Trypsin-Like Activity in Subgingival Plaque*

A Diagnostic Marker for Spirochetes and Periodontal Disease?

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TAXONOMIC SCREENING OF SUBGINGIVAL PLAQUE organisms with various enzyme assays have shown that *Treponema denticola, Bacteroides gingivalis* and an unspeciated *Capnocytophaga* species possess a trypsin-like enzyme (TLE) that can be detected by the hydrolysis of N-benzoyl-DL-arginine-2-naphthylamide (BANA). As these organisms can be considered to be periodontopathic, it was of interest to determine whether this BANA hydrolyzing enzyme could be detected directly in subgingival plaque samples. Subgingival plaque samples were collected from single sites of known pocket depth, and after dispersal by vortexing, aliquots were incubated overnight with BANA and were counted microscopically. The color reactions were developed with fast garnet, read by the eye and classified as positive (red to red-orange), negative (yellow) and questionable. In the BANA-positive plaques, the spirochetes averaged 43% of the microscopic count, whereas in the BANA negative plaques the spirochetes averaged 8% of the microscopic count. The average pocket depth of BANA-positive plaques was 6.7 mm, whereas the average pocket depth of BANA-negative plaques was 4.5 mm. When both of these parameters were combined, the presence of a positive BANA reaction was usually indicative of subgingival plaques containing >34% spirochetes removed from sites that had probing depths of 7 mm or more. Seventy-one per cent of the plaques removed from untreated periodontal patients were BANA-positive, while only 8% of the plaques removed from successfully treated patients seen at maintenance recall visits were BANA-positive.

These data indicate that the ability of subgingival plaque to hydrolyze BANA is a reliable marker for the presence of high proportions of spirochetes in the plaque sample and possibly could be used clinically to identify those sites and/or individuals who might require treatment to reduce this spirochetal overgrowth.

Periodontitis results in a loss of tooth-supporting tissue that is associated with the subgingival plaque being colonized by a preponderance of gram-negative anaerobic bacteria. Spirochetes,[1,2] black pigmented *Bacteroides* (BPB), especially *B. gingivalis*,[2,3] *Wolinella recta*[4] and in some cases *Haemophilus (Actinobacillus) actinomycetemcomitans*[3,5] are particularly prominent when there is evidence of clinical disease, i.e., bleeding upon probing, gingival inflammation, suppuration. These bacteriological observations suggest that increased proportions of the above organisms in plaque samples could have diagnostic implications. Thus, a clinician who could demonstrate elevated levels or proportions of these organisms could make a diagnosis of an active periodontitis (active in the sense that treatment is needed), advise the patient of such, and commence treatment so as to reduce or suppress these organisms from the plaque. Alternately, the detection of elevated levels of these organisms in plaque removed from an otherwise clinically healthy site might indicate a preclinical infection and could lead to simple, cost-effective interceptive treatments. The need for such diagnostic aids has been given high priority by a recent NIDR workshop[6] and repeated again in reviews concerned with periodontal disease activity.[7,8]

BIOCHEMICAL DIAGNOSIS

The taxonomic screening of periodontopathic organisms, such as *B. gingivalis, B. intermedius, B. melaninogenicus, Capnocytophaga ochracea, C. gingivalis, A. actinomycetemcomitans,* and *T. denticola* among others, have shown that only *T. denticola, B. gingivalis*

and an unspeciated *Capnocytophaga* possess a trypsin-like (TL) activity.[9-11] This activity is measured as the hydrolysis of the colorless substrate N-benzoyl-DL-arginine-2-naphthylamide (BANA) releasing a chromophore (β-naphthylamide) which turns a bright orange-red when a drop of fast garnet is added to the solution. This suggested that if these species were prominent in the subgingival plaque, their presence could be detected by the ability of the plaque to directly hydrolyze BANA. Furthermore, if the magnitude of this BANA-hydrolyzing activity could be correlated with the plaque levels and/or proportions of these putative periodontopathogens, and with the periodontal status of the patient, then this enzyme activity might have diagnostic significance.

In this present investigation, subgingival plaques obtained from single pocket sites in successfully treated and in untreated periodontal patients were examined for their bacteriological composition and for their ability to hydrolyze BANA. The findings suggest that BANA hydrolysis is an indicator of the spirochetal load in the subgingival plaque and possibly of periodontal disease, if disease is judged by the clinician's assessment as to whether the patient does or does not need mechanical-surgical procedures.

MATERIALS AND METHODS

Patients. The patient population was drawn from the university graduate periodontal clinic. These patients, on the basis of clinical and radiographic examination, were judged to have a periodontitis that required some form of periodontal surgery as part of the initial treatment plan. The untreated patients were sampled prior to the beginning of root instrumentation, whereas the treated patients had successfully completed active periodontal treatment and were sampled at the beginning of a regularly scheduled maintenance visit. The sites chosen in the untreated patients appeared from a clinical and radiographic examination to be the most severely involved site in each quadrant, i.e., bleeding upon probing, bone loss, attachment loss and probing depth >5 mm and were usually about molar teeth. The sites chosen in the treated patients were usually those that exhibited the greatest bone and attachment loss and had been treated by surgical procedures. The clinical characteristics of these sites and the bacteriological profile of the plaques have been described.[2,17]

Bacteriological Procedures. Subgingival plaque was usually removed from one pocket per quadrant in either untreated periodontal patients or treated patients who were examined at recall visits. The supragingival plaque about the sampled site was removed and discarded. The root surface was then scaled with a curette, and the plaque on the scaler tip was transferred to a vial containing 0.4 ml of reduced transport fluid (RTF) without EDTA.[12] The plaque samples were immediately placed into an anaerobic chamber and after dispersing for 20 seconds with a vortex mixer, a 50-μl portion of each sample was removed for microscopic examination, and another 50- or 100-μl portion was removed to measure the enzymatic hydrolysis of BANA. The remaining plaque sample was diluted to 4 ml with RTF, sonically dispersed for 20 seconds with a Kontes sonifier,* serially diluted in RTF and plated automatically with a spiral plater on a variety of media. In this manner enzyme hydrolysis, microscopic and cultural counts were obtained on the same plaque samples.

The bacteriological procedures have been reported in detail elsewhere[2] and will not be further described. For the microscopic counts, 10 μl of the aliquot removed for microscopic examination were placed on a glass slide, covered with a 22 × 30 mm cover slip, sealed and viewed by dark-field microscopy. Either 200 organisms or the number of organisms in 20 high-power fields (hpf) were enumerated, depending on which event occurred first. The single cells were identified as spirochetes, selenomonads (curved motile rods), vibrio-like motile rods (comma shaped rods with helter-skelter movement), fusiforms, nonmotile rods or cocci. The spirochetes were further subdivided into large, intermediate and small-sized organisms.

Enzyme Procedures. Preliminary studies revealed that the hydrolysis of BANA by pure cultures of *B. gingivalis* and *T. denticola* could be demonstrated in phosphate or tris buffers, or in RTF at various pH's. For the analysis of plaque samples, a Sorensen buffer 0.15 M/L KH₂PO₄, 0.15 M/L Na₂HPO₄ at pH 7.2 was used. A stock solution of BANA (44 mg in 1 ml dimethyl sulfoxide (DMSO) was diluted 1 to 100 in the Sorenson buffer to give a working solution of 0.67 mM BANA.[10] One hundred microliters of this working solution were added to 50 μl of the plaque suspension and incubated in air at 37°C overnight. Thus the period of incubation ranged from 17 to 23 hours. A drop of fast garnet[10] was added and the intensity of the red-orange color was read by eye and assigned a number from 1 (yellow or negative) to 4 (red or strongly positive). Intermediate yellow-orange and orange-red colors were scored as a 2 (weakly positive) or as a 3 (positive).

Previous studies using a spectrophotometer (Artek ELISA reader) had shown that an absorption of 0.1 at 480 mm could be easily discerned by eye and given a weak positive score of 2. Accordingly, the spectrophotometric readings were dispensed with, and the results were determined by eye and a color chart so as to simulate conditions that would exist in a dental office. The 3 and 4 scores were contracted to give a single positive score for the statistical analysis.

Polymorphonuclear neutrophils (PMNs). Some PMNs which exited from the gingiva into the sulcus or pocket would be present in the plaque sample and could

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contribute to the hydrolysis of the BANA if they possessed the necessary enzyme. This possibility was determined by incubating BANA overnight with PMNs obtained from the peripheral blood or from crevicular washings. Two hundred to 300 μl of blood, obtained by finger puncture from 7 volunteers, was added over a Ficoll/Hypaque solution.[13] The PMN layer was removed and resuspended in 250 μl of a pyrogen-free Dulbecco phosphate buffer saline (PBS) without Mg++ or Ca++ to which had been freshly added a 1% (V/V) solution containing 10% gelatin, 200 mM EDTA and 500 mM glucose (9.9 ml PBS plus 0.1 ml gelatin-EDTA-glucose solution [PBS-GEG]).[14]

Fifty microliters of this suspension were used to count PMNs in a hemocytometer and to determine the viability of the cells by trypan blue dye exclusion. One hundred microliters of cells were either incubated directly with 100 μl of the BANA solution or were vortexed for 20 seconds, so as to mimic the dispersal procedure used for the plaque and then incubated with BANA.

Crevicular or pocket washings were obtained from diseased periodontal sites by flushing the pocket with 10 μl of the PBS-GEG solution and then recovering the wash solution by aspiration.[15] An Eppendorf pipette was placed at the crevicular orifice and six or seven consecutive 10-μl washings were obtained during a 2- to 4-minute interval and pooled to give a single PMN sample. Each sample was diluted to 250 μl with the PBS-GEG solution and duplicate 100 μl aliquots were incubated with BANA. The PMNs in the remaining suspension were counted in the hemocytometer and stained for viability using trypan blue.

Statistical Analysis. The counts for each organism enumerated culturally and microscopically, and the BANA hydrolysis score at pH 7 were recorded on forms suitable for computer processing, using a program specifically developed for our data. Proportions of the various organisms were not calculated if there were less than 20 colony-forming units (CFU) in the nonselective ETSA agar medium,[16] or if on the microscopic slide there was less than one CFU per hpf. This procedure assured that proportional values obtained from very small plaque samples, where method errors are high, would not be included in the analyses.

The bacterial count per hpf and proportions of organisms in each plaque sample were statistically analyzed by an analysis of variance (ANOVA) using the Scheffe test to compare between the various enzyme reactions. The data were also analyzed as a function of spirochetal proportions and pocket depth using both the ANOVA and the nonparametric Kruskal-Wallis test. These statistical analyses were performed using MIDAS, the statistical program of the Michigan Terminal System.

RESULTS

Fifty three per cent of the plaque samples exhibited either a weak positive (10%) or a positive (43%) BANA reaction. Of the known BANA positive species, *T. denticola* (measured indirectly as a small spirochete) was detected in 70% of all the plaques, and in 93% of the BANA-positive plaques. *B. gingivalis* and the *Capnocytophaga* species were detected in 15% and 9%, respectively, of all plaques, but in only 27% and 6% of the BANA-positive plaques (Table 1). This frequency distribution indicated that among these known BANA-positive species, *T. denticola* was the organism most likely responsible for the plaque hydrolysis of BANA.

This BANA hydrolysis could not be associated with PMNs. Thus the PMNs obtained from the peripheral blood of seven volunteers or from the pocket washings of 10 diseased sites, i.e., probing depths of 6 mm or more which bled upon probing, were unable to hydrolyze BANA. These PMN suspensions contained from 5 to 25×10^6 PMNs in the enzyme assay and were either 95% (peripheral blood) or 85% (pocket washings) viable. These levels of PMNs were considerably higher than the levels of PMNs seen in the microscopic examination of the plaque.

When the data were stratified according to the BANA reaction of the plaque, it was found that the BANA-positive and weakly positive plaques were removed from pockets that averaged 6.7 mm in depth, whereas the BANA-negative plaques were removed from pockets that averaged 4.5 mm in depth (Table 2). The BANA-positive plaques averaged significantly more bacteria per hpf, spirochetes per hpf (data not shown) and per cent spirochetes than either the BANA weakly

Table 1
Percentage of Subgingival Plaques and BANA-Positive Plaques in Which Known BANA-Positive Species Were Detected

BANA-positive species	All plaques		BANA-positive plaques*	
	Detected	Not detected	Detected	Not detected
T. denticola† (small spir)	70	30	93	7
B. gingivalis	15	85	27	73
Capnocytophaga	9	91	6	94

* 53% of the plaques were either positive or weakly positive.
† Measured indirectly as a small spirochete.

Table 2
Relationship Between BANA Hydrolysis, Pocket Depth and Certain Bacterial Parameters

BANA hydrolysis	Pocket depth (mm)	Bacteria /hpf*	% Spirochetes†	% B. gingivalis‡	% Capno-cytophaga
Positive	6.7 ± 1.8§	20.5 ± 16.7	43 ± 21	2.3 ± 8.0	0.04 ± 0.2
Weakly positive	6.7 ± 3.4	12.7 ± 12.0	23 ± 22		
Negative	4.5 ± 1.9	6.8 ± 10.4	8 ± 15	0.1 ± 0.6	0.05 ± 0.2
Significance	n = 306‖	n = 406	n = 406	n = 114	n = 65
ANOVA	p <0.0001	p <0.0001	p <0.0001	p = 0.05	NS
Kruskal-Wallis	p <0.0001	p <0.0001	p <0.0001	NS^d	NS

* Hpf = high power microscopic field.
† Would include *T. denticola*.
‡ *B. gingivalis* was detected in 38% of positive plaques and 27% of negative plaques.
§ Mean ± standard deviation.
▢ Values in box are significantly different from other values in column, $P < 0.01$ Scheffe test.
‖ Number of plaque samples.

positive or BANA-negative plaques. In turn the BANA weakly positive plaques had significantly more bacteria, more spirochetes and per cent spirochetes than did the BANA-negative plaques. The BANA-positive plaques had higher proportions of *B. gingivalis* than did the BANA-negative plaques primarily because in the few instances when *B. gingivalis* was present in high proportions, i.e., >5%, the plaques were always BANA-positive. The *Capnocytophaga* species, when present, were in low proportions in both the BANA-positive and BANA-negative plaques.

The relationship between BANA hydrolysis and spirochetal proportions was further analyzed by comparing the plaque BANA response with the various spirochetal morphotypes that could be identified by microscopic examination. The proportions of small spirochetes, which would include *T. denticola*, exhibited a highly significant correlation with the BANA response of the plaque (Table 3). A similar highly significant relationship was also observed with the proportions of intermediate and large sized spirochetes. From this it would appear that spirochetes representative of all the morphotypes contributed to the ability of the plaque to hydrolyze BANA (Table 3). The converse of this situation was also examined, i.e., if spirochetes were not detected, what proportions of the plaques were BANA-negative? When no spirochetes could be detected, 89% of the plaques were BANA-negative. When no small spirochetes were detected, 88% of the plaques were BANA-negative, whereas the corresponding values for intermediate sized spirochetes were 61% BANA-negative, and for large sized spirochetes, 80% BANA-negative.

These data indicate that the spirochetes could be associated with about 90% of the BANA-positive or weakly positive reactions exhibited by the plaque and that the other BANA-positive bacterial species, in the absence of the spirochetes, accounted for only 10% of the BANA-positive or weakly positive reactions. Thus BANA hydrolysis could be considered as a measure of the presence of spirochetes in the plaque.

The data were then analyzed to determine whether

BANA hydrolysis by the plaque would be indicative of the spirochetal proportions present in the plaque (Table 4). If no spirochetes were detected, only 5% of the plaques gave a positive BANA reaction, and 6% gave a weak positive reaction. When spirochetes were present at less than 10% of the microscopic count, about 20% of the plaques were weakly positive and another 10% were positive. However, when spirochetes accounted for more than 10% of the microscopic count, from 59% to 87% of the plaques were either BANA-positive or weakly positive. As the spirochetal proportions increased, the overwhelming majority of the plaques were BANA positive. Thus, the frequency and intensity of the BANA reaction were significantly associated with the plaque proportions of spirochetes.

Ten per cent spirochetes seem to be an inflection point below which the BANA reactions were mostly negative and above which they were mostly positive (Table 4). Below this point the pockets tended to be less than 6 mm in probing depth, while above it the pockets were mostly 6 mm or more. This point did not seem to be associated with any marked changes in the amount of plaque that had been removed from the pocket, as the number of bacteria per hpf was comparable both below and above 10% spirochetes, except in those plaques where no spirochetes were detected. This point did reflect a change in the absolute numbers of spirochetes in the plaque sample, as below 10% spirochetes, the plaques had less than one spirochete per hpf, while above 10% spirochetes, the plaques usually had more than one spirochete per hpf. Thus BANA-positive reactions generally indicated that the plaque sample had more than 10% spirochetes and more than one spirochete per hpf.

It was next determined whether the ability of the plaque to hydrolyze BANA had any relationship to probing depth. Sites with probing depths of 2 or 3 mm had significantly lower proportions of spirochetes and only 10% positive BANA reactions (Table 5). Sites with probing depths of 4 mm had 16% spirochetes but were only associated with 26% BANA-positive reactions, and half of these were of the weakly positive type. Five- or

Table 3
Relationship Between BANA Hydrolysis and Spirochetal Morphotype

BANA hydrolysis	Spirochete morphotype		
	% Small	% Intermediate	% Large
Positive (165)*	25.6 ± 14.6 †	11.2 ± 8.0	6.1 ± 9.3
Weakly positive (53)	14.0 ± 14.6	6.9 ± 7.7	1.9 ± 6.9
Negative (188)	6.7 ± 12.3	1.1 ± 2.9	0.2 ± 1.4
ANOVA	P < 0.0001	P < 0.0001	P < 0.0001
Kruskal-Wallis	P < 0.0001	P < 0.0001	P < 0.0001

* Number of samples.
☐ Value in box significantly different from other values in column.
† Mean ± standard deviation.

Table 4
BANA Hydrolysis as a Function of Percentage Spirochetes in Plaque

% Spirochetes	No. of plaques	Probing depth	% BANA hydrolysis			Plaque size	
			Weak	Positive	Total*	Spirochetes /hpf	Bacteria /hpf
Not detected (ND)	101	3.8 ± 1.8mm †	6%	5%	11%	0.05‡	4.0 ± 10.4
<5% (2.7%)§	49	4.3 ± 1.9	18	9	27	0.3 ± 0.4	10.0 ± 11.6
5 to <10% (7.0)	27	5.4 ± 2.1	22	11	33	0.8 ± 0.7	11.7 ± 10.0
10 to <20% (14)	39	6.0 ± 1.8	23	36	59	1.9 ± 1.7	13.3 ± 11.3
20 to <30% (25.5)	33	6.5 ± 1.3	18	52	70	3.9 ± 3.5	15.0 ± 12.9
30 to <40% (34.7)	45	6.9 ± 1.6	16	71	87	6.1 ± 4.3	17.4 ± 12.2
>40% (45.4)	38	6.7 ± 1.9	8	76	84	7.4 ± 6.8	16.4 ± 15.6
Significance							
ANOVA		P < 0.0001				<0.0001	<0.0001
Kruskal-Wallis				<0.0001			

* Positive plus weakly positive BANA reactions were combined.
† Mean ± standard deviation.
☐ Values in box significantly different from all other values in column, Scheffe test $P < 0.05$.
‡ The level of detection was about 0.1 spirochete/hpf. If no spirochetes were detected, they were either absent or present at levels less than 0.05 spirochetes/hpf.
§ Actual mean percentage of spirochetes in each level.

6-mm deep sites exhibited a significant increase in proportions of spirochetes, but now the average value of 30% spirochetes was associated with 52% to 57% positive BANA reactions with the majority being of the positive type. Sites ≥7 mm deep had 34% to 46% spirochetes and 80% to 92% positive BANA reactions (Table 5).

The relationships between increasing probing depth and per cent positive BANA hydrolysis, per cent spirochetes, spirochetes per hpf and the numbers of bacteria per hpf were all highly significant. No relationship between percentage of *B. gingivalis* and probing depth was evident (Table 5). Of those parameters which significantly increased with increasing probing depth, the best correlation was seen between per cent positive BANA hydrolysis and per cent spirochetes. Thus, the presence of a positive BANA reaction in the plaque was usually indicative of plaques containing >34% spirochetes removed from sites that had probing depths of 7 mm or more.

The patients from whom these plaques were removed were either untreated individuals diagnosed clinically as having adult periodontal disease (ADA IV), or were similar ADA Type IV patients who had completed all

necessary phases of periodontal treatment including surgery and who were now being seen for maintenance treatments at recall visits. The ability of their plaque samples to hydrolyze BANA was then determined as a function of whether the patients were either untreated or were treated and maintained.

Seventy-one per cent of the plaques removed from the untreated patients were BANA positive, while only 8% of the plaques removed from the treated and maintained patients were BANA positive (Table 6). Within the untreated group those sites which were BANA positive had significantly higher proportions of spirochetes, spirochetes per hpf and bacteria per hpf, but not higher proportions of *B. gingivalis* or *Capnocytophaga*. Within the treated and maintained group, the BANA-positive plaques had significantly higher proportions of spirochetes which was due primarily to having significantly higher proportions of small spirochetes (Table 6).

DISCUSSION

These data indicate that the ability of subgingival plaque to hydrolyze BANA is a reliable marker for the presence of high proportions of spirochetes in the

Table 5

BANA Hydrolysis and Bacterial Parameters as a Function of Pocket Depth

Pocket depth	BANA Hydrolysis			% Spirochetes	Spirochetes /hpf	Bacteria /hpf	% B. gingivalis
	Negative	Positive	% Negative				
2mm (20)*	18	2	(90%)†	3.9 ± 9.8‡	1.0 ± 1.4‡	11.5 ± 22.6‡	ND§
3 (42)	38	4	(90)	5.5 ± 14.2	2.9 ± 7.6	6.0 ± 8.5	0.02 ± 0.04
4 (27)	20	7	(74)	15.7 ± 24.5	2.8 ± 3.9	5.7 ± 6.1	0.3 ± 0.7
5 (31)	15	16	(48)	30.2 ± 30.0	3.7 ± 3.9	12.0 ± 13.1	0.6 ± 1.4
6 (88)	38	50	(43)	31.6 ± 24.6	6.3 ± 8.2	13.5 ± 13.0	2.7 ± 8.1
7 (40)	7	33	(18)	33.8 ± 20.7	6.0 ± 5.9	15.6 ± 12.6	3.2 ± 11.7
8 (28)	3	25	(11)	46.2 ± 20.5	13.2 ± 15.5	24.2 ± 23.8	0.1 ± 0.3
9 (13)	1	12	(8)	42.5 ± 19.2	7.4 ± 7.4	16.7 ± 9.0	ND
10 (8)	1	7	(13)	38.9 ± 20.2	13.7 ± 14.0	27.5 ± 22.9	ND
11 (5)	1	4	(20)	44.3 ± 30.9	10.0 ± 11.5	16.0 ± 13.1	ND
n = 302	n = 302			n = 308	n = 242	n = 308	n = 103
Significance Kruskall-Wallis	P = 0.0001						
ANOVA				P < 0.0001	P < 0.0001	P < 0.0001	P = 0.4

* Number of pockets in parentheses.

† % Samples which are negative.

‡ Mean ± standard deviation.

§ ND = not detected.

Table 6

Relationship Between BANA Hydrolysis and Clinical Status of Patient

BANA hydrolysis	Pocket depth (mm)	Per cent (%) Spirochete	% Large Spirochete	% Int Spirochete	% Small Spirochete	Spirochete /hpf	Bacteria /hpf	% B. gingivalis	% Capno-cytophaga
Untreated Active Periodontitis (ADA IV)									
Positive (94)*	6.9 ± 2.0†	40.3 ± 20.8	5.4 ± 8.6	10.6 ± 8.3	24.3 ± 16.0‡	8.3 ± 10.8	18.3 ± 17.4‡	3.5 ± 10.8	0.3 ± 1.2
Negative (37)	5.4 ± 2.3	11.6 ± 16.1	0.1 ± 0.5	1.9 ± 3.5	9.6 ± 13.9	3.3 ± 6.0	8.4 ± 11.2	0.4 ± 1.0	ND§
Treated and Maintained Patients (Former ADA IV)									
Positive (8)	5.6 ± 0.5	20.3 ± 20.7	0.1 ± 0.2	0.4 ± 0.9	19.8 ± 21.1	5.2 ± 7.8	28.8 ± 24.0	ND	ND
Negative (90)	4.0 ± 1.6	6.2 ± 11.9	0.1 ± 0.4	0.3 ± 1.5	5.8 ± 11.4‡	1.2 ± 2.7	6.5 ± 12.0‡	0.1 ± 0.2	0.1 ± 0.2
ANOVA	P < 0.0001	P < 0.0001	P < 0.0001	P < 0.0001	P < 0.0001	P < 0.0001	P < 0.0001	NS	NS

* Number of plaque samples.

† Mean ± standard deviation.

‡ Values with this superscript in same column are significantly different, Scheffe test P < 0.01.

§ ND = not detected.

↕ Values connected by arrow are significantly different, Scheffe test P < 0.01.

☐ Values in box are significantly different from all other values in column, Scheffe test P < 0.01.

plaque sample and, possibly, could be used clinically to identify those sites and/or individuals who might require treatment to reduce this spirochetal overgrowth.[17] This latter possibility is suggested because the ability of the subgingival plaque to hydrolyze BANA was significantly associated with (1) increased probing depth (Table 5) and (2) untreated periodontal disease (Table 6). Thus, untreated ADA Type IV patients who presented with the clinical symptoms of chronic periodontitis had plaques that were mostly BANA positive, whereas successfully treated ADA Type IV patients, seen at periodic recall visits, were essentially BANA negative. Within each patient category the ability of the plaque to hydrolyze BANA identified those sites which had elevated levels and proportions of spirochetes (Table 6).

These findings suggest that the BANA hydrolysis by the plaque has the potential to be an objective indicator of periodontal disease activity and could be used in combination with clinical criteria both to initiate therapy and as a means to monitor the efficacy of treatment. However, before this is accepted, additional information is needed concerning the source(s) of the BANA hydrolyzing activity of the plaque, as it is possible that when this is known, the BANA test can be made both more specific and sensitive for periodontal disease activity.

The present results indicate that the spirochetes are the major contributor to the plaque BANA hydrolyzing activity. Thus, when spirochetes were not detected, 89% of the plaques were BANA negative, whereas when spirochetes were detected, 90% of the plaques were weakly positive or positive for BANA (Tables 1 and 3). This indicates that about 10% of the BANA-positive reactions could not be associated with the spirochetes and most likely reflected contributions from *B. gingi-*

valis, the unspeciated *Capnocytophaga* species, the recently described new species *B. forsythus*[18] and other yet to be identified species. There is no evidence that host enzymes contributed significantly to the plaque BANA hydrolyzing activity as PMNs, which account for about 90% of the leukocytes seen in gingival crevicular fluid[19] or washings,[20] were unable to hydrolyze BANA. We have not determined whether shed epithelial cells possess BANA hydrolyzing activity, although crevicular washings that contain some of these cells were BANA negative. The increased frequency of BANA hydrolysis with increased probing depths was associated with the increased proportions and numbers of spirochetes (Table 5). Evian *et al.*[21] have also shown that the proportions of spirochetes increase as a function of pocket depth. This selection for spirochetes can in part be explained by a lower pO_2 in the deeper pockets.[22]

Plaque BANA hydrolyzing activity could be associated with all morphotypes of spirochetes (Table 3). As intermediate and large sized spirochetes cannot yet be routinely cultivated, it is not possible to associate this enzyme activity with any particular species within these types of organisms. Among the cultivable small sized spirochetes, only *T. denticola* out of *T. pectinovorum*, *T. socranskii* and *T. vincentii* exhibited BANA hydrolyzing activity (unpublished results). This *T. denticola* enzyme is cell bound, has a molecular weight of about 55,000 and is novel in that it does not degrade such common proteins as albumin, hemoglobin, gelatin, azocol and casein.[23] We are currently preparing a monoclonal antibody to this enzyme to determine whether such an antibody can inhibit the ability of plaque to hydrolyze BANA.

The enzyme most likely responsible for BANA hydrolysis in *B. gingivalis* has been characterized[24] and found to be a cell-bound alkaline protease which degrades albumin, azocoll and gelatin as well as the synthetic substrate benzoyl L-arginine p-nitroanilide (BAPNA). This enzyme is stimulated by reducing agents such as dithiothreitol, whereas the *T. denticola* enzyme is unaffected by reducing agents. Thus the *B. gingivalis* enzyme, which is a true protease, differs markedly from the *T. denticola* enzyme which apparently is not a protease. However, both enzymes are cell bound and would not be present in gingival crevicular fluid unless released by cell lysis. In this regard, plaque then is a more suitable specimen for BANA analysis than either crevicular fluid or pocket washings.

The observation that crevicular fluid may not contain certain bacterial enzymes might account for the emphasis given to the presence of host enzymes in crevicular fluid.[20,25] Several investigators have reported the presence of collagenase,[26,27] β-glucuronidase,[28] lactic dehydrogenase,[29] lysozyme,[30] acid and alkaline phosphatases[31,32] and myeloperoxidase[33] in the crevicular fluid. However, no convincing evidence that these

enzymes or their levels could be of diagnostic value in periodontal disease was found by Cimasoni in his 1983 review of crevicular fluid.[20] This may in part reflect a lack of standardized methodology both to sampling crevicular fluid and enzyme measurements.[25] Thus, when standard precedures were used, the crevicular fluid levels of collagenase, β-glucuronidase and arylsulfatase were shown to increase in the absence of oral hygiene using the experimental gingivitis model.[25] However, the detection of these enzymes was not as simply performed as was the detection of the BANA hydrolyzing enzyme described here. Nor is it known whether these host enzymes will show the same discrimination between healthy and diseased sites as was observed with the BANA hydrolyzing enzyme.

Other bacterial enzymes or combination of enzymes may also have diagnostic potential for periodontal disease activity. We have shown, using the experimental gingivitis model, that the number of sites containing plaque capable of hydrolyzing BANA increased from 3% prior to the period of no oral hygiene to 32% after 3 weeks of no oral hygiene.[34] Several bacterial enzymes responsible for the hydrolysis of the β-naphthylamide derivatives of L-pyrrolidine, valine, serine, arginine and alanine also increased.

It is possible that these subgingival bacterial enzymes might be detected in saliva. Thus, in a small number of patients, the salivary levels of several of these enzymes, including the BANA enzyme, were shown to decrease following periodontal treatment.[35] This procedure requires that the saliva be centrifuged and the testing performed on the salivary sediment. This is more laborious than sampling subgingival plaque directly and involves a dilution of the subgingival enzymes with enzymes from all other oral sources.

Collectively, the present findings and the data of others indicate that the detection of bacterial[23,24] or possibly host enzymes[20,25-31] in subgingival plaque or crevicular fluid may provide an objective means of detecting periodontal infections. The ability to detect BANA hydrolyzing activity in subgingival plaques appears to be a simple, sensitive and inexpensive means of monitoring spirochete levels and/or proportions in subgingival plaques. This BANA hydrolyzing activity may, upon further evaluation, prove to be a means of monitoring periodontal disease activity in any given pocket or patient.

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