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 Treponema denticola is resistant to polymyxin B, a cationic peptide antibi-

-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.

T. denticola strains ATCC 35405, ATCC 35404, ATCC 35521, 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 10⁷ 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 polystyrene 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 H₂O 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, 10⁵/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% CO₂. 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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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% CO₂ 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 hβD-2 is unknown.

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% CO₂ 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 mM) buffer was equal to that seen in phosphate-buffered saline.

REFERENCES