Correlation of the Hydrolysis of Benzoyl-Arginine Naphthylamide (BANA) by Plaque with Clinical Parameters and Subgingival Levels of Spirochetes in Periodontal Patients

E. F. SCHMIDT, W. A. BRETZ; R. A. HUTCHINSON, and W. J. LOESCHE

University of Detroit School of Dentistry, Detroit, Michigan 48207; and University of Michigan School of Dentistry, Ann Arbor, Michigan 48109-1078

Recent studies have shown that the extent of hydrolysis by plaque of the trypsin substrate, N-benzoyl-DL-arginine-2-naphthylamide (BANA), correlates with the numbers and proportions of bacteria in subgingival plaque samples, and appears to be an indicator of clinical disease. In this study, BANA hydrolysis by subgingival plaque was evaluated in a blind manner for its ability to reflect both clinical parameters and subgingival levels of bacteria and spirochetes. Subgingival plaque samples were collected from periodontally healthy and diseased sites in 23 untreated periodontal patients and in 13 treated and maintained periodontal patients. In untreated patients, BANA hydrolysis was statistically associated with the total number of spirochetes and bacteria in the plaque sample, but in the treated patients BANA hydrolysis was statistically associated only with the spirochetes. Most BANA-positive reactions in both patient groups were from sites which were clinically diagnosed and high in spirochetes. The majority of the negative reactions for BANA hydrolysis in both patient groups were among the sites which were periodontally healthy and low in spirochetes. Specificity and sensitivity of the test were above 80% for disease status in untreated patients. The predictive value of a positive and negative test was above 83%. Slightly lower sensitivity, specificity, and predictive value were found in the treated group. The BANA reaction appears to be an accurate and simple indicator of both clinical disease status and plaque levels of spirochetes in individual tooth sites in untreated and treated periodontal patients.


Introduction.

Probing depth, attachment loss, and bleeding upon probing have traditionally been used as indicators of periodontal diseases. However, these clinical parameters reflect past disease experience and the inflammatory status of the tissues, and do not provide information about the bacterial composition of the subgingival plaque. Cultural microbiological procedures are effective in identifying periodontal pathogens in the plaque, but they are time-consuming and require specialized personnel. Darkfield microscopy (Listgarten and Hellden, 1978) allows for organism identification with a characteristic morphology or motility to be viewed, counted, and correlated with clinical parameters. However, the clinician often lacks the time to make a microscopic examination cost-effective. Therefore, a simple bacteriological test that could be reliably used in everyday practice to identify the presence or dominance of disease indicator organisms would be highly desirable.

Three of the most important bacteria associated with periodontitis—Bacteroides gingivalis (Loesche et al., 1985), Treponema denticola (Moore et al., 1985; Simonson et al., 1988), and Bacteroides forsythus (Tanner et al., 1985)—share the unique ability of hydrolyzing the trypsin substrate, N-benzoyl-DL-arginine-2-naphthylamide (BANA) (Loesche, 1986). Previous studies have shown that the extent of BANA hydrolysis by subgingival plaque correlates best with the number and proportions of spirochetes present in those plaque samples, removed from both pooled (Bretz and Loesche, 1987) and individual (Loesche et al., 1987) tooth sites. The BANA activity could be detected when there were about 1 or 2 spirochetes per microscopic high-power field (hpf) (approximately 1 to 3 million spirochetes). This trypsin-like enzyme activity may also be detected by the hydrolysis of the fluorescent substrate Z-arginine-7-amino-4-trifluoromethyl Coumarin (Z-Arg-AFC) (Pickett et al., 1986).

In the present investigation, we sought to determine how well BANA or Z-Arg-AFC hydrolysis correlated with clinical judgment and with clinical parameters in recognizing periodontally healthy or diseased sites in periodontal patients. Both the clinical and enzyme parameters were correlated with selected microscopic parameters. In this manner, we could assess how reliable BANA hydrolysis was in identifying clinical disease.

Materials and methods.

Population sample.—The subjects were 23 untreated and 13 treated patients from the Periodontics Clinic of the University of Detroit School of Dentistry. Criteria for acceptance into the study for the untreated periodontitis patients included a clinical diagnosis of at least generalized mild periodontitis (ADA II) (Schluger et al., 1977), no history of antibiotic therapy in the previous six months, no systemic condition which might be directly responsible for the periodontal breakdown, and that the females should not be pregnant. The treated patients required a history of successful treatment, i.e., no evidence of loss of clinical attachment following the completion of the initial therapy (scaling, root planing and/or surgery) for at least one year (with a recall interval of 3-6 months). No patients with the initial diagnosis of localized juvenile periodontitis (Liljenberg and Lindhe, 1980), early onset periodontitis (Loesche et al., 1985), or pre-pubertal periodontitis (Page et al., 1983) were accepted into the study.

The clinician who examined the patients (EFS) assigned a diagnosis of "clinically healthy" or "diseased" to the sample site based upon probing depth, bleeding upon probing, and his evaluation of tissue tone and appearance. This was done so as to provide a clinical diagnosis against which the BANA reaction could be evaluated with regard to its sensitivity (being positive when the clinician judged disease to be present) and its specificity (being negative when the clinician judged that there was no disease). Each untreated patient provided four plaque samples: two from sites diagnosed as healthy and two from sites diagnosed as diseased. The diseased sites were al-

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To whom correspondence and reprint requests should be addressed.
ways the most severely involved in the mouth and were usually around the molar teeth. In the treated patients, the sites that had been considered as diseased prior to treatment were characterized as responsive or unresponsive to treatment based on their need for retreatment, as judged by the clinical examiner. Sites that were unresponsive bled upon being probed and had probing depths 6 mm. Healthy sites were the same as described in the untreated patients. The frequency distribution of sampled sites is shown in the Fig.

**Bacteriological procedures.**—All sampling was performed prior to the beginning of root instrumentation in the untreated patients, whereas the treated patients were sampled at the beginning of a regularly scheduled maintenance visit. Prior to the sampling, supragingival plaque was removed and discarded, and the subgingival sample was collected with a sterile curette. The plaque samples were immediately suspended in 0.6 mL of Sorensen buffer at a pH of 7.2 and dispersed for 20 sec in a vortex mixer. For the microscopic counts, a 10-μL aliquot of the plaque suspension was removed and placed on a glass slide, covered with a 2 × 30 mm coverslip, sealed, and viewed by darkfield microscopy. Twenty high-power fields (hpf) were examined for each sample, and the total numbers of both bacteria and spirochetes per hpf were calculated.

**Enzyme assays.**—For the analysis of the BANA hydrolysis, 250-,11 aliquots of the plaque suspension were added to 50 pi of a BANA solution, yielding a final concentration of 16.6 mmol/L BANA. After an average of 18 hr of overnight incubation at 37°C, two drops of fast garnet were added to the solution, which produced the chromogenic reaction. The intensity of the chromogenic reaction was read visually by a calibrated operator and scored as negative for yellow color and positive for an orange/reddish or red color.

In a separate experiment, pooled subgingival plaque was placed in 0.6 mL of Sorensen buffer, dispersed, and counted microscopically. One hundred 1.1L of the plaque suspension was added to 100 μL of BANA. At the same time, 10 μL of the plaque suspension was placed on a paper strip which was overlaid onto another strip containing 10 III, of Z-Arg-AFC (Pickett et al., 1986). Plaque hydrolysis of Z-Arg-AFC was observed under a UV light after 15 min, and a shift from blue over to green indicated a positive result. Results of the BANA reaction were read according to the methods described above.

**Statistical analysis.**—Each of the data sets—i.e., clinical, enzymatic, microscopic—was determined independently and then compiled for statistical analysis by means of the chi-square test. The unit of reference was the individual tooth, since sufficient studies have indicated that while indeed teeth are nested within individuals, the pathophysiological event manifests itself around individual teeth, which are either clinically diseased or healthy (for review, see Haffajee and Socransky, 1986).

The reliability of the diagnostic usefulness of the spirochete levels and BANA hydrolysis should be measured. The traditional measures of the diagnostic value of a test are its sensitivity and specificity. Sensitivity measures the proportion of those with the disease who are correctly identified by the test, i.e., true positive. Specificity measures the proportion of those who do not have the disease who are correctly called disease-free by the test, i.e., true negative. We have analyzed test results using these measures and also the predictive value of the test, i.e., the probability of the disease being present or absent, after obtaining the results of the test (Riegelman, 1985).

**Results.**

Color development is a function of the enzyme levels in the plaque capable of hydrolyzing the BANA substrate and should reflect the levels of the BANA-positive species in the plaque sample. BANA hydrolysis was better able to detect spirochete levels in plaque than the fluorescent test, which used Z-Arg-AFC (Table 1). All plaque samples with > one spirochete/hpf exhibited a positive BANA reaction, whereas all samples with undetectable or < one spirochete/hpf were BANA-negative.

In contrast, there was no significant relationship between spirochete levels and a positive fluorescence with Z-Arg-AFC used. This led us to choose the BANA substrate for further evaluation in treated and untreated periodontal patients.

The microscopic examination of the subgingival plaque in the untreated patients showed that the positive BANA reactions were produced by samples harboring more than one spirochete per hpf, i.e., 37 of 38 samples, and by plaque harboring 5 or more bacteria per hpf, i.e., 34 of 38 samples (Table 2). This relationship was to be expected, since both total bacteria and spirochetes increase in numbers in untreated periodontal sites as a function of pocket depth (Loesche, 1987). In the subgingival plaque removed from the treated patients, where less plaque was present, spirochetes per hpf, but not bacteria per hpf, could be significantly associated with BANA hydrolysis (Table 2). Thus, 8 of 9 BANA-positive reactions were associated with plaque that harbored one spirochete per hpf, whereas 41 of 43 BANA-negative reactions occurred in plaque that had undetectable or less than one spirochete per hpf.

The next evaluation was the comparison of the BANA reactions from single sites with the clinical judgment rendered by the clinician. Clinically healthy sites had probing depths

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Positive</th>
<th>Negative</th>
<th>Signif.</th>
</tr>
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<tbody>
<tr>
<td><strong>CO</strong></td>
<td><strong>BANA Hydrolysis</strong></td>
<td><strong>AND Z-Arg-AFC REACTIONS</strong></td>
<td><strong>AND THEIR RELATION TO THE NUMBER OF SPIROCHETES</strong></td>
</tr>
<tr>
<td><strong>BETWEEN BANA AND THEIR RELATION TO THE NUMBER OF SPIROCHETES</strong></td>
<td><strong>BANA Hydrolysis</strong></td>
<td><strong>AND Z-Arg-AFC REACTIONS</strong></td>
<td><strong>AND THEIR RELATION TO THE NUMBER OF SPIROCHETES</strong></td>
</tr>
<tr>
<td><strong>Per hpf</strong></td>
<td>3</td>
<td>21</td>
<td><strong>Signif.</strong></td>
</tr>
<tr>
<td><strong>Spiro/hpf</strong></td>
<td><strong>&lt;1</strong></td>
<td><strong>3</strong></td>
<td><strong>&gt;3</strong></td>
</tr>
<tr>
<td><strong>Z-Arg-AFC Hydrolysis</strong></td>
<td><strong>&lt;1</strong></td>
<td>10</td>
<td>11</td>
</tr>
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</table>

Fig.—Frequency distribution of sampled sites. We have assumed that the clinical judgment of disease is the standard against which...
<table>
<thead>
<tr>
<th>1-3</th>
<th>6</th>
<th>1</th>
<th>non-signif.</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;3</td>
<td>11</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>TOTAL</td>
<td>27</td>
<td>15</td>
<td>=</td>
</tr>
</tbody>
</table>

*Spirochetes were undetected in 15 of the 21 samples.
*Number of plaque samples.
### Table 2

<table>
<thead>
<tr>
<th>BANA Score</th>
<th>Bacteria/hpf</th>
<th>Untreated Periodontal Patients (23)</th>
<th>Spirochetes/hpf</th>
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<tr>
<td>&lt;5</td>
<td>5-20</td>
<td>&gt;20 total</td>
<td>&lt;1</td>
</tr>
<tr>
<td></td>
<td>47</td>
<td>4 54</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>19 38</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>51</td>
<td>23 92</td>
<td>41</td>
</tr>
<tr>
<td>Total</td>
<td>Chi Square = 52.85</td>
<td>p &lt; 0.0001</td>
<td>Chi Square = 73.6</td>
</tr>
</tbody>
</table>

### Table 3

<table>
<thead>
<tr>
<th>Sites from</th>
<th>Probing Depth</th>
<th>% Bleeding</th>
<th>n</th>
<th>Probing Depth</th>
<th>% Bleeding</th>
</tr>
</thead>
<tbody>
<tr>
<td>all patients</td>
<td>72</td>
<td>2.5 ± 0.5 mm</td>
<td>10%</td>
<td>72</td>
<td>5.9 ± 1.2 mm</td>
</tr>
<tr>
<td>untreated patients</td>
<td>56</td>
<td>2.4 ± 0.5 mm</td>
<td>4</td>
<td>56</td>
<td>6.1 ± 1.3 mm</td>
</tr>
<tr>
<td>treated</td>
<td>26</td>
<td>2.7 ± 0.5 mm</td>
<td>22</td>
<td>26</td>
<td>5.6 ± 1.1 mm</td>
</tr>
</tbody>
</table>

*All differences between healthy and diseased sites were significant at p<0.01 by an independent t test.*

that averaged 2.5 mm, and bleeding upon being probed occurred in 10% of these sites (Table 3). In contrast, diseased sites had significantly higher probing depths, *i.e.*, 5.9 mm, and 95% of these sites bled upon being probed. The distinction between healthy and diseased sites was most evident in the untreated patients, since all diseased sites exhibited bleeding (Table 3). In the treated patients, about 20% of the healthy sites and 80% of the diseased sites exhibited bleeding upon being probed.

The majority of the negative BANA reactions were obtained from subgingival plaque samples collected from the healthy sites. In the untreated patients, 45 of the 54 negative BANA reactions were from healthy sites, and in the treated patients, 39 of the 43 negative BANA reactions were from either healthy sites or from formerly diseased sites that had been responsive to treatment (25 and 14, respectively) (Table 4). Conversely, 37 of the 38 positive BANA reactions were from diseased sites in the untreated patients, and 6 of the 9 positive reactions in the treated patients were from sites that were judged unresponsive to treatment. This translated into high values for true-negative results (98.8% specificity), for true-positive results (80.4% sensitivity), and a predictive value for a positive test of 97.4% in the untreated patients (Table 5).

In the treated patients, plaque removed from the formerly diseased sites that did not respond to treatment exhibited positive BANA reactions in 60% of the samples, whereas plaque removed from sites that had responded to treatment yielded positive reactions in only 12.5% of the samples. This indicated that the majority of sites judged clinically responsive were no longer dominated by BANA-positive species, and were more like the samples removed from the healthy sites in these individuals (Table 5). These healthy sites had a true-negative reaction in 96% of the cases (Table 5). The responsive sites exhibited mainly negative BANA reactions, *i.e.*, specificity =87.5%, whereas the unresponsive sites tended to be BANA-positive, *i.e.*, sensitivity = 60%.

The most likely explanation for the relationship between BANA reaction and clinical status would be that the diseased sites in the untreated patients and the unresponsive sites in the treated patients harbored a higher number of spirochetes than did the healthy sites in either patient group. As is shown in Table 6, most of the diseased sites in the untreated patients (39 of 46) and the unresponsive sites in the treated patients (9 of 10) presented with at least one spirochete *per* hpf, while less than one spirochete *per* hpf was found to be typical of the healthy sites of the untreated patients and the responsive sites of the treated patients. The frequency distribution between number of spirochetes *per* hpf and clinical status was significant by chi-square analysis.

### Discussion

Periodontal disease is described and/or measured by clinical descriptors of morbidity and inflammation. These descriptors/measurements are subjective and highly variable (Listgarten, 1980), yet they remain the standard against which any more objective measurement(s) will have to be compared. Periodontal disease reflects the host’s response to the types and amounts of bacteria present in the subgingival plaque, to the extent that one may consider periodontal disease a chronic infection. Very few descriptors or measurements of the bacterial component of plaque have entered into clinical practice, although the usefulness of microscopic examinations of plaque has been advocated for years (Armin, 1964; Listgarten and Henan, 1978; Keyes *et al.*, 1978), and cultural identification for most putative pathogens is now a reality (Slots, 1986).

It is in this context that the hydrolysis of BANA by plaque should be evaluated. We had shown previously that the pos-
The relationship between chromogenic reaction for BANA hydrolysis in subgingival plaque samples and clinical status is presented in Table 4. The table shows a significant association between the BANA score and clinical status, with higher BANA scores correlating with diseased sites in untreated patients and unresponsive sites in treated patients.

Table 5 provides specificity and sensitivity values of BANA reactions relative to clinical status. It indicates that a positive BANA test is more indicative of spirochetal load than bacterial load, as shown by its ability to identify subgingival plaque with elevated spirochetes, but not elevated bacteria, in treated patients (Table 2). This agrees with previous data showing that a positive BANA test in supragingival plaque occurred only when appreciable levels of spirochetes were present (Bretz and Loesche, 1987).

Table 6 shows the relationship between clinical status and spirochetes/hpf. A positive BANA test was more indicative of spirochetal load than bacterial load, as indicated by the ability of the BANA test to identify subgingival plaque with elevated spirochetes, but not elevated bacteria, in the treated patients (Table 2). This agrees with previous data which showed that a positive BANA test in supragingival plaque occurred only when appreciable levels of spirochetes were present (Bretz and Loesche, 1987).

These data indicate that subgingival plaque samples which are BANA-positive are associated with sites that are judged to be in need of clinical treatment. This is in agreement with bacteriological studies which showed that spirochetes, *B. gingivalis*, and *B. forsythus* are present in increased proportions in patients with clinical signs of periodontal disease (Loesche et al., 1987).
et al., 1985; Dzink et al., 1985; Slots et al., 1985), and that these micro-organisms are reduced following successful treatment and maintenance (Loesche et al., 1984). Therefore, this enzyme test could help the clinician to make an objective diagnosis of an anaerobic infection associated with these BANA-positive species. It also has the potential of enabling the clinician to monitor the adequacy of treatment procedures, since a negative BANA test would indicate that those micro-organisms have been significantly suppressed or eliminated from the subgingival plaque, as was observed in the responsive sites in the treated patients (Table 2 and 6).

Once the patient is placed on a regular maintenance program, a positive BANA test could identify plaque which had become repopulated in high numbers by these bacteria. Whether a positive BANA test would precede clinical signs of disease activity, such as bleeding upon being probed and color and morphological change in the tissues, remains to be determined. In this study, one of the 26 healthy sites in the untreated patients and two of the 16 responsive sites in the treated patients tested BANA-positive. We do not know whether these sites will in time exhibit periodontal disease.

Some sites showed a negative BANA reaction but were diagnosed clinically as diseased in the untreated patients (nine of 54) and as unresponsive in the treated patients (four of 43) (Table 4). These false-negative results could not be explained by too little plaque in the sample, since in the diseased sites there was always enough plaque, i.e., plaque was visible on the curette. Another possibility would be that the bleeding upon probing was due to a marginal gingivitis associated with BANA-negative bacteria. Alternately, there could have been an error in clinical judgment or in the recording of the data. Finally, those sites may be clinically diseased, but the microorganisms involved would be BANA-negative, such as A. actinomycetemcomitans, Wolinella recta, B. intermedius, and others yet to be identified. However, these false-negatives were rare, as reflected by the very high marks for the true-negative (specificity) and predictive values for a negative test in both types of patients.

In the unresponsive sites in the treated patients, there was a sensitivity (true-positive) of 60%. This relatively lower value may have been due to the fact that only 10 sites were clinically diagnosed as unresponsive—a number perhaps too low to warrant an adequate statistical analysis. Also, since the clinical examiner considered all sites with probing depths greater than 6 mm as unresponsive, there is the possibility that while exhibiting clinically unacceptable probing depths, these sites were in reality no longer infected with periodontopathogens, as indicated by the negative BANA reaction. In this case, the BANA reaction may more accurately reflect the clinical health of the site.

In conclusion, this blind clinical investigation demonstrated that the BANA hydrolytic activity in subgingival plaque may be a potential diagnostic tool which could be employed:

1. As a reliable indicator of BANA-positive species in plaque;
2. As a simple, objective means of determining diseased sites, i.e., sites requiring some form of periodontal treatment; or
3. To confirm or possibly determine the need for retreatment in patients who are being treated on a regular basis.

REFERENCES


