The Utility of the BANA Test for Monitoring Anaerobic Infections due to Spirochetes (*Treponema denticola*) in Periodontal Disease

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Treponema denticola, Porphyromonas gingivalis, and Bacteroides forsythus each possesses an enzyme(s) that hydrolyzes the synthetic substrate benzoyl-DL-arginine-naphthylamide (BANA). The presence of these organisms in a subgingival plaque sample can be determined by the ability of the plaque to hydrolyze BANA. In the present study, we describe the usefulness of the BANA test at various stages of a clinical trial of the efficacy of metronidazole in the treatment of periodontal disease. A BANA-positive test was significantly associated with high levels and proportions of spirochetes in the plaque, so that it provided information comparable with that which could be obtained by a microscopic examination of the plaque. Patients with such anaerobic spirochetal infections were randomly assigned to a group receiving either metronidazole or placebo (250 mg, three times a day) for one week and whose teeth were scaled and root-planed. The advantages of the decision that metronidazole be used were apparent from the comparison with the results obtained in the patients who received only the scaling and root planing. The initially BANA-positive teeth in the patients treated with metronidazole, scaling, and root planing gained attachment and exhibited a significant reduction in the need for periodontal surgery, when compared with the BANA-positive teeth in the patients who received only placebo, scaling, and root planing. After the conclusion of this therapy, those teeth with persistent BANA-positive plaques had significantly higher proportions and levels of spirochetes than did the teeth with BANA-negative plaques. A tooth site which tested BANA-positive after the initial therapy lost significantly more attachment in the year following active treatment than did a tooth site that tested BANA-negative, *i.e.*, a mean difference of 0.48 mm *per* year. The ability of the BANA test to detect an anaerobic infection due to the BANA-positive species and possibly to predict future attachment loss indicates that this test may be helpful in the management of the periodontally diseased patient.

Introduction.

The demonstration that most forms of periodontal disease can be associated with the presence and/or proportional overgrowth of a finite number of bacterial species has opened the possibility that these "infections" can be treated with specific antimicrobial therapies (Loesche, 1987). Any treatment paradigm involving antimicrobial agents should require that an infection be diagnosed and that the efficacy of treatment be monitored. The diagnosis of an infection by cultural methodologies is complicated by the anaerobic nature of the flora and the fastidious growth requirements of certain putative pathogens, such as the spirochetes (Loesche, 1988) and *Bacteroides forsythus* (Wyss, 1989). Alternative methods to culturing, which may be more cost-efficient, have been suggested. They would include the microscopic examination of plaque for spirochetes and motile organisms (Listgarten and Hellдén, 1978; Keyes and Rams, 1983); the use of highly specific polyclonal or monoclonal antibodies to the putative pathogens (Zambon *et al.*, 1985; Bragd *et al.*, 1987; Simonson *et al.*, 1988); the use of whole genomic probes or oligodeoxynucleotide probes to ribosomal RNA to these organisms (Savitt *et al.*, 1988; Giovannoni *et al.*, 1988); or the detection of marker molecules, such as various enzymes, which are characteristic of some of the organisms (Syed *et al.*, 1984; Loesche, 1986; Suido *et al.*, 1987).

Treponema denticola (a small spirochete), *Porphyromonas (Bacteroides) gingivalis,* and *Bacteroides forsythus* each possesses an enzyme(s) that hydrolyzes the synthetic substrate benzoyl-DL-arginine-naphthylamide (BANA) (Loesche, 1986). Fifty-one other plaque species do not hydrolyze BANA, and several *Capnocytophaga* and non-pigmented *Bacteroides* species are variable in giving a weak BANA reaction (Loesche *et al.*, 1990a). Because the three BANA-positive species are frequently cited as potential periodontal pathogens (Loesche, 1987; Moore, 1987; Dzink *et al.*, 1988), and all are anaerobic organisms, the ability of a given subgingival plaque sample to hydrolyze BANA could be a measure of an anaerobic periodontal infection due to one or more of these species. BANA hydrolysis will detect *P. gingivalis* and/or *T. denticola* with a sensitivity of 92% and a specificity of 70% with highly specific polyclonal antibodies to these organisms in an ELISA assay being used as the reference standard (Loesche *et al.*, 1990b). In untreated periodontal patients, BANA hydrolysis by subgingival plaques showed a sensitivity of 80% and a specificity of 98%, when compared with a clinical judgment as to whether the sampled tooth site was periodontally healthy or diseased (Schmidt *et al.*, 1988).

The present report is concerned with the possible usefulness of the BANA test in improving the diagnosis and/or treatment of periodontal disease. In particular, we were interested in the applications of the BANA test at various stages of a longitudinal clinical trial of the efficacy of metronidazole in periodontal disease. Could the BANA test be used for detection of a preponderance of the putative anaerobic pathogens in the plaque, and thereby allow the clinician to diagnose an anaerobic infection associated with these organisms? What would be the benefits and/or consequences if that diagnosis led to the use of metronidazole? Finally, what is the significance of a BANA-positive plaque sample after the teeth have been rigorously scaled and root-planed? Do such plaques reflect persistent infections and represent sites that will deteriorate in the foreseeable future?

Materials and methods.

Subjects. —Patients seen at the Graduate Periodontal Clinic who had an advanced form of adult periodontitis that would normally require periodontal surgery (and who had an anaer-

obic periodontal infection, as judged by high proportions of spirochetes in three or four plaque samples), were entered into two double-blind studies involving the unsupervised one-week use of systemic metronidazole (Loesche *et al.*, 1987a, 1988). Prospective patients who were pregnant, or had a history of drug allergies, or who were taking medication that could influence their periodontal condition (*i.e.*, antibiotics, non-steroidal anti-inflammatory agents), were excluded from the study. Seventy-two patients completed the treatment phase of the study, and 55 of these patients were available for the one- and two-year follow-up examinations.

Experimental protocol. —All patients received the treatment that is standard in the graduate clinic, *i.e.*, rigorous debridement (scaling and root planing) of the teeth that had 3 mm or more probing depth, detailed oral hygiene instructions, and occlusal adjustment when indicated. In addition, the patients were randomly assigned to receive either metronidazole or placebo (250 mg, three times a day for one week). The medication was given either at the first week of scaling/root planing (first double-blind study) or after all scaling/root planing was completed (second double-blind study). The placement of the week of medication, either before or after debridement, did not have a significant effect on the microbiological outcomes (Loesche *et al.*, 1990c). The experimental protocol was ethical, since subjects in the placebo group received all the scaling and root planing that would normally be delivered to patients attending our graduate clinic. In this sense, the placebo group was a positive control group, reflecting the clinical benefits that can be achieved by root-surface debridement, plus any benefits that might be associated with the participation in a double-blind clinical study.

All patients were evaluated for various clinical and bacteriological parameters prior to any treatment, again four to six weeks after the completion of all debridement and medication, and at annual intervals during the recall program. At each of these evaluations, the following information was collected: full-mouth probing depths and attachment levels about five sites (the disto-lingual site was not examined) of each tooth in the dentition (Ramfjord *et al.*, 1973); cultural and microscopic analyses of the subgingival plaque removed from the most diseased tooth site in each quadrant; and the ability of these plaques to hydrolyze BANA. After the second treatment-planning session, any teeth that required periodontal surgery were given this treatment, and then these patients entered the recall program. In the recall program, the patients were given a prophylaxis and oral hygiene instructions by a dental hygienist at three-month intervals.

Furthermore, a periodontist who was not involved in the treatment of the patients, and who was unaware of which treatment group the patient was in, examined the patient, and, using the above clinical parameters and radiographs, determined the number of teeth that either needed periodontal surgery or needed to be extracted for periodontal purposes, at each of the four treatment planning sessions. The criteria used to determine the need for surgery at the second treatment planning session were: (1) presence or absence of bleeding; (2) presence or absence of exudate; (3) change in probing depth and attachment levels from those measured at the first treatment planning sessions; (4) root topography; (5) nature of bony defect or furcation involvement, as shown by radiographs as well as probing; and (6) whether surgical intervention was necessary so that better access for additional root instrumentation could be gained. In a separate investigation, two periodontists not involved in this study used these criteria to evaluate independently the periodontal surgical needs of 64 patients who were also not involved in the present study. The correlation coefficient for agreement between these two examiners was 0.82, which was

significant at the $p < 0.0001$ level, indicating that the surgical criteria used were reproducible. The detailed clinical results of the effect of metronidazole on periodontal surgery will be reported elsewhere (Loesche *et al.*, unpublished data).

Bacteriology. —The bacteriological procedures have been described (Loesche *et al.*, 1984, 1985) and will be summarized. A periodontist selected the most periodontally-involved tooth in each quadrant, and these teeth were designated as bacteriological test teeth (Schmidt *et al.*, 1988). The subgingival plaque from a discrete site on these test teeth was removed with a curette, added to a vial containing 0.5 mL of reduced transport fluid without EDTA, placed immediately into an anaerobic chamber, and dispersed for 20 s with a Vortex mixer. A 50-μL portion was removed, and 10 μL of this was placed on a glass slide, viewed by dark-field microscopy, and the number of spirochetes, selenomonads, motile rods, fusiforms, non-motile rods, and cocci was determined. The remaining 40 μL was incubated overnight at 37°C in the presence of BANA, and any released naphthylamide was developed by the addition of fast garnet (Laughon *et al.*, 1982). The remaining 450 μL of the plaque suspension was diluted to 4 mL, sonically dispersed for 20 s with a Kontes sonifier (Kontes Glass Co., Vineland, NJ), serially diluted in RTF, and plated automatically with a spiral plater (Spiral Systems Inc., Cincinnati, OH) on a variety of selective and non-selective media.

Statistical analysis. —A two-factor ANOVA, with one fixed factor being the BANA result and one random factor being the patient, was performed in Table 1 for comparison of the bacteriological profiles of the plaque samples. For Tables 2 and 4, the correlated binomial model was used to obtain the estimates of the proportion of bacteriological test sites where an event of interest (*i.e.*, change in teeth needing surgery) occurred. This method takes into account the correlated nature of the observations made on test sites within patients, and the heterogeneity of total number of sites measured *per* patient

TABLE 1

MICROBIOLOGICAL PROFILE OF BANA-POSITIVE AND BANA-NEGATIVE PLAQUES AT ENTRY TO CLINICAL STUDY OF METRONIDAZOLE

	BANA-Positive n = 224	Significance	BANA-Negative n = 35
Microscopic			
% Spirochetes	62.1	p = 0.0001[a]	39.5
% Rods	16.3	p = 0.0001	32.9
% Cocci	3.6		5.9
% Fusiforms	4.7	p = 0.009	9.5
% Selenomonads	9.3		7.7
% Motile	3.2		4.5
Bacteria/hpf	26	p = 0.006	12
Spirochetes/hpf	16.1	p <0.001	4.7
Cultural			
% *A. naeslundii*	1.1		1.7
% *A. viscosus*	2.2		3.0
% *A. odontolyticus*	0.4		0.4
% *S. sanguis*	0.9		2.1
% *S. mutans*	0.7		3.0
% *Veillonella*	0.5		0.8
% *F. nucleatum*	3.9		6.4
% *Selenomas* sp.	0.8		0.2
% *B. melaninogenicus*	1.0		0.6
% *B. intermedius*	9.6		7.4
% BANA neg *Capnocytophaga* sp.	0.2		0.1
% BANA pos *Capnocytophaga* sp.	0.2		0.1
% *P. gingivalis*	0.9		0.1

(a) Difference between groups was significant when the two-factor ANOVA was used.

TABLE 2

PROPORTION OF BANA-POSITIVE PLAQUES CONVERTING TO BANA-NEGATIVE PLAQUES FOLLOWING TWO DIFFERENT
TREATMENT MODALITIES

Treatment Modality	No. of Subjects	Proportion Converting to BANA-negative		Within-patient Correlation	Ho: sites are independent Chi-square	Significance
Metronidazole + Scaling + Root Planing	33	74/106[a]	(70%)	r = 0.191[b] ±0.10	5.3	p = 0.02
Placebo + Scaling + Root Planing	39	63/119[a]	(53%)	r = 0.30[b] ±0.13	6.5	p = 0.01
Treatment Effect: significance		chi-square = 4.54 p = 0.03				

(a) Number of plaque samples.
(b) r = within-patient correlation coefficient ± standard error.

(Hujoel et al., 1990). Estimates of the proportions and their standard errors were obtained by use of maximum likelihood routines. The outcome of interest in Table 6 was the slope of the simple regression line obtained from the regression of attachment level measurements on time; a positive slope indicates a gain of attachment and a negative slope, a loss of attachment. A two-factor analysis of variance was performed, with the dependent variable being the regression coefficient, and the independent factors being the BANA result (positive or negative) (fixed factor) and the patient (random factor).

Results.

Table 1 shows the microbiological profile of BANA-positive and BANA-negative plaques removed from periodontally diseased teeth in patients who were recruited into the clinical trial of metronidazole. Eighty-six percent of these plaques were BANA-positive, and they differed from the BANA-negative plaques in that they had significantly higher proportions and levels of spirochetes per high-power microscopic field (hpf) and in total numbers of bacteria per hpf. The BANA-negative plaques had significantly higher proportions of rods and fusiforms. Within the spirochetes, the BANA reaction was significantly associated with the small-size spirochete (p = 0.009) and the intermediate size spirochete (p = 0.04). T. denticola would be found in both of these groupings (Cheng et al., 1985). The proportions of P. gingivalis and BANA-positive Capnocytophaga species were low in both groups (Table 1). A positive BANA test in a plaque sample thus indicated high levels of spirochetes, presumably high levels of T. denticola, and provided the same information about spirochetes as could be obtained by the microscopic examination of plaques.

A positive BANA test could be used so that an anaerobic

infection associated with spirochetes and/or T. denticola could be diagnosed. Metronidazole is an antimicrobial agent whose spectrum of activity is limited to anaerobes (Tally et al., 1978) and would be an indicated treatment choice for infections involving anaerobic organisms such as the spirochetes. If metronidazole were the drug of choice in anaerobic periodontal infections, then those patients receiving it should exhibit benefits (in regard to both their anaerobic infection and the clinical appearance of their periodontium) that would be above and beyond those associated with routine scaling and root planing. We then sought to determine what magnitude of benefits would accrue to those patients who received systemic metronidazole based on a positive BANA reaction. So that these determinations would be made objectively, the observations were made in conjunction with a double-blind clinical trial of metronidazole, in which the patients were randomly assigned to a metronidazole or placebo medication superimposed upon a regimen of scaling and root planing.

In patients treated with metronidazole plus scaling and root planing, 70% of the BANA-positive plaques converted to BANA-negative, whereas in the positive control group, 53% of the BANA-positive plaques converted to BANA-negative (Table 2). There was a significant within-patient correlation in both groups, indicating that if one tooth site converted from BANA-positive to BANA-negative, then there was an increased probability that other sites in the same mouth would also go from positive to negative (Table 2). After the within-patient correlation was accounted for, the conversion to BANA-negative plaques was significantly greater in the metronidazole group, indicating a treatment effect (chi-square, 4.54; p = 0.032) (Table 2). This conversion was associated with a significantly greater reduction in proportions of spirochetes in the subgingival plaques in the metronidazole group, relative to the

TABLE 3

EFFECT OF SCALING AND ROOT PLANING PLUS EITHER METRONIDAZOLE OR PLACEBO MEDICATION ON LEVELS OF BANA-POSITIVE PLAQUE ORGANISMS

BANA-Positive Organisms	1st Treatment-planning Session		2nd Treatment-planning Session		
	Metronidazole n = 33[c]	Placebo n = 39	Metronidazole n = 33		Placebo n = 39
Spirochetes[a]	55.1%	59.5%	27.7%	p = 0.002[d]	41.3%
P. gingivalis[b]	0.3	1.8	0.3		0.2
Capnocytophaga sp.[b]	0.2	0.1	0.1		0.5

(a) Percentage of the microscopic count.
(b) Percentage of the viable count.
(c) n = number of patients.
(d) Difference between groups was significant when the two-sample test for promotions was used.

TABLE 4

PROPORTION OF BANA-POSITIVE SITES THAT EXPERIENCED A REDUCTION IN SURGICAL NEED FOLLOWING TWO DIFFERENT TREATMENT MODALITIES

Treatment Modality	No. of Subjects	Proportion not Needing Surgery		Within-patient Correlation	Ho: sites are independent Chi-square	Significance
Metronidazole + Scaling + Root Planing	32	49/108[a]	(45%)	r = 0.301[b] ± 0.10	8.84	p<0.003
Placebo + Scaling + Root Planing	34	19/91[a]	(21%)	r = 0.35[b] ± 0.10	10.07	p<0.001
Treatment Effect: significance		chi-square = 7.93 p = 0.005				

(a) Number of teeth.
(b) r = within-patient correlation coefficient ± standard error.

positive control group (Table 3). Thus, the combination of scaling/root planing and metronidazole reduced the proportion of spirochetes from 55% to 28%, whereas the combination of scaling/root planing and placebo reduced the proportion of spirochetes from 60% to 41%. The BANA-positive *P. gingivalis* and *Capnocytophaga* species were minor members of the cultivable flora in both treatment groups, and their proportions did not change appreciably as a result of treatment (Table 3).

We next looked for clinical correlates of this metronidazole effect only in those teeth that were initially BANA-positive. One approach was the determination of whether the treatment affected the need for periodontal surgery. At baseline, a total of 225 sites tested BANA-positive, and 199 of these were judged to be in eventual need of periodontal surgery. Many of these teeth improved as a result of the various treatments, to the extent that they did not need any surgical intervention at the second treatment-planning session. In the metronidazole-treated patients, 45% of the teeth that were initially BANA-positive and recommended for surgery no longer required surgery at the second treatment-planning session, whereas in the positive control patients, 21% of similar teeth no longer required surgery (Table 4). This difference between treatment groups was highly significant at the p = 0.005 level (Table 4). When the analysis was performed on those teeth that were initially BANA-negative, there was no statistically significant difference between the treatment groups. Thus, the reduction

in surgery was from 12 to five teeth in the metronidazole group and from 14 to eight teeth in the positive control group.

A BANA-positive plaque at the second treatment-planning session, regardless of the treatment group to which the patient belonged, indicated high proportions and levels of spirochetes (Table 5). In the 83 plaques that were BANA-positive at both the first and second treatment-planning sessions, the treatments caused a slight reduction in the proportions of spirochetes and reduced the levels of spirochetes *per* hpf by about 55%. But in the 114 plaques that were BANA-positive at the first treatment-planning session and that converted to BANA-negative at the second treatment-planning session, the treatment caused a highly significant reduction in proportions of spirochetes and a 90% reduction in the levels of spirochetes *per* hpf (Table 5). Thus, a BANA-positive plaque at the completion of treatment indicated an inadequate resolution of the spirochetal infection.

We were interested in the future periodontal health of these BANA-positive teeth relative to the BANA-negative teeth, given the fact that there were about six times more spirochetes remaining on the surfaces of the BANA-positive teeth, compared with the BANA-negative teeth (Table 5). The average probing depths and attachment levels were plotted over time for the BANA-positive and BANA-negative teeth, *i.e.*, for those teeth present at the second, third, and fourth annual treatment-planning sessions. There were no significant differences in the probing-depth measurements over time for the 175 teeth that

TABLE 5

EFFECT OF TREATMENT UPON THE BANA-POSITIVE STATUS OF PLAQUES REMOVED FROM DISCRETE TOOTH SITES

	Sites which Convert to BANA-negative at Second Treatment-planning Session			Sites which Remain BANA-positive at Second Treatment-planning Session	
	BANA-positive 1st Treatment-planning Session n = 114	BANA-negative 2nd Treatment-planning Session n = 114		BANA-positive 1st Treatment-planning Session n = 83	BANA-positive 2nd Treatment-planning Session n = 83
% Spirochetes Difference	62.5	25.8[a] 37.7%	p<0.0001	61.5	48.6[a] 12.9
% *P. gingivalis* Difference	0.4	0.06 0.34		1.2	0.6 0.6
% *Capnocytophaga* sp. Difference	0.12	0.08 0.04		0.05	0.05
Bacteria/hpf Difference	22.4	5.9[a] 16.5		33.6	19.1[a] 14.5
Spirochetes/hpf Difference	14.0	1.5[a] 12.5		20.7	9.3[a] 11.4

(a) Values between groups at second treatment-planning session are significantly different (p<0.01).

TABLE 6

THE RELATIONSHIP BETWEEN BANA STATUS AFTER INITIAL
THERAPY AND FUTURE ATTACHMENT LOSS

	First Year Only		
	df	ss	
BANA Status	1	0.0084	F value = 5.21
Patient	53	0.1770	p = 0.024
Error	121	0.1944	
			Mean loss *per* Year
BANA-positive sites		n = 57	−0.48 mm
BANA-negative sites		n = 119	0.00
			0.48 mm

	First, Second, or Third Year		
	df	ss	
BANA Status	1	0.0069	F value = 5.14
Patient	54	0.1698	p = 0.025
Error	123	0.1665	
			Mean loss *per* year
BANA-positive sites		n = 59	−0.33 mm
BANA-negative sites		n = 120	0.05
			0.38 mm

df = Degrees of freedom; ss = sum of squares.

were monitored in 54 patients. However, there was a significant difference in the attachment levels between BANA-positive and BANA-negative teeth. The teeth that were BANA-positive at the second treatment-planning session lost, on the average, 0.48 mm of attachment during the year following active treatment, whereas in the teeth that were BANA-negative, there was no discernible loss (Table 6). When the analyses were extended so that data could be taken in from the first and second, and, if available, the third annual treatment-planning sessions, the BANA-positive teeth lost, on the average, 0.38 mm of attachment (*per* year) more than the BANA-negative teeth. This difference was significant (p = 0.025, Table 6).

Discussion.

A diagnostic test should be useful, ideally leading to a choice of treatment that would confer benefits upon the patient. Very few diagnostic tests exist for periodontal disease. Microscopic examinations of plaque samples have been used in the detection of high levels and/or proportions of spirochetes (Listgarten and Helldén, 1978), which can then be used for diagnosis of an anaerobic spirochetal infection (Loesche, 1988), or to monitor the need for periodontal treatment (Listgarten and Levin, 1981). In this study, we sought to determine whether the BANA test could substitute for a microscopic count in yielding diagnostic information regarding the levels and proportions of spirochetes and other BANA-positive species in plaques removed from tooth sites that had been judged by a periodontist as being the worst and/or the most representative of the periodontally diseased sites in each quadrant.

T. denticola, as measured indirectly as a small/intermediate-sized spirochete, was the only known BANA-positive species monitored that could be associated with a BANA-positive reaction in the plaque. *P. gingivalis* and the BANA-positive *Capnocytophaga* species were present in low and similar proportions in both the BANA-positive and BANA-negative plaques (Table 1), and could not be associated with the BANA reaction. *B. forsythus* was not monitored in this investigation because of the difficulty in the detection of this organism on primary isolation plates (Gmür *et al.*, 1989), and because specific antibodies and/or DNA probes to this organism were not available at the time of this investigation. Recent studies in which plaques were monitored by immunological reagents to *T. denticola*, *P. gingivalis*, and *B. forsythus* suggest that *B. forsythus* can be a contributor to the BANA reaction (Bretz *et al.*, unpublished results).

Although the spirochetes were significantly elevated in the BANA-positive plaques relative to the BANA-negative plaques, there still were impressive numbers of spirochetes in the BANA-negative plaques, *i.e.*, 39.5% spirochetes and 4.7 spirochetes *per* hpf (Table 1). The absence of a positive BANA reaction in the presence of what would appear to be adequate numbers of spirochetes would suggest that *T. denticola*, the only spirochete known to be BANA-positive, was not present in high numbers in these plaques. If this be true, and if *T. denticola* is more of a periodontopathogen than the other plaque spirochetes (Loesche, 1988), then the BANA test could provide more diagnostic information than can be obtained by a microscopic examination for spirochetes.

The present findings agree with previous findings that showed a strong relationship between a BANA-positive reaction and high levels of plaque spirochetes (Bretz and Loesche, 1987; Loesche *et al.*, 1987b). However, there is the possibility that other plaque species, and possibly host enzymes, could be contributing to this reaction. We have screened approximately 244 strains representing 51 species (including *T. vincentii*, *T. pectinovorum*, an unspeciated treponemal species, and four black-pigmented bacteroides species that could be present in plaque), and found them uniformly BANA-negative (Loesche *et al.*, 1990a). A variable reaction was observed with certain *Bacteroides* and *Capnocytophaga* species in which 36 of 152 separate determinations were positive or weakly positive, but these reactions usually occurred with numbers of organisms, *i.e.*, 10^8 CFU, that were beyond their expected levels in a mg of plaque. In the present investigation, the *Capnocytophaga* species were monitored and did not seem to be contributing to the BANA-positive reaction in the plaque (Tables 1, 3, and 5). There was no convenient way to monitor the BANA-variable *Bacteroides* species, such as *B. capillosus*, *B. denticola*, and *B. oralis*, so their contributions to the observed BANA-positive reactions are not known. Thus, we think it unlikely that other plaque species were contributing to the BANA reaction.

The plaque sample is often contaminated with gingival crevicular fluid (GCF) and/or blood, and these fluids contain proteolytic enzymes of host origin that could potentially hydrolyze BANA. We have not found blood, saliva, or neutrophils isolated from the blood or from gingival crevicular washings to be able to hydrolyze BANA (Bretz and Loesche, 1987; Loesche *et al.*, 1987b). Recent reports have shown that GCF that was pooled from multiple patients had a trypsin-like activity, as measured by the hydrolysis of peptide derivatives of 7-amino-4-trifluoromethyl coumarin (AFC) (Cox and Eley, 1989a, b). This AFC-hydrolytic enzyme(s) is presumably of host origin and could be present in plaque samples. We, however, have found that when portions of the same plaque sample are exposed to either BANA or z-Arg-AFC, only the hydrolysis of BANA could be significantly associated with the plaque proportions of spirochetes (Schmidt *et al.*, 1988). Thus, the enzyme in the plaque that hydrolyzed the AFC substrate and is possibly the tryptase enzyme (Cox and Eley, 1989a) bore no relationship to the plaque levels of spirochetes.

Another reason for the suspicion that the host's proteolytic enzymes would not be important contributors to the proteolytic activity of subgingival plaque samples is simply one of quantitation. These host enzymes were detected in a GCF sample that was pooled from 20 patients, with from about 2 to 13 μL of GCF collected *per* patient (Cox and Eley, 1989b). This

would amount to from 40 to 260 mg of GCF. Our plaque samples weighed between 1 and 2 mg, and if they contained as much as 10% GCF, this would amount to from 0.1 to 0.2 mg of GCF. Since our BANA reactions were performed on about one-tenth of the plaque sample, at most, from 10 to 20 μg of GCF would be present, which is from 4000- to 13,000-fold less GCF than had been used for the demonstration of the host's proteolytic enzymes in GCF (Cox and Eley, 1989b).

The utility of the BANA test was shown in the double-blind study involving metronidazole. A positive BANA test provided the scientific basis for the selection of this particular antimicrobial agent. The advantages of our decision to use metronidazole in the presence of an anaerobic infection are apparent from the comparison with the clinical results obtained in the positive control patients (Tables 2 and 4). The positive control patients received the type of therapy that is routinely given to patients classified as American Dental Association types III and IV periodontal patients, and their clinical response should reflect the improvements that are typical of this standard form of treatment. Any improvements beyond this level should reflect the metronidazole, given the double-blind nature of the study. Since three to four plaques were sampled in each patient, it was necessary to take into account the fact that sites nested within a patient could be influenced by a host factor. This was done by use of the correlated binomial model, which takes into account the correlated nature of sites within a patient and the heterogeneity of the total number of sites measured *per* patient (Hujoel *et al.*, 1990). The highly significant effect of the host factor on the conversion of BANA-positive plaques to BANA-negative plaques (Table 2) and the reduction in surgical needs (Table 4) illustrate the need for the host factor to be taken into account. It indicates that certain intrinsic (immunological factors) or extrinsic (compliance, oral hygiene) host factors determine the tooth-specific response to a certain extent.

After the host factor and the positive effects of scaling and root planing were accounted for, a treatment effect due to the unsupervised usage of metronidazole was evident. The metronidazole was associated with a significant reduction in the proportions and levels of spirochetes (Table 3), a significant conversion of BANA-positive plaques to BANA-negative plaques (Table 2), and a significant reduction in the need for periodontal surgery (Table 4). Thus, an important utility of the BANA test would be its ability to allow the clinician to make a diagnosis of an anaerobic infection, which in turn would enable the clinician, by prescribing metronidazole as part of the treatment, to achieve clinical benefits for the patient that were beyond those that could be achieved by scaling and root-planing alone.

A tooth site with a BANA-positive plaque at the conclusion of the medication and the scaling/root planing had significantly higher proportions of spirochetes than did a tooth site with a BANA-negative plaque (Table 5). Such BANA-positive teeth could possibly experience more periodontal disease in the foreseeable future than teeth with BANA-negative plaques, if one assumes that the high proportions of spirochetes indicated a residual infection with these putative periodontopathogens. In the present investigation, the tooth sites that were BANA-positive at the second treatment-planning session lost significantly more attachment than did the BANA-negative tooth sites when examined during the recall period at one year, and at two and three years after active treatment (Table 6).

Other investigators have been unable to show that clinical parameters such as probing depths, attachment levels, and bleeding on probing, among others, were of value in the prediction of future attachment loss in untreated periodontal patients (Haffajee *et al.*, 1983; Lang *et al.*, 1986; Badersten *et al.*, 1987). Listgarten and Levin (1981) were able to show that the proportions

of spirochetes in pooled plaque samples, but not clinical parameters, facilitated the prediction of which patients, in the absence of maintenance therapy, would relapse following active periodontal therapy. Our finding that a positive BANA test, following initial therapy, is indicative of future attachment loss during recall, is similar to the Listgarten and Levin report, since the BANA test was positively correlated with plaque levels and proportions of spirochetes (Table 5) (Loesche *et al.*, 1987b).

The possibility that a positive BANA test is associated with future attachment loss is a finding of potentially great clinical significance and needs to be carefully scrutinized. Among the criticisms that can be offered are those relating to the statistical model that was used. In this model, a complex, correlated observation vector of attachment loss over time is reduced to a unidimensional response, the regression slope. A linear model implies that periodontal disease progression is linear, whereas it may be exponential, or occur in random bursts of activity (Goodson, 1986) or as asynchronous multiple bursts (Haffajee and Socransky, 1986). The statistical methodology used may not be efficient for showing the effect of time-dependent covariates, such as the host-flora interactions that can occur in the pocket ecosystem. Also, the results discussed in this paper make inference toward the most diseased sites in each quadrant. The sites were selected by expert choice of a periodontist as being the most representative of the periodontally diseased sites in each quadrant. The sampling technique is a "purposive" or "judgment" sample, and care should be taken in extrapolation of these results to other sites. For instance, these results should not be extrapolated to the BANA results obtained in gingivitis sites or to sites that were randomly chosen in the dentition.

The other models that have been proposed to explain progression of periodontal disease, *i.e.*, the random burst and/or the asynchronous multiple burst model, also have problems in interpretation (Fleiss *et al.*, 1988). Attachment loss that occurs in bursts of 2 mm or more have large error rates to the extent that one-third of the bursts may be false-positive (Gunsolley and Best, 1988; Cohen and Ralls, 1988). A high cut-off point, such as 2 mm or more attachment loss, results in high specificity, low sensitivity, and rare disease (Aeppli *et al.*, 1985). Thus, while the linear progression model used for prediction of attachment loss in this study is not without problems, it does represent a useful way for detection of low magnitude changes in attachment that may reflect how some attachment is lost in periodontal disease.

Also, the role of surgery in this outcome needs to be evaluated. Approximately 66% of the BANA-positive test teeth and 54% of the BANA-negative test teeth went on to have Widman flap surgery, and then they, and the non-surgically treated test teeth, were followed for one or more years. Surgery and concomitant root planing presumably should have converted the remaining BANA-positive teeth to BANA-negative, and this would mitigate against the BANA-positive teeth exhibiting future attachment loss. On the other hand, surgery, even though it was conservative and did not involve osseous re-contouring, could have had an adverse effect on attachment levels that would appear at the annual treatment-planning sessions during the recall period. Since, there was a higher proportion of BANA-positive teeth than BANA-negative teeth experiencing surgery, there is the possibility that if surgery had an adverse effect on attachment levels, this could contribute to why the BANA-positive teeth tended to lose more attachment than the BANA-negative teeth. However, since the Widman flap surgery that was performed in these patients is reported to maintain or actually increase attachment levels in pockets initially $\geq$ 6 mm (Knowles *et al.*, 1979; Lindhe *et al.*, 1982; Pihlstrom *et al.*, 1984), this possibility would not appear likely.

The results reported in this investigation imply that the BANA test has multiple utilities in periodontal disease that would facilitate patient management. A direct estimate of this usefulness ideally would require a randomized clinical trial in which one group was managed with access to the BANA data, whereas the other group was managed without such information (Begg, 1987). A trial such as this would provide estimates on the influence of the diagnostic test on the subsequent patient management and on the disease progression. The BANA test as described in this paper is not convenient for such field tests, but a solid-state modification of the BANA test, which provides comparable results within 15 min at chairside (Loesche *et al.*, 1990b), could be evaluated in this regard.

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