Characteristics of Trypsin-like Activity in Subgingival Plaque Samples

W.A. BRETZ and W.J. LOESCHE

School of Dentistry, The University of Michigan, Ann Arbor, Michigan 48109

Previous studies have demonstrated that the hydrolysis of the trypsin substrate N-benzoyl-DL-arginine-2-naphthylamide (BANA), by subgingival plaque obtained from a single site, correlates best with the numbers and proportions of spirochetes in plaque samples and may serve as an indicator of clinical disease. In this investigation, we determined whether the association between BANA hydrolysis and spirochetes could be obtained in pooled subgingival plaque samples. Concomitantly, the characteristics of this reaction in terms of substrate type and concentration, microbial numbers needed to give a positive reaction as assessed by microscopic counts, rapidity of hydrolysis, and the effect of pH and various additives on the plaque BANA hydrolytic activity have been studied in pooled plaque samples from patients who were periodontally healthy or diseased. In addition, it was determined whether BANA hydrolytic activity found in subgingival plaque reflected contributions from saliva and supragingival plaque. Results indicated that the assay can best be performed with 0.67 mmol/L BANA at pH 7.0. EDTA and $CaCl_2$ gave a slight inhibition and DTT a slight enhancement of the BANA reaction by the pooled plaque suspensions. The majority of the reactions (85%) developed their full color after overnight incubation. BANA hydrolysis was not found in saliva and occurred with much greater frequency in subgingival plaque as opposed to supragingival plaque. Analysis of the data indicated that BANA hydrolysis by pooled subgingival plaque samples is a suitable test for the detection of spirochetes when two or three spirochetes per *high microscopic field are present in the sample.*

J Dent Res 66(11):1668-1672, November, 1987

Introduction.

In recent years, evidence has been presented which suggests a specific bacterial etiology in many forms of periodontal diseases. The association of a limited number of bacterial species—*i.e.*, *Haemophilus (Actinobacillus) actinomycetemcomitans* (Zambon, 1985), *Bacteroides gingivalis* (Slots *et al.*, 1985; Loesche *et al.*, 1985), *Bacteroides forsythus, Wolinella recta* (Dzink *et al.*, 1985), and spirochetes (Loesche *et al.*, 1985)—with various forms of periodontal disease allows for the development of diagnostic tools that are based upon the detection of one or more of these organisms and/or their overgrowth in plaque. The detection and/or enumeration of these species by cultural or microscopic methods is time-consuming and labor-intensive. An ideal approach would be the development of a diagnostic test for the presence and/or levels of these organisms that is simple, inexpensive, and reliable.

B. gingivalis, a *Capnocytophaga* species phenotypically similar to *C. gingivalis*, *Treponema denticola*, a small spirochete (Laughon *et al.*, 1982a), and *B. forsythus* (Tanner *et al.*, 1985) possess a trypsin-like enzyme which can be detected by a biochemical chromogenic reaction. Previous studies (Loesche *et al.*, 1987) have demonstrated that the hydrolysis of the trypsin substrate N-benzoyl-DL-arginine-2-naphthylamide (BANA) by subgingival plaque obtained from a single site correlates best with the numbers and proportions of spirochetes in plaque samples and may serve as an indicator of clinical disease. In this investigation, we determined whether the same association between BANA hydrolysis and spirochetes could be obtained in pooled subgingival plaque samples, since it is likely in practice that a clinician would pool such samples so as to obtain information from one test *per* patient. In addition, the characteristics of this reaction in terms of substrate type and concentration, microbial numbers needed to give a positive reaction, rapidity of hydrolysis, pH optimum, and the effects of additives (enhancers and/or inhibitors) on the plaque BANA hydrolytic activity have been studied in patients who were periodontally healthy or diseased.

Received for publication July 23, 1986
Accepted for publication July 29, 1987
This research was supported by Public Health grants DE 02731 and DE 06030 from the National Institute of Dental Research.

Materials and methods.

Subgingival plaque samples were collected and pooled from three or four sites *per* patient by means of a curette after the supragingival plaque had been removed and discarded. In a separate experiment, using a second group of individuals, supragingival plaque samples were removed from teeth that were periodontally healthy or diseased. These pooled samples were transferred to a vial containing 0.6 mL of Sorensen phosphate buffer (pH 7.2, 0.15 mol/L monopotassium phosphate, 0.15 mol/L disodium phosphate) and dispersed for 20 seconds by means of a vortex mixer; 0.05 mL of this plaque suspension was removed for microscopic count, with the remainder used for the various experiments.

For the microscopic counts, 0.01 mL of the aliquot used for the microscopic analysis was placed on a glass slide, covered with a cover slip, sealed, and examined by dark-field microscopy. The remaining plaque suspension was further divided into several 0.05-mL aliquots and added to 0.1 mL BANA to give a final concentration of 0.67 BANA mmol/L. The BANA stock solution was prepared by the addition of 44 mg to 1 mL of dimethyl sulfoxide and was diluted 1:100 in Sorensen phosphate buffer. The samples were incubated overnight at 37°C, and the color was developed by the addition of a drop of 0.1% fast garnet (Laughon *et al.*, 1982a). The results were read by eye with the aid of a color chart and with scoring methods as described elsewhere (Loesche *et al.*, 1987).

Optimal concentration of BANA.—The purpose of this experiment was to determine optimal concentration for plaque assay. Previous studies (Laughon *et al.*, 1982b) using pure strains of *Bacteroides gingivalis* showed 1 mmol/L BANA in the final reaction mixture to be the optimal concentration. We wanted to determine whether or not this concentration was also optimal for plaque assays. The concentrations tested ranged from 0.67 mmol/L to 13.4 mmol/L BANA in the final reaction mixture.

Rapidity of hydrolysis.—Plaque samples were incubated at 37°C, and the color reactions were read after 30 minutes, one hour, and 18 hours (overnight incubation). The first two time intervals were chosen to duplicate a time interval that might occur during a patient's visit to the dentist.

Microbial numbers needed to give a positive reaction.—The number of bacteria *per* high-power microscopic field (hpf) and the number of spirochetes *per* hpf required to give a positive reaction were determined by dark-field microscopy. Each plaque suspension was examined until either 200 organisms or the

number of organisms in 20 hpf was enumerated. The single cells were identified as spirochetes, selenomonads, vibrio-like, motile rods, fusiforms, non-motile rods, or cocci. The numbers of spirochetes and bacteria *per* hpf were correlated with the BANA reaction.

In separate experiments, the BANA reagent (0.67 mmol/L) was added to undiluted plaque suspension (0.1 mL) and to 1:2 and 1:10 dilutions thereof. After overnight incubation and addition of the fast garnet color developer, the amount of color was determined and correlated with the number of bacteria and/or spirochetes *per* hpf. In this manner, it was possible to estimate the minimal number of bacteria or spirochetes needed to give a positive BANA reaction.

Substrate type. —Recently, Ohta *et al.* (1986) isolated and characterized a trypsin-like enzyme from *Treponema denticola* which was capable of hydrolyzing N-benzoyl-L-arginine-p-nitroanilide (BAPNA) as well as BANA. This substrate, unlike BANA, does not require an additional reagent to develop its color. BANA and BAPNA substrates were compared at different concentrations using the same plaque suspensions.

Effect of pH on BANA hydrolysis. —The plaque samples were normally suspended in Sorensen phosphate buffer (pH 7.2) and added to the BANA solution, which was also at pH 7.2. In this study, the plaque suspensions were added to BANA stock solutions that had been prepared in Sorensen phosphate buffer at pHs 6.0, 7.0, and 8.0, so as to mimic those pHs which could be found in resting subgingival plaque. These plaque-BANA mixtures were adjusted to yield the respective pHs by addition of 0.1 mol/L HCl or KOH.

Additives. —The previous study used plaque suspended in reduced transport fluid (RTF) for the measurement of BANA hydrolysis (Loesche *et al.*, 1987). Since RTF contains $CaCl_2$, EDTA, and dithiothreitol (DTT) as additives, the effects of these compounds on the plaque BANA hydrolytic activity were determined by addition of 0.01, 0.1, or 1 mmol/L of these reagents to the BANA-plaque-Sorensen buffer mixture previously described.

Statistical analysis. —The data were entered, *via* a customized program, into files suitable for computer processing. The bacterial counts, as well as the total spirochetal counts and their relation to test results, were submitted to analysis of variance (ANOVA) and the nonparametric Kruskal-Wallis and chi-square tests. These statistical analyses were performed by means of MIDAS, the statistical program of the Michigan Terminal System.

Results.

The majority of the samples hydrolyzed BANA over a 20-fold concentration range (0.67 to 13.4 mmol/L) (Table 1). The highest proportions of positive reactions and the lowest proportions of weak positive reactions were seen at 0.67 mmol/L BANA, and this concentration was chosen for the remaining studies. Fifteen percent of the positive reactions at these concentrations developed their maximal color in one hour, while 85% of the reactions required overnight incubation for maximal color development. The samples which exhibited a rapid color change had high levels of spirochetes *per* hpf, *i.e.*, above 20 spirochetes *per* hpf.

There was a strong positive relationship between BANA hydrolysis and both the number of spirochetes *per* hpf and the number of bacteria *per* hpf in the subgingival plaques (Table 2). An average of 6 spirochetes *per* hpf and 15 bacteria *per* hpf was associated with weakly positive reactions, suggesting that these values were near the detection limit for BANA hydrolysis. A distribution analysis of the numbers of spirochetes and bacteria *per* hpf in the BANA-positive and BANA-negative plaque samples indicated that the detection limit was lower, being about 2 spirochetes and 6 bacteria *per* hpf, respectively. Thus, 90% of the BANA-positive plaque samples had 2.4 spirochetes or more *per* hpf, whereas only 25% of the BANA-negative samples had more than 1.8 spirochetes *per* hpf (Table 3). Ninety percent of the BANA-positive samples had 6.4 bacteria or more *per* hpf, whereas only 25% of the BANA-negative samples had more than 6.5 bacteria *per* hpf.

A similar detection limit was estimated *via* a different approach. Plaque suspensions and various dilutions thereof were incubated with the BANA reagent. BANA-positive reactions occurred in samples that averaged 27 bacteria and 15 spirochetes *per* hpf (Table 4). A 1-to-2 dilution of these samples did not change the proportions of BANA-positive or weakly positive plaque, but a 1-to-10 dilution converted 90% of these samples to BANA-negative. The remaining BANA-positive or weakly positive samples had at least 7 bacteria and 2 spirochetes *per* hpf, whereas in the now-BANA-negative samples, the bacteria averaged 2.1 *per* hpf and the spirochetes 1.3 *per* hpf (Table 4).

TABLE 1
EFFECT OF BANA CONCENTRATION ON ABILITY OF PLAQUE TO HYDROLYZE BANA

BANA hydrolysis (n = 17)	(0.67 mmol/L)[a]	(3.3 mmol/L)	(6.7 mmol/L)	(13.4 mmol/L)
% Positive	76*	64*	64*	47*
% Wk Pos.	6	24	12	18
% Negative	18	12	24	35

[a]Concentration in the final reaction mixture.
*Differences among various concentrations were not statistically significant.

TABLE 2
TEST RESULTS AND THEIR RELATIONSHIP TO THE NUMBER OF BACTERIA AND SPIROCHETES *PER* HIGH-POWER MICROSCOPIC FIELD (hpf) IN SUBGINGIVAL PLAQUES

BANA Hydrolysis	n	Bact/hpf (mean ± SD)	Spir/hpf (mean ± SD)
Positive	113	24.9 ± 24.1	13.5 ± 15.1
Wk Pos.	65	15.3 ± 10.3	6.2 ± 5.4
Negative	123	5.7 ± 5.3	1.4 ± 1.6
ANOVA		$p < 0.0001$	$p < 0.0001$
Kruskal-Wallis		$p < 0.0001$	$p < 0.0001$

TABLE 3
FREQUENCY DISTRIBUTION OF SPIROCHETES *PER* HIGH-POWER FIELD (hpf) AND BACTERIA *PER* hpf IN BANA-POSITIVE AND BANA-NEGATIVE PLAQUE SAMPLES

Percentile	BANA Positive	BANA Negative	Ratio Pos./Neg.
	Spirochetes/hpf		
0.10	2.4	0.25	9.2
0.25	4.5	0.35	12.8
0.50	7.2	0.7	10.4
0.75	15.2	1.8	8.4
0.90	38.7	3.75	10.3
	Bacteria/hpf		
0.10	6.4	1.4	4.6
0.25	10.0	2.7	3.7
0.50	15.4	4.0	3.9
0.75	25.6	6.5	3.9
0.90	41.6	11.9	3.5

TABLE 4
EFFECT OF DILUTION ON THE ABILITY OF POOLED SUBGINGIVAL PLAQUE SAMPLES TO HYDROLYZE BANA

BANA hydrolysis (n = 68)	Undiluted Plaque	Diluted Plaque 1/2	1/10
% positive	67.5	67.5	2.5
bacteria/hpf	27.0	13.5	8.0
spirochetes/hpf	15.0	7.5	3.0
% weak positive	32.5	30.0	7.5
bacteria/hpf	19.0	9.5	7.0
spirochetes/hpf	7.0	4.0	2.0
% negative		2.5	90.0
bacteria/hpf		2.0	2.1
spirochetes/hpf		1.0	1.3

These experiments showed that increased numbers of spirochetes and bacteria *per* hpf were significantly related to BANA hydrolysis. The spirochetes were a major component of the bacteria *per* hpf, comprising about 40% of the flora, *i.e.*, average 44.1%, median 42.8%. Thus, it is possible that the relationship between bacteria *per* hpf and BANA hydrolysis could reflect in large part the contributions of the spirochetes to the total bacterial count. We reasoned that if the nonspirochetal bacteria were making a contribution to the BANA hydrolysis that was equal to that of the spirochetes, there would be no difference in the number of spirochetes and bacteria *per* hpf when a ratio of BANA-positive to BANA-negative plaque samples was calculated.

In the data presented in Table 3, the number of spirochetes *per* hpf, when the ratio of positive to negative BANA samples was determined, ranged from 8 to 13, with a median value of 10. The corresponding ratio for the number of bacteria *per* hpf ranged from 3.5 to 4.6, with a median value of 3.9. This suggested that the spirochetes were making the greater contribution to the positive BANA reactions, since the ratio between a positive and a negative reaction was 10, as opposed to 4 when all the bacteria were considered. If the nonspirochetal organisms were making an equal contribution, then their ratio would also have been about 10. In reality, given the fact that the spirochetes comprised about half the bacteria in the plaque, then the decrease in the ratio from 10 to 4 almost reflects the dilution of the spirochete count by BANA-negative bacteria. These calculations suggest that the spirochetes, rather than the nonspirochetal organisms, were making the major contribution to the BANA-positiveness of the plaque.

The relative contribution of the spirochetes to the positive BANA activity of the plaque samples was also assessed by measurement, in separate experiments, of the BANA activity of supragingival plaque samples that would not be expected to have the same dominant spirochetal composition as subgingival plaque. The supragingival plaque samples were removed, but instead of being discarded, were incubated with the BANA reagent. The subgingival samples collected at the same time from the same teeth were incubated independently with the BANA reagent. Sixty-two percent of the subgingival samples were BANA-positive, whereas only 15% of the supragingival samples were BANA-positive (Table 5). This indicates that the microbes responsible for BANA hydrolysis resided primarily in the subgingival plaque.

Additional studies were performed to see whether the spirochetes could contribute to the BANA hydrolysis observed in the supragingival plaque. These supragingival plaque samples were mainly BANA-negative, despite the presence of high numbers of bacteria *per* hpf (Table 6). However, there was a significant positive relationship between BANA hydrolysis and

TABLE 5
EFFECT OF SUPRAGINGIVAL AND SUBGINGIVAL PLAQUE ON BANA HYDROLYSIS

No. of Samples	Supragingival[a] 26 %	Subgingival[a] 26 %
Positive	15	62
Wk Pos.	12	3
Negative	73	35

[a]Supragingival and subgingival test results significantly different by chi-square analysis, $p < 0.005$.

TABLE 6
RELATIONSHIP BETWEEN BANA HYDROLYSIS AND THE NUMBER OF BACTERIA/hpf AND SPIROCHETES/hpf IN SUPRAGINGIVAL PLAQUES

BANA Hydrolysis	No. of Samples	Bact/hpf	Spir/hpf	% Spirochetes
Positive	1	9.4	1.3	13.8
Wk Pos.	4	10.3 ± 2.5[a]	1.0 ± 0.2	10.2 ± 1.2
Negative	35	15.9 ± 11.4	0.4 ± 0.3	1.5 ± 1.8
ANOVA		NS	$p < 0.001$	$p < 0.0001$

[a]Average plus or minus standard deviation.
NS = Not significantly different.

TABLE 7
EFFECT OF pH ON BANA OR BAPNA HYDROLYSIS BY POOLED PLAQUE*

	pH		
BANA hydrolysis (n = 27)	6.0 (%)	7.0 (%)	8.0 (%)
Positive	44	52	52
Wk Pos.	19	19	19
Negative	37	29	29
BAPNA hydrolysis (n = 15)	(%)	(%)	(%)
Positive	47	47	47
Wk. Pos.	20	20	20
Negative	33	33	33

*Differences in results are not statistically significant.

the absolute number of spirochetes, *i.e.*, spirochete *per* hpf, and relative number of spirochetes, *i.e.*, % spirochetes. In those few samples that were positive or weakly positive, the spirochetes averaged about 1 organism *per* hpf but comprised about 10% of the flora.

There were no differences in hydrolysis of BANA and BAPNA substrates at the tested concentrations. Both BANA and BAPNA were equally hydrolyzed by plaque suspensions over the pH range of 6.0 to 8.0 (Table 7). The addition of EDTA and $CaCl_2$ over a 100-fold concentration range gave a slight inhibition and DTT a slight enhancement of BANA hydrolysis by subgingival plaque samples (Table 8).

There is the possibility that proteases produced by PMNs present in crevicular fluid and/or by bacteria present in saliva or supragingival plaque could be contributing to the BANA hydrolysis observed in these subgingival plaque samples. Loesche *et al.* (1987) have shown that peripheral blood and crevicular fluid PMNs did not hydrolyze BANA. We were unable to detect BANA hydrolysis in 0.1 mL of saliva collected from 15 patients who were either healthy or periodontally diseased (data not shown). This volume of saliva is about 20 times the amount of saliva that could have contaminated

TABLE 8
EFFECT OF VARIOUS ADDITIVES ON BANA HYDROLYSIS BY POOLED PLAQUE SAMPLES

BANA hydrolysis	pH 7.0 Control	0.01 mmol/L[a]	0.1 mmol/L	1 mmol/L
		$CaCl_2$ addition (n = 14)		
% Positive	14	14	14	14
% Wk Pos.	29	7	7	7
% Negative	57	79	79	79
		EDTA addition (n = 36)		
% Positive	39	39	36	36
% Wk Pos.	28	19	22	22
% Negative	33	42	42	42
		DTT addition (n = 21)		
% Positive	52		43	62
% Wk Pos.	29		38	29
% Negative	19		19	10

[a]Concentration in the final reaction mixture.

the plaque samples and indicates that the saliva was not contributing to the BANA hydrolysis of our subgingival samples.

Discussion.

Several studies have demonstrated that the number and proportions of spirochetes can be used to distinguish between healthy or periodontally diseased patients (Listgarten and Hellden, 1978; Loesche *et al.*, 1985; Slots *et al.*, 1985). However, spirochetes may not be the exclusive microbial indicator of periodontal disease, since *B. gingivalis, B. forsythus, H. actinomycetemcomitans*, and others have also been associated with periodontal diseases. Thus, it may be necessary to develop a test that will detect and quantitate most if not all of these species. This BANA assay, while correlating significantly with plaque levels and proportions of spirochetes, has the potential to detect the presence in high numbers of other species, such as *B. gingivalis* and *B. forsythus*, that are capable of degrading BANA (Loesche *et al.*, 1986). In this capacity, the BANA test may provide a simple means of diagnosing an anaerobic infection involving these periodontopathogens.

The characteristics of the BANA reactions from subgingival plaque samples were investigated in this study in order to optimize the conditions for this assay. The concentration of BANA over the range of 0.67 mmol/L to 13.4 mm/L was not critical, and the level of 0.67 mmol/L was chosen for further testing, since it yielded the maximal amount of hydrolysis *per* amount of substrate used (Table 1).

The importance of spirochetes was demonstrated when the ratio of positive and negative BANA results was compared as a function of the number of spirochetes or bacteria *per* hpf (Table 3). The importance of spirochetes was also shown by the findings obtained with the supragingival plaque samples. With these, there was no relationship between bacterial numbers and BANA hydrolysis, while there was a highly significant positive association between BANA hydrolysis and spirochetes *per* hpf and % spirochetes (Table 6). In this respect, we concluded that the BANA hydrolysis was directly related to the number of spirochetes in both subgingival and supragingival plaque samples.

This analysis showed that the detection limit for the spirochetes was in the vicinity of 2 spirochetes *per* hpf, since 90% of the positive reactions had 2 or more spirochetes *per* hpf (Table 3). This value was also suggested by the dilution experiments, since BANA-positive samples converted to BANA-negative samples when the samples were diluted to 1.3 spirochete and 2.7 bacteria *per* hpf (Table 4). These spirochetal numbers are comparable with the 2 to 3 spirochetes *per* hpf that had previously been observed with single plaque samples taken from the most diseased sites *per* quadrant (Loesche *et al.*, 1987). Thus, by including multiple subgingival plaque samples that were not necessarily taken from the most diseased sites, we could obtain results similar to those obtained when only single sites were sampled.

Among the cultivable spirochetes—*i.e., T. socranskii, T. vincenti, T. pectinovorum*, and *T. denticola*—only *T. denticola* hydrolyzes BANA (Syed *et al.*, 1987, unpublished data). This would suggest the importance of *T. denticola* in the diseased sites. In this regard, Simonson *et al.* (1987) have shown, using monoclonal antibodies to *T. denticola*, that the numbers of this species increased significantly both in absolute numbers and relative numbers in samples taken from sites exhibiting periodontitis as opposed to gingivitis.

These results do not rule out the possibility that other bacteria were contributing to the positive BANA results. A previous investigation had shown that *B. gingivalis* and *C. gingivalis* were minor contributors to the BANA positiveness of plaque (Loesche *et al.*, 1987). This leaves the recently described species, *B. forsythus*, and other unrecognized bacteria, as well as uncultivable spirochete species, as potential contributors to the BANA reaction.

BANA and BAPNA substrates were both readily hydrolyzed by plaque. However, BANA was the substrate of choice, because any blood in the sample gave a pale yellow color which was indistinguishable from the pale yellow color associated with a weak BAPNA result. Accordingly, in the presence of any blood, it would be difficult to recognize a weak BAPNA reaction by the plaque.

There was no significant effect on the BANA reactions of the various pHs that would be encountered in the plaque. The trypsin-like enzyme recently isolated from *B. gingivalis* (Yoshimura *et al.*, 1984) is stimulated by DTT and EDTA and unaffected by $CaCl_2$, whereas the *T. denticola* enzyme (Ohta *et al.*, 1986) is unaffected by DTT and EDTA and activated by $CaCl_2$. In our study, EDTA and $CaCl_2$ gave a slight inhibition and DTT a slight enhancement of the BANA reaction by the pooled plaque suspensions (Table 8). Thus, the ingredients in the RTF did not seem to affect the results, nor did their addition suggest whether the *T. denticola* or *B. gingivalis* enzyme was the dominant enzyme in the plaque.

Analysis of these data indicates that BANA hydrolysis by pooled subgingival plaque samples is a suitable test for the detection of spirochetes when at least 2 or 3 spirochetes *per* hpf are present in the sample. The assay can best be performed with 0.67 mmol/L BANA at pH 7.0, although there appears to be a wide range of substrate concentrations and pHs at which the enzyme is active.

The following instances are examples of when or where the BANA test could be applied in periodontal therapy: (a) at initial diagnosis, in conjunction with clinical parameters, so as to establish treatment tactics; (b) to determine whether initial treatment has been adequate or whether additional modalities are called for; (c) at recall visits, to determine whether retreatment is necessary; and (d) to predict possible future development of periodontal inflammation or periodontal breakdown. In this regard, the predictive value of clinical parameters is minimal in anticipating attachment loss in untreated patients (Haffajee *et al.*, 1983).

Loesche *et al.* (1987) have shown that BANA hydrolysis by plaque samples has the potential to be a marker of periodontal morbidity as assessed by probing depth measurements and by plaque proportions of spirochetes. Syed *et al.* (1984) have shown that BANA hydrolysis can be used to measure the efficacy of antimicrobial treatment in the experimental gingivitis

model. Gusberti *et al.* (1986) have shown that BANA hydrolysis along with other enzyme markers can be used to diagnose and to monitor treatment efficacy in refractory patients. Thus, the clinical management of patients with periodontal disease can be based on criteria given by both bacterial and clinical parameters that can be compared at different time intervals, as was suggested by Listgarten (1986).

In summary, the BANA test is simple to perform, of low cost, and could be useful for the dental profession as a parameter for diagnosing and monitoring the levels of spirochetes in subgingival plaque samples. The sensitivity and specificity of the BANA test are being evaluated in ongoing clinical studies in our laboratory.

Acknowledgments.

We would like to thank Drs. E. Schmidt, E. Chaves, J. Johnston, J. Schwartz, J. Giordano, B. Steffenson, G. Anderson, and Ms. Debra Stults for the collection of plaque samples.

REFERENCES

DZINK, J.L.; TANNER, A.C.R.; HAFFAJEE, A.D,; and SOCRANSKY, S.S. (1985): Gram-negative Species Associated with Active Destructive Periodontal Lesions, *J Clin Periodontol* 12:648–659.

GUSBERTI, F.A.; SYED, S.A.; HOFMANN, T.; and LANG, N.P. (1986): Diagnostic Methods for the Assessment of Potential Periodontal Activity: Enzymatic Activities of Bacterial Plaque and their Relationship to Clinical Parameters. In: **The Borderland Between Caries and Periodontal Disease III**, T. Lehner and G. Cimasoni, Eds., Geneva: Editions Médécine et Hygiene, pp. 383–390.

HAFFAJEE, A.D.; SOCRANSKY, S.S.; and GOODSON, J.M. (1983): Clinical Parameters as Predictors of Destructive Periodontal Disease Activity, *J Clin Periodontol* 10:257–265.

LAUGHON, B.E.; SYED, S.A.; and LOESCHE, W.J. (1982a): API-ZYM System for Identification of *Bacteroides spp.*, *Capnocytophaga spp.*, and *Spirochetes* of Oral Origin, *J Clin Microbiol* 15:97–102.

LAUGHON, B.E.; SYED, S.A.; and LOESCHE, W.J. (1982b): Rapid Identification of *Bacteroides gingivalis*, *J Clin Microbiol* 15:345–346.

LISTGARTEN, M.A.(1986): A Perspective on Periodontal Diagnosis, *J Clin Periodontol* 13:175–181.

LISTGARTEN, M.A. and HELLDEN, L. (1978): Relative Distribution of Bacteria at Clinically Healthy and Periodontally Diseased Sites in Humans, *J Clin Periodontol* 5:115–132.

LOESCHE, W.J. (1986): The Identification of Bacteria Associated with Periodontal Disease and Dental Caries by Enzymatic Activity Methods, *Oral Microbiol Immunol* 1:65–70.

LOESCHE, W.J.; SYED, S.A.; SCHMIDT, E.; and MORRISON, E.C. (1985): Bacterial Profiles of Subgingival Plaques in Periodontitis, *J Periodontol* 56:447–456.

LOESCHE, W.J.; SYED, S.A.; and STOLL, J. (1987): Trypsin-like Activity in Subgingival Plaque — A Diagnostic Marker for Spirochetes and Periodontal Disease?, *J Periodontol* 58:266–273.

OHTA, K.; MAKINEN, K.K.; and LOESCHE, W.J. (1986): Purification and Characterization of an Enzyme Produced by *Treponema denticola* Capable of Hydrolyzing Synthetic Trypsin Substrates, *Infect Immun* 53:213–220.

SIMONSON, L.G.; GOODMAN, C.H.; MORTON, H.E.; and ROUSE, R.F. (1987): Quantitative Estimation of *Treponema denticola* in Adult Subgingival Plaque, *J Dent Res* 66:169, Abst. No. 499.

SLOTS, J.; EMRICH, L.J.; GENCO, R.J.; and ROSLING, B.G. (1985): Relationship Between Some Subgingival Bacteria and Periodontal Pocket Depth and Gain or Loss of Periodontal Attachment After Treatment of Adult Periodontitis, *J Clin Periodontol* 12:540–552.

SYED, S.A.; GUSBERTI, F.A.; LOESCHE, W.J.; and LANG, N.P. (1984): Diagnostic Potential of Chromogenic Substrates for Rapid Detection of Bacterial Enzymatic Activity in Health and Disease Associated Periodontal Plaques, *J Periodont Res* 19:618–621.

SYED, S.A.; SALVADOR, S.L.; MAKINEN, K.K.; MAKINEN, P.-L.; and LOESCHE, W.J. (1987): Biochemical Comparison of Peptidases in Oral *Treponema sp.*, *ASM Abst* 87:No. K137.

TANNER, A.C.R.; STRZEMPKO, M.N.; BELSKY, C.A.; and McKINLEY, G.A. (1985): API ZYM and API An-Ident Reactions of Fastidious Oral Gram-negative Species, *J Clin Microbiol* 22:333–335.

YOSHIMURA, F.; NISHIKATA, M.; SUZUKI, T.; HOOVER, C.I.; and NEWBRUN, E. (1984): Characterization of a Trypsin-like Protease from the Bacterium *Bacteroides gingivalis* Isolated from Human Dental Plaque, *Arch Oral Biol* 29:559–564.

ZAMBON, J.J. (1985): *Actinobacillus actinomycetemcomitans* in Human Periodontal Disease, *J Clin Periodontol* 12:1–20.