Oral Microbiology Immunology 2004: 19: 403–407
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Oral Microbiology and Immunology

Resistance to human β-defensins is common among oral treponemes

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Brissette CA, Simonson LG, Lukehart SA. Resistance to human β-defensins is common among oral treponemes.
Oral Microbiol Immunol 2004: 19: 403–407. © Blackwell Munksgaard, 2004.

Background/aims: Oral treponemes are implicated in the pathogenesis of periodontal disease. We have previously shown that *Treponema denticola* ATCC type strains and strain GM-1 are resistant to killing by human β-defensins (hβD)-1 and -2. We hypothesize that resistance to β-defensins is a common feature of oral treponemes, which allows colonization and persistence in the oral cavity. In this study, we tested additional isolates of *T. denticola*, as well as six other species of treponemes, for resistance to hβD-1, -2 and -3. We also examined the four ATCC strains of *T. denticola* and strain GM-1 for resistance to hβD-3.
Methods: Resistance was determined by motility and Alamar Blue assays for metabolic activity.
Results: All *T. denticola* strains tested were resistant to hβD-1, -2 and -3, with the exception of strain Ambigua, which was sensitive to hβD-2 and -3. All other treponemes except *Treponema vincentii* were resistant to hβD-1. *Treponema pectinovorum* was sensitive to hβD-2, while *T. vincentii*, *T. pectinovorum* and *Treponema maltophilum* were sensitive to hβD-3. *Escherichia coli* was used as a control organism and was killed by all three defensins.
Conclusion: Resistance to the constitutively expressed hβD-1 may assist treponemes in initial colonization of epithelial surfaces, while resistance to the inducible hβD-2 and -3 would allow some treponemes to survive in active periodontal lesions.

Key words: *Treponema*; defensins; periodontal disease

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Accepted for publication August 2, 2004

Humans produce several antimicrobial peptides, including the α and β defensins and the cathelicidin LL-37. α-defensins HNP 1–4 are expressed by neutrophils, and α-defensins 5 and 6 are expressed by Paneth cells of the small intestine. β-defensins are epithelium-derived; four have been characterized to date. LL-37, the only human cathelicidin, is produced by both neutrophils and epithelium (35). There has been great interest in using synthetic antimicrobial peptides as an adjunct to traditional therapies for oral diseases (15, 30, 44), but naturally occurring antimicrobial peptides likely play a role in protection from periodontal disease (1, 23). Severe periodontal disease is seen in patients with morbus Kostmann, an inherited neutrophil disorder, and is associated with a deficiency of antimicrobial peptides including LL-37 and α-defensins HNP 1–4 (36).

Both epithelial- and neutrophil-derived antimicrobial peptides from nonhuman mammals have proven effective against periodontal pathogens such as *Porphyromonas gingivalis*, *Prevotella intermedia*, *Actinobacillus actinomycetemcomitans*, *Eikenella corrodens*, and *Capnocytophaga* spp. *in vitro* (27–29). Recently, β-defensins were demonstrated to have activity against *P. gingivalis*, actinomycetes, streptococci, and *Candida* species (17, 25, 33). β-defensins are found in saliva and gingival crevicular fluid, and are expressed by the oral epithelium, tongue, and salivary glands (3, 9, 10, 12, 13, 26, 37). Human β-defensin-1 is expressed constitutively in gingival tissues; hβD-2 and -3 are induced in response to some periodontal microorganisms and inflammatory stimuli (18–20).

Our previous studies indicate that *Treponema denticola* ATCC type strains and strain GM-1 are resistant to hβD-1 and -2 (6). However, there are more than 40

additional oral treponemal species, many uncultivated, and their sensitivities to β-defensins have not been determined (11). In this study we investigate the susceptibility of additional isolates of *T. denticola* and several species of oral treponemes to hβD-1, -2, and -3.

Material and methods

Bacterial culture

T. denticola strains ATCC 35405, 35404, 33521, 33520 and GM-1 were obtained from Pamela Braham (University of Washington, Seattle, WA) and maintained as previously described (2). *Escherichia coli* strain ML35 was obtained from ATCC (American Type Culture Collection, Rockville, MD) and maintained in Luria–Bertani medium at 37°C. All other *Treponema* species utilized in this study are listed in Table 1. *Treponema lecithinolyticum* and *Treponema maltophilum* were maintained in OMIZ-P4 (45) with the following additions: for *T. lecithinolyticum*, 100 mg/l asialofetuin, 2 g/l D-trehalose, 2 g/l L-rhamnose (Becton Dickinson and Company, Cockeysville, MD), 2 g/l D-sucrose; for both *T. lecithinolyticum* and *T. maltophilum*, 1% v/v yeast extract (Becton Dickinson and Company), 1% v/v neopeptone (Becton Dickinson and Company), and 1% heat-inactivated human serum. *Treponema medium*, *Treponema socranskii* and *Treponema vincentii* were maintained in NOS media as modified by Walker *et al*. (43). *Treponema pectinovorum* was maintained in GM-1 medium supplemented with 2% v/v heat-inactivated rabbit serum, 150 mM ACES buffer, 0.6% w/v D-galacturonic acid and 0.75% v/v yeast extract. Because defensin sensitivity may be affected by growth phase, growth curves for all oral treponeme species were established (data not shown), and testing was conducted with log phase organisms. All treponemes were grown anaerobically. Unless otherwise stated, all chemicals and reagents were from the Sigma Chemical Company (St. Louis, MO).

Defensin killing assay

Four-day log-phase cultures of *Treponema* species and log-phase cultures of *E. coli* were centrifuged at 10,000 × *g* for 10 min at 20°C. Bacteria were washed once and resuspended in modified chemically defined medium (OMIZ-P4 without phenol red and sugars). 1×10^8 motile treponemes/ml were added to quadruplicate wells of a 96-well polypropylene plate (Corning Incorporated Life Sciences, Acton, MA) and incubated with 10 μg/ml of hβD-1, -2 or -3 (Peprotech, Rocky Hill, NJ) or 0.2% SDS (positive control for killing). Previously, we determined that *T. denticola* was insensitive to a range of concentrations of hβD-1 and -2, up to 100 μg/ml (6). Motility (% motile) was determined by dark-field microscopy. After 4 h of incubation at 37°C and 5% CO_2, a 1/10 vol. of Alamar Blue (Biosource, Camarillo, CA) was added and

Table 1. Bacterial strains and sources

Bacterial species	Strain	Source
Treponema denticola	Ambigua	L. Simonson
Treponema denticola	T32A	L. Simonson
Treponema denticola	D65BR1	L. Simonson
Treponema denticola	7	L. Simonson
Treponema medium		ATCC 700293
Treponema vincentii		ATCC 33580
Treponema lecithinolyticum		ATCC 700332
Treponema maltophilum		ATCC 51940
Treponema socranskii ssp. *socranskii*		ATCC 35536
Treponema pectinovorum		ATCC 33768

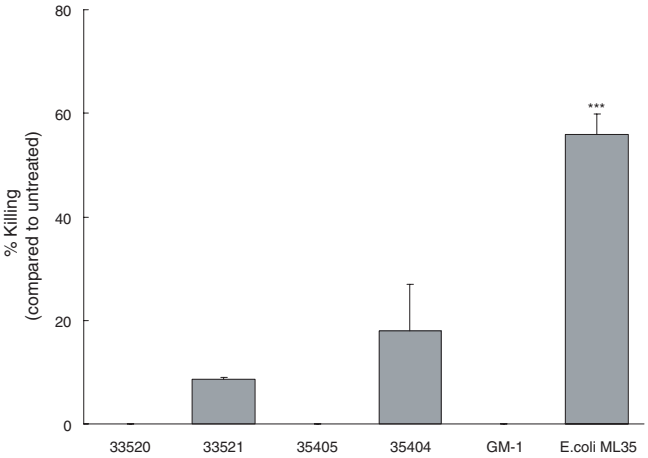

Fig. 1. T. denticola ATCC type strains are resistant to killing by hβD-3. 1×10^8 mid-log phase treponemes/ml were incubated with 10 μg/ml of hβD-3 for 4 h. A 1/10 vol of Alamar Blue was added and bacteria were incubated for an additional 20 h. Reduction of Alamar Blue indicates treponemal viability. Data represent the means (percent killing) and standard errors from four or more experiments. Similar results were shown by motility assay. Student's *t*-test assuming unequal variances was used to determine significance; ***$P<0.001$.

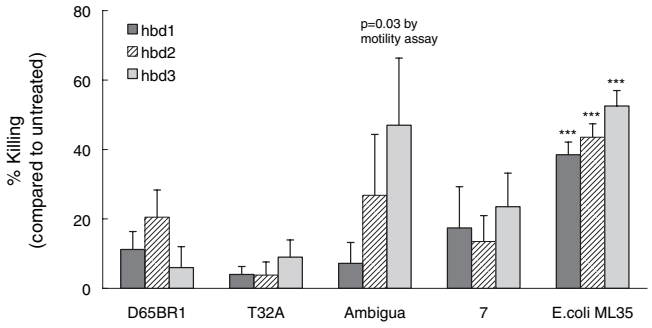

Fig. 2. Other *T. denticola* strains are resistant to killing by hβD-1, -2, and -3. 1×10^8 mid-log phase treponemes/ml were incubated with 10 μg/ml of hβD-1, -2, or -3 for 4 h. A 1/10 vol of Alamar Blue was added and bacteria were incubated for an additional 20 h. Reduction of Alamar Blue indicates treponemal viability. Data represent the means (percent killing) and standard errors from four or more experiments. Similar results were shown by motility assay. Student's *t*-test assuming unequal variances was used to determine significance of killing compared to untreated bacteria; ***$P<0.001$.

bacteria were incubated for an additional 20 h. The optical density for each well was read on a Dynatech colorimetric plate reader at 570 and 600 nm. Percent reduction of Alamar Blue was calculated according to manufacturer's instructions. Percent killing was determined by the formula:

(% reduction in presence of peptide)/(% reduction in absence of peptide) × 100

As a control for hβD activity, *E. coli* ML35 was incubated in the same manner, and viability was determined by plate count. Student's *t*-test assuming unequal variances was used to determine significance. Previously, we demonstrated that the Alamar Blue assay for metabolic activity correlated with both motility of *T. denticola* as visualized by dark-field microscopy, and viability as determined by colony forming units on semisolid medium (6). Viability as measured by Alamar Blue reduction and treponemal motility correlate in both stationary and log phase growth ((6) and data not shown).

Results

T. denticola ATCC type strains are resistant to killing by hβD-3

Previously, we determined that *T. denticola* type strains ATCC 35404, 35405, 33520, 33521, as well as strain GM-1, are resistant to a range of concentrations of hβD-1 and -2, that no killing could be observed up to 24 h, and that resistance was present in both stationary and mid-log phase (6). To determine whether these strains are also resistant to hβD-3, *T. denticola* strains were incubated with hβD-3 and resistance was determined by motility and Alamar Blue assay. All four ATCC strains and strain G^{-1} were resistant to hβD-3 under these assay conditions, whereas significant killing of *E. coli* occurred ($P<0.001$, Fig. 1). hβD-3 concentrations as high as 230 μg/ml had no effect on *T. denticola* viability (data not shown).

Other *T. denticola* strains are resistant to killing by hβD-1, -2, and -3

To determine whether resistance to β-defensins is common among more recent isolates of *T. denticola*, clinical isolates representing three serovars were tested for susceptibility to hβD-1, -2, and -3. Strains D65BR1, T32A, and 7 were resistant to hβD-1, -2 and -3 as determined by both Alamar Blue assay (Fig. 2) and motility determination (data not shown). Strain Ambigua demonstrated some susceptibility to hβD-2 and -3; whereas the level of killing was not significantly different than the control in the absence of peptide, it was considerably higher than any other *T. denticola* strains tested, on a par with the observed *E. coli* killing (Fig. 2). In the motility assay, Ambigua was significantly killed by hβD-3 ($P=0.03$, data not shown). Data for all *T. denticola* strains tested are summarized in Table 2A.

Susceptibility of oral treponemes to killing by hβD-1, -2, and -3

Six oral treponeme species were tested in mid-log phase for susceptibility to hβD-1, -2, and-3. As demonstrated in Fig. 3, only *T. vincentii* was killed by hβD-1 after 4 h ($P<0.05$). *T. pectinovorum* was killed by hβD-2 ($P<0.05$), while *T. vincentii*, *T. pectinovorum*, and *T. maltophilum* were killed by hβD-3 ($P<0.01$, 0.05, 0.05, respectively). Many of the oral *Treponema* spp. are quite fastidious and do not remain viable in the absence of antimicrobial peptides for longer than a few hours, so we were unable to determine if extended incubation in the presence of β-defensins might demonstrate killing. Taken together, these results suggest that most oral treponemes are resistant to hβD-1 and -2, although three species of treponemes are

Table 2. Summary of treponemal susceptibility to hβD-1, -2, and -3

A	Serovar	hβD-1	hβD-2	hβD-3
35405	A	–	–	–
7	B	–	–	–
33521	B	–	–	–
GM-1	B-like	–	–	–
35404	C	–	–	–
33520	C	–	–	–
T32A	C	–	–	–
D65BR1	D	–	–	–
Ambigua	D	–	+/–	+/–

B	Phylogenetic group	hβD-1	hβD-2	hβD-3
T. vincentii	1	+	–	+
T. medium	1	–	–	–
T. lecithinolyticum	4	–	–	–
T. maltophilum	4	–	–	+
T. socranskii	6	–	–	–
T. pectinovorum	8	+/–	+	+

Data are summarized from Fig. 1–3. Some of the data on *T. denticola* ATCC 35405, 35404, 33520, 33520, and G^{-1} were previously published (6). –, no killing. +, statistically significant killing ($P<0.05$). +/–, killing not significant, but strong trend towards susceptibility.

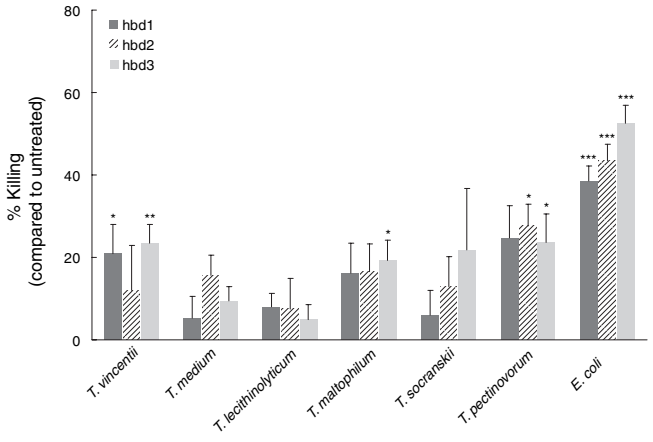

Fig. 3. Susceptibility of oral treponemes to killing by hβD-1, -2, and -3. 1×10^8 mid-log phase treponemes/ml were incubated with 10 μg/ml of hβD-1, -2, or -3 for 4 h. A 1/10 vol of Alamar Blue was added and bacteria were incubated for an additional 20 h. Reduction of Alamar Blue indicates treponemal viability. Data represent the means (percent killing) and standard errors from four or more experiments. Similar results were shown by motility assay. Student's *t*-test assuming unequal variances was used to determine significance; * $P<0.05$. ** $P<0.01$, *** $P<0.001$.

sensitive to hβD-3. *T. medium*, *T. lecithinolyticum*, and *T. socranskii* were resistant to all three defensins, just like *T. denticola*. Data for all treponeme strains tested are summarized in Table 2B.

Discussion

Oral treponemes are closely linked with periodontal disease, and make up the bulk of the microflora present in diseased sites (21, 22). To thrive in this inflammatory environment, oral *Treponema* must have evolved mechanisms for avoiding the host's innate immune response. Recent studies have elegantly demonstrated the importance of antimicrobial peptides such as β-defensins *in vivo* (7, 31, 32, 34, 38). We have previously shown that *T. denticola* ATCC strains 35405, 35404, 33520, 33521, and strain GM-1 are resistant to a range of concentrations of hβD-1 and -2. We now demonstrate that these five *T. denticola* strains are also resistant to hβD-3. Resistance to hβD 1-3 is common both among ATCC strains and more recent isolates. Sensitivity to a given hβD does not appear to correlate with *T. denticola* serovar, as only one of nine isolates demonstrated any sensitivity to β-defensins (Strain Ambigua, Fig. 2).

Treponema have been placed into 10 phylogenetic groups based on 16s rRNA analysis; seven of these groups have cultivatable members, and all groups have representative species or clones present in the mouth (11). We examined the sensitivity of several treponema to β-defensins. Only *T. vincentii*, one of seven species tested, is sensitive to hβD-1. Only *T. pectinovorum* is sensitive to hβD-2, and three species tested are sensitive to hβD-3. Interestingly, hβD-1 is the least potent human β-defensin, while hβD-3 has the broadest spectrum of antimicrobial activity (16, 17, 42), which correlates with our findings. Phylogenetic grouping did not appear to correlate with sensitivity or resistance: *T. medium* and *T. vincentii* are closely related Group 1 treponemes, but only *T. vincentii* demonstrates sensitivity to hβD-1 and -3. *T. maltophilum* and *T. lecithinolyticum* are Group 4 treponemes, but only *T. maltophilum* is sensitive to hβD-3.

The mechanism of β-defensin antimicrobial activity is unclear, but may involve membrane disruption and interference with negatively charged macromolecules such as DNA (14). Thus, the slow growth and unique membrane composition of treponemes may help explain the resistance of *T. denticola* and other oral treponemes to human β-defensins. However, other spirochetes have demonstrated sensitivity to cathelicidins and neutrophil-derived defensins from humans and rabbits, suggesting another mechanism of resistance is present (4, 5, 8, 24, 39). In addition, we have previously demonstrated that resistance to β-defensins is evident for *T. denticola* in both stationary and mid-log phase, and no killing was observed with incubation times of up to 24 h; this suggests the slow growth rate of oral treponemes is not responsible for their resistance (6). We have previously shown that proteolytic activity of *T. denticola* is not responsible for resistance to β-defensins (6). An intriguing possibility for a resistance mechanism arises from the newly completed *T. denticola* genome, which shows the presence of 84 efflux pump-related genes (40). Efflux of antimicrobial peptides has been demonstrated to account for the resistance of another mucosal pathogen, *Neisseria gonorrhoeae* (41). We are further exploring this mechanism in *T. denticola*.

In conclusion, most oral *Treponema* are resistant to human β-defensins. Resistance to the constitutively expressed hβD-1 may enable treponemes to associate closely with the gingival epithelium and to establish themselves early in the periodontal lesion. Resistance to the inducible hβD-2 or -3 may dictate which treponemes are prevalent in the inflammatory environment of the active periodontal lesion.

Acknowledgments

C.A.B. was supported by NIDCR Training Grant DE07023. This work was supported by Public Health Service Grant DE015354 and University of Washington Research Royalty Fund proposal no. 2681.

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