfate for 4 h at 37°C and 5% CO_2. A 1/10 volume of Alamar Blue (Biosource, Camarillo, Calif.) was then added, and the bacteria were incubated for an additional 20 h. *E. coli* was used as a control for hβD-2 activity. Optical density for each well was read on a Dynatech colorimetric plate reader at 570 and 600 nm. Percent reduction was calculated as described previously (8). Results from four experiments confirm that *T. denticola* ATCC 35404 remains metabolically active after incubation with hβD-2. In a representative experiment, incubation in medium alone showed 72.06% ± 10.01% (mean ± standard error of the

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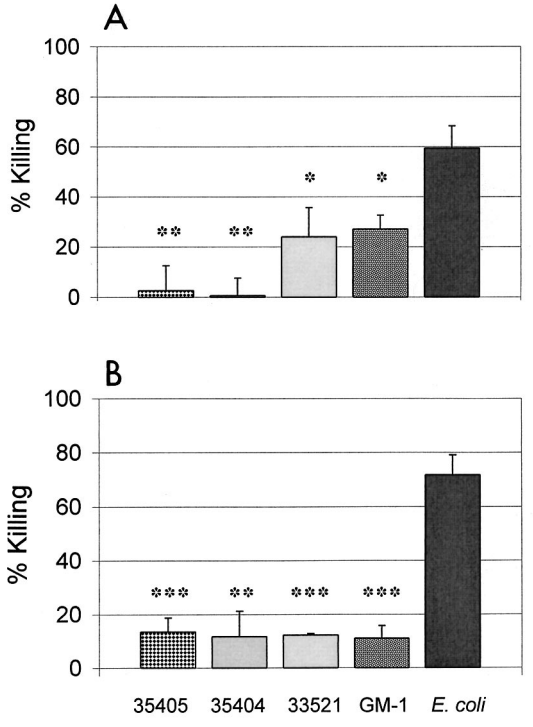

FIG. 1. Sensitivity of *T. denticola* and *E. coli* to human β-defensins. *T. denticola* strains representing three serotypes (ATCC 35405, ATCC 33521, and ATCC 35404), as well as one clinical isolate (GM-1), remain motile in the presence of 10 μg of hβD-1 (A) and hβD-2 (B) per ml. *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$, compared to killing of *E. coli*. Percent killing was calculated as described in the text. Data are means ± SEM from multiple 4-h experiments. *P* values were determined by Student's *t* test for two samples, assuming unequal variances.

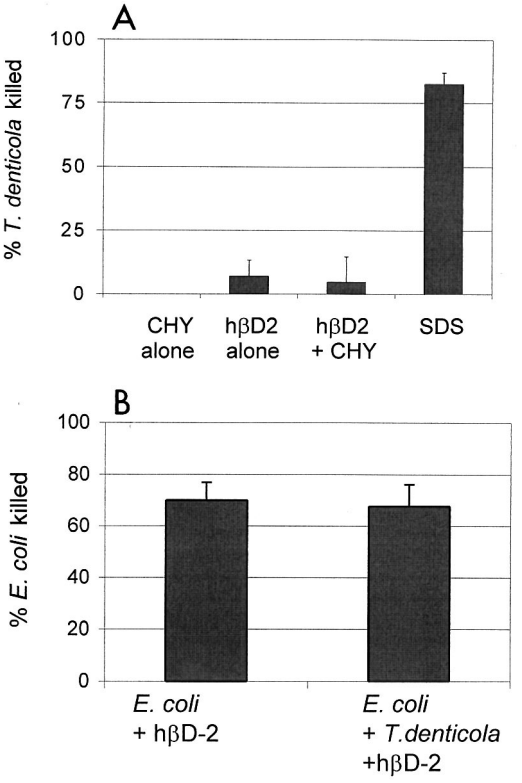

FIG. 2. *T. denticola* proteases are not responsible for resistance to hβD-2. (A) *T. denticola* ATCC 35404 incubated in the presence or absence of 100 μM chymostatin (CHY) remained resistant to 10 μg of hβD-2 per ml over a 4-h period. (B) *E. coli* was incubated in the presence of 10 μg of hβD-2 per ml for 4 h, with or without equal numbers of *T. denticola* ATCC 35404. Data are means ± SEM from five (A) and three (B) experiments. SDS, sodium dodecyl sulfate.

mean [SEM]) reduction, while incubation with 10 μg of hβD-2 per ml resulted in 71.57% ± 9.88% reduction. Dead bacteria yielded 12.91% ± 1.49% reduction. These results confirm that under these assay conditions, *T. denticola* remains metabolically active after treatment with hβD-2.

The precise mechanism of killing by β-defensins is unknown. Research with other cationic peptides suggests that secondary targets, such as cytochromes or components of DNA synthesis, may be necessary for killing (14, 29). To determine the ability of *T. denticola* to replicate following exposure to hβD-2, *T. denticola* ATCC 35404 cultures at 10^7 organisms/ml were incubated with and without 10 μg of hβD-2 per ml in GM-1 medium at 37°C and 5% CO_2 for 4 h. Cultures were diluted serially in semisolid GM-1 medium (with 0.5% Noble agar and 0.5% gelatin) and incubated anaerobically at 37°C for 1 to 2 weeks (5). *E. coli* incubated in GM-1 medium was used as a control for hβD-2 activity and was quantitated by serial dilution on LB agar. Two independent experiments demonstrate that *T. denticola* was not killed by hβD-2 under these assay conditions: untreated treponemes produced $7.2 \times 10^6 \pm 4.9 \times 10^6$ CFU/ml, while treponemes incubated in the presence of peptides produced $1.8 \times 10^7 \pm 1.4 \times 10^7$ CFU/ml ($P = 0.55$, Student's two-tailed *t* test assuming unequal variances). Further, colony size for the hβD-2-treated treponemes was equivalent to that for untreated treponemes, suggesting that there was no sublethal effect on growth. *E. coli* incubated with hβD-2 in this medium was killed as readily as in phosphate-buffered saline, suggesting that the GM-1 medium itself did not inhibit the activity of hβD-2 (data not shown).

One reason for *T. denticola*'s resistance to β-defensins may be that the peptides are degraded or inactivated. The proteases of *T. denticola* are known to degrade several host proteins and bioactive peptides (3, 7, 10, 11, 23, 27). To test the hypothesis that *T. denticola* can degrade hβD-2, thus preventing its activity, we examined the effect of protease inhibitors on survival of *T. denticola* exposed to hβD-2. *T. denticola* ATCC 35404 and 10 μg of hβD-2 per ml were incubated in the presence or absence of final concentrations of 100 μM chymostatin (Sigma Chemicals, St. Louis, Mo.) at 37°C and 5% CO_2 for 4 h. Viable bacteria were enumerated by dark-field microscopy (*T. denticola*) or plate counts (*E. coli*). Killing of *T. denticola* was not enhanced in the presence of chymostatin, which inhibits the major outer membrane-associated protease of *T. denticola*, dentilisin (Fig. 2A). Similar results were obtained with other strains of *T. denticola* and with aprotinin (Sigma) and Complete protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, Ind.) (data not shown). The activity of the protease inhibitors was confirmed by measuring the degradation of recombinant interleukin 8 (R&D Systems, Minneapolis, Minn.) by *T. denticola* in the presence or absence of the

protease inhibitors; chymostatin effectively inhibited >90% of interleukin 8 degradation by *T. denticola* (data not shown). These results suggest that resistance of *T. denticola* to hβD-2 is not due to proteolytic destruction of defensin peptides. To examine the possibility that hβD-2 is inactivated by *T. denticola* by an independent method, coculture killing assays were performed. If *T. denticola* inactivates hβD-2 by proteolytic degradation, then the presence of *T. denticola* should protect *E. coli* from killing by hβD-2 in these experiments. Killing of *E. coli* by 10 μg of hβD-2 per ml was unaffected by the presence of equal numbers of *T. denticola*, confirming that *T. denticola* does not inactivate hβD-2 or decrease its biological activity for sensitive organisms (Fig. 2B). These results suggest that mechanisms other than degradation by protease are responsible for resistance to defensins.

In conclusion, we have demonstrated that *T. denticola* is resistant to killing by human β-defensins, that this resistance exists across strains, and that this resistance is not due to inactivation of the defensins by treponemal proteases. Spirochetes are frequently seen in close association with the epithelium, and resistance to the elevated concentrations of β-defensins at this interface may explain the preponderance of spirochetes observed at those sites in periodontal lesions. The resistance of *T. denticola* to β-defensins may confer a survival advantage for *T. denticola*, allowing colonization and persistence in the periodontal pocket.

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ADDENDUM IN PROOF

The resistance to human β-defensin 2 by *Treponema denticola* tested in low-sodium (10 μM) buffer was equal to that seen in phosphate-buffered saline.

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