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Quantitative Relationship of *Treponema denticola* to Severity of Periodontal Disease

LLOYD G. SIMONSON,[1]* CHARLES H. GOODMAN,[2] JOHN J. BIAL,[1] AND HAROLD E. MORTON[1]

Naval Dental Research Institute, Naval Training Center, Great Lakes, Illinois 60088-5259,[1] and Department of Periodontics, Northwestern University Dental School, Chicago, Illinois 60611[2]

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The *Treponema denticola* content of plaque was quantitatively estimated for samples taken from periodontitis patients as well as periodontally healthy subjects among two separate human populations. The populations studied included military volunteers and civilians at a university dental clinic. The plaque samples from each population were grouped according to pocket depth measurements at the collection site. A biotin-avidin enzyme-linked immunosorbent assay procedure was developed with a monoclonal antibody specific for a serovariety of *T. denticola*. *T. denticola* was present at significantly elevated levels in plaque samples collected from deep-pocket sites of patients with severe periodontitis relative to the healthy controls and a group with moderate disease. The ratio of *T. denticola* content per milligram of plaque in the deep pocket groups to that of the other two groups was about 2:1 for both populations. This is the first quantitative evidence of a positive relationship between a specific spirochete species and severe periodontitis.

Spirochetes have been implicated as potential etiologic agents of severe periodontal disease in adults (1, 4, 6, 7). Moore et al. (9) found that certain specific spirochetes were more closely associated with severe periodontitis than they were with healthy sites or gingivitis. They identified *Treponema denticola* as one of the spirochete species more frequently isolated from severely diseased sites in young adults. In a comparison of moderate and severe periodontitis, Moore et al. (8) suggested that *T. denticola* may have clinical significance in mature adults and is one of the most likely etiologic agents of severe periodontitis. Since it is difficult to isolate and identify spirochetes from clinical plaque samples, quantitative studies of specific spirochete populations present in periodontal disease sites have not been reported. In the present investigation, we report the use of immunochemical assay methods with monoclonal antibodies for the detection and quantitation of the level of *T. denticola* in human clinical samples. We estimated the quantities of *T. denticola* in plaque samples from healthy and diseased sites from two separate human populations.

MATERIALS AND METHODS

Clinical samples from two separate human populations were studied. A military population sample consisted of 65 volunteers at a Navy dental clinic. The military population parameters are presented in Table 1. A university population sample included 36 volunteers at the periodontics clinic of Northwestern University Dental School. The parameters of the university volunteers are shown in Table 2. Each population was divided into three groups according to periodontal disease severity and pocket depth at the sample site. Periodontally healthy subjects exhibited gingival sulcus depths of less than 4 mm, little or no gingival inflammation, and no radiographic evidence of alveolar bone loss. The remaining two groups within both populations were subjects with American Dental Association case type III and IV periodontitis. The moderate periodontitis patients exhibited moderate gingival inflammation, bleeding upon probing, 4- to 6-mm pockets, and radiographic evidence of early to moderate alveolar bone loss. The advanced periodontitis patients exhibited moderate to severe gingival inflammation, bleeding upon probing, multiple sites probing greater than 6 mm, and radiographic evidence of generalized, moderate to severe alveolar bone loss. None of the subjects had a history of significant medical problems as determined by a health questionnaire, and none had received dental treatment including scaling and root planing or antibiotic therapy for at least 6 months before sampling.

Clinical indices recorded for each patient included the plaque index (10) and the gingival index (5). The pocket depth at the sample site was measured to the nearest millimeter with a Michigan O probe. Total plaque (both subgingival and supragingival) was studied in the military population, whereas only subgingival plaque samples were obtained from the university population. Plaque specimens from healthy subjects were pooled from interproximal sites to have sufficient amounts for assay measurements. Plaque samples from the disease sites were from single sites. All plaque samples were collected with sterile curettes and placed immediately into vials containing formalinized coating buffer. Two clinicians, one for each population, performed the diagnoses and collected the plaque samples.

Immunoassay. Each plaque sample was dispersed by sonicating for 5 s at 32 to 39 W (25 to 30% of the 130-W average output) with a Heat Systems-Ultrasonics model WI40 sonifier. The wet weight was determined spectrophotometrically by measuring absorbance of the plaque suspension at 420 nm. The absorbance values were then compared with a standard curve constructed from serial dilutions of preweighed pooled plaque samples. An enzyme-linked immunosorbent assay (ELISA) was performed as described previously (11), with some modifications. A biotin-avidin system was used to enhance the ELISA values and provide greater sensitivity. In this system, each assay plate containing the clinical plaque samples also had a duplicate set of serially diluted *T. denticola* whole cell standards ranging from 0 to 20 μg of wet cell weight. The standards were prepared from a mixture of equal amounts of six *T. denticola* strains that had been washed with 0.02 M $MgCl_2$–phosphate-buffered saline and fixed in formalinized saline. The six strains of *T.*

* Corresponding author.

TABLE 1. Quantitative estimation of *T. denticola* antigen in plaque from male military volunteers

Test group[a]	No. of patients	No. of samples	Pocket depth[b] (mm)	Age[b] (yr)	Age range (yr)	*T. denticola* content (μg/mg of plaque)	
						Mean (±SD)	Range
Normal	26	26	2.8 (0.6)	20.8 (3.6)	17–33	2.7 (2.9)	0.5–15.5
Intermediate	24	24	4.9 (0.8)	28.7 (2.8)	25–34	4.4 (5.2)	1.1–26.6
Deep	15	15	7.7 (0.9)	25.9 (3.8)	19–33	13.4 (9.3)[c]	1.5–33.4

[a] Test groups are indicated by pocket depth as follows: normal, <4 mm; intermediate, 4 to 6 mm; deep, >6 mm.
[b] Mean (± standard deviation).
[c] Significantly different ($P < 0.05$, Fisher least significant difference post hoc multiple comparison).

denticola included ATCC 33521, FM, N-39, Ichelson, Ambigua, and D39DP1. The primary antibody in the ELISA system is murine monoclonal antibody IAA11, which is specific for *T. denticola* only as described previously (12). Monoclonal antibody IAA11 was able to detect 8 of 15 *T. denticola* strains by ELISA and was used undiluted as the cell-free culture medium supernatant. This monoclonal antibody reacts with the outer sheath of the *T. denticola* cell wall and is an immunoglobulin G3 isotype. The second antibody in the ELISA procedure was an affinity-purified biotin-labeled rabbit anti-mouse immunoglobulin G (Miles Laboratories, Inc.). The enzyme label consisted of an alkaline phosphatase avidin conjugate (Miles). Substrate incubation times were typically 30 min at 25°C.

Statistical analysis. The mean *T. denticola* antigen equivalents per milligram of plaque was determined for each of the three pocket depth groups in the two human populations studied. A one-way analysis of variance *F* test was used to test the hypothesis that the mean *T. denticola* content per milligram of plaque was different for each of the pocket depth groups in each population. Each analysis of variance generated an *F* value which was converted to a significance level by using a table of programmed *F* values. Significant outcomes ($P < 0.05$) were further compared by a Fisher least-significant-difference, post hoc multiple-range test. Only within-population comparisons were made, since each of the populations was studied separately with no attempt to normalize subjects between the two distinct population samples.

RESULTS

The sensitivity of the biotin-avidin-enhanced ELISA is shown in Fig. 1. Each assay plate included a similar standard curve over the 0- to 20-μg range of *T. denticola* whole cell antigen standards. The assay was essentially linear over this range, and the lower limit of sensitivity was in the 2- to 4-μg range. Although the sensitivity increased about fivefold (from 5 μg to 1 μg), the specificity of monoclonal antibody TDII,IAA11 was as reported previously (12) with an earlier indirect ELISA procedure. Negative control wells, incubated with the hybridoma growth medium, showed very little background color, and this background was used to blank the automated plate reader to compensate for extraneous color development in the absorbance measurements.

The microgram quantities of *T. denticola* antigen equivalents (estimated from the antigen standard mixture) per milligram of plaque from the military population are shown in Table 1. Only male volunteers ranging from 17 to 34 years of age were studied. The mean micrograms of *T. denticola* per milligram of plaque increased with pocket depth. The analysis of variance for the three groups was significant at the $P < 0.001$ level ($F = 16.5$). The Fisher least-significant-difference post hoc multiple comparison indicated that the deep-pocket group, with >6-mm pockets at the sampling site, had a significantly elevated level of *T. denticola* antigen ($P < 0.05$). The range of *T. denticola* content was quite variable within each group. All of the sites were found to have some trace of *T. denticola* antigen, although the immunoassay cannot distinguish between living and dead cells.

The quantitative estimations of *T. denticola* antigen content in subgingival plaque samples from volunteers at a university clinic are shown in Table 2. This population ranged from 27 to 72 years of age, and included both women and men. The overall analysis of variance for the three groups was significant ($P < 0.01$). The mean micrograms of *T. denticola* antigen equivalents was again significantly elevated ($P < 0.05$, Fisher least-significant-difference multiple comparison test) in the deep-pocket subject group (>6 mm) compared with the intermediate-pocket-depth group and the healthy control group. The range within each group was again quite variable. All sites were also found to be positive for some quantity of *T. denticola* antigen. Several members of the intermediate-pocket-depth group were found to have *T. denticola* antigen quantities greater than the mean value for the deep-pocket group. The mean gingival index values for the intermediate and deep-pocket groups were quite similar to those for the healthy control group. Other population parameters are summarized in Tables 1 and 2. The ratios of micrograms of *T. denticola* per milligram of

TABLE 2. Quantitative estimation of *T. denticola* antigen content in subgingival plaque from university volunteers

Test group[a]	No. of patients (men/women)	No. of samples	Pocket depth[b] (mm)	Age[b] (yr)	Age range (yr)	Gingival index[b]	Plaque index[b] (%)	*T. denticola* content (μg/mg of plaque)	
								Mean (± SD)	Range
Normal	10 (8/2)	11	2.6 (0.5)	41.5 (10.3)	27–63	0.8 (0.4)	24.8 (13.4)	74.3 (48.7)	8.6–165.9
Intermediate	15 (8/7)	28	5.1 (0.7)	50.1 (14.1)	32–71	1.4 (0.5)	59.0 (16.5)	68.2 (61.1)	5.5–283.6
Deep	11 (7/4)	21	7.5 (1.0)	47.8 (16.3)	33–72	1.7 (0.4)	71.7 (26.9)	139.5 (122.8)[c]	5.2–361.9

[a] See footnote *a* of Table 1.
[b] Mean (± standard deviation).
[c] Significantly different ($P < 0.05$, Fisher least significant difference post hoc multiple comparison).

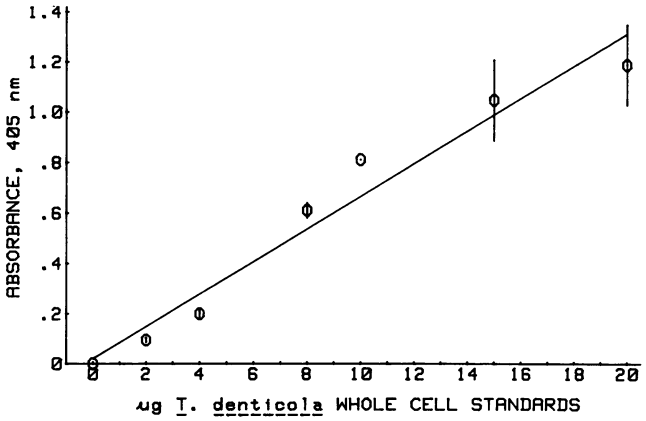

FIG. 1. Biotin-avidin-enhanced ELISA absorbance measurements after a 30-min substrate incubation period in a 96-well polystyrene plate. A linear regression plot of the absorbance values is shown over a typical range of *T. denticola* standard antigen mixture used.

plaque in the deep-pocket groups to those of the healthy and intermediate groups were about 2:1 in both populations.

DISCUSSION

This investigation presents the first quantitative evidence of a positive relationship between a specific spirochete and the morbidity of periodontitis. The specific spirochete is a serotype of *T. denticola*. This spirochete was present at a significantly elevated level in plaque samples collected from deep-pocket (>6 mm) sites of severe periodontitis patients. Listgarten and Levin (4) found that only spirochetes appeared to be positively correlated with probing depth. We extend these findings by identifying and quantitating a specific component of the spirochete flora which increases with periodontal disease severity. Armitage et al. (1) also found that subgingival spirochetes increased significantly when pocket depth and attachment loss exceeded 3 mm. Although they did not identify the spirochetes, they similarly noted that spirochetes were present in healthy sites but only in low percentages. They also found that spirochetes in general were two- to threefold higher in severely diseased sites. In the present investigation, we found an approximately twofold increase in *T. denticola* from severe sites relative to healthy and intermediate disease sites. This was consistent for both the military and university clinic populations studied. Although the two populations were studied separately and independently, some comparisons can be made to explain the much lower quantities of *T. denticola* antigen present in the military population versus the university population. The most probable reason for lower values in the military population is that the samples included both supragingival and subgingival plaque, whereas the university samples were only subgingival plaque. This had the effect of greatly diluting the relative *T. denticola* content, since spirochete populations are usually associated with subgingival, not supragingival, plaque. In addition, the mean ages of the military patient groups were much below those of the corresponding university population groups (Tables 1 and 2). However, it should be noted that it was possible to detect *T. denticola* differences even with total (subgingival plus supragingival) plaque samples. Also, the periodontists were consistent within each study but differed between the two study populations.

Cheng et al. (2) studied the serological heterogeneity among the species *T. denticola*. It is possible that some serotypes may be more related to disease than others. Our approach should facilitate studies related to detecting differences in disease associations for the various spirochete serovarieties. The immunochemical approach that we have employed in this study overcomes many of the technical difficulties encountered by traditional culture and identification studies of the bacterial species present in plaque samples of pocket scrapings. Listgarten and Hellden (3) noted that the cultivable microorganisms may bear little relationship to the content of the original sample because of distortions introduced by the sampling technique, sample dispersion, growth medium, and many other factors. The immunochemical approach can identify both living and dead cells and readily allows the study of bacterial serotypes in a quantitative manner. The quantity of *T. denticola* serotypes in subgingival plaque may be an indicator of disease activity. The diagnostic and prognostic value of measuring changes in *T. denticola* populations must yet be determined by longitudinal research studies. Future studies could relate the specific spirochete content with progressive changes in attachment levels.

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