Blastogenic Response of Human Lymphocytes to Oral Bacterial Antigens: Comparison of Individuals with Periodontal Disease to Normal and Edentulous Subjects

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Cell-mediated immunity in humans to antigens derived from oral plaque bacteria was investigated by using the lymphocyte blastogenesis assay. Subjects with varying severities of periodontal disease including normal, gingivitis, periodontitis, and edentulous were compared. Mononuclear leukocytes were separated from peripheral blood and cultured with antigens prepared by sonication of Actinomyces viscosus (AV), Actinomyces naeslundii (AN), Veillonella alcalescens (VA), Leptotrichia buccalis (LB), Bacteroides melaninogenicus (BM), and homologous dental plaque (DP). The lymphocyte response of subjects with gingivitis or periodontitis was significantly greater than that of normal subjects to antigens of AV, AN, and DP, but did not differ from the response to edentulous subjects. Periodontitis subjects were significantly more reactive than edentulous and normal subjects in response to VA, LB, and BM. These findings suggest that the tested gram-negative bacteria and the host response they evoke are associated with advanced periodontal destruction.

It is clear from recent studies that immune reactions occur between antigens of bacterial dental plaque (DP) and the host, and that these reactions contribute to the development and progression of periodontal disease. The possible role of antibody-mediated effects on the periodontium has been described (4), and immediate hypersensitivity (16) to antigens of DP bacteria has been demonstrated. Cellular immune reactions between DP bacterial antigens and human lymphocytes in vitro have also been investigated. Using lymphocyte blastogenesis as an in vitro correlate of delayed hypersensitivity, Ivyani and Lehner (7, 8) and Horton et al. (5) demonstrated that lymphocytes from patients with moderate periodontal disease were stimulated to undergo blastogenesis by antigens derived from DP bacteria. In contrast, no such response was observed in subjects free from periodontal disease. The present study was designed to clarify further the lymphocyte blastogenic response of various dental populations to antigens derived from DP bacteria. Specifically we have studied the peripheral blood mononuclear cell blastogenic response of persons who differed with respect to periodontal health status. The subjects were tested with a battery of sonically disrupted oral bacteria as stimuli to determine: (i) whether these preparations contain components capable of stimulating peripheral blood mononuclear cells; (ii) whether there is any specific oral organism or group of oral organisms to which humans with periodontal disease are sensitized; and (iii) whether in the absence of, or in the less severe forms of periodontal disease, this specific lymphoproliferative response is absent or weaker. The results of this study reveal that there is a specific lymphoproliferative response, in patients with moderate to severe periodontal disease, to antigens of certain gram-negative oral bacteria and that persons with little or no periodontal disease exhibited little or no response to these antigens. This finding suggests that the host's response to these gram-negative oral organisms plays a special role in the inflammatory processes seen in moderate to severe periodontal disease.

MATERIALS AND METHODS

Preparation of antigens. Actinomyces viscosus (AV; ATCC 19246), Actinomyces naeslundii (AN; ATCC 12104), Streptococcus sanguis (SS; ATCC 10556), and Streptococcus mutans (SM; strain 6715-T2) were grown in Trypticase soy broth for 48 h. Veillonella alcalescens (VA), isolated from human DP, was grown in FM-3 medium (15) for 72 h. Bacteroides melaninogenicus (BM; strain K110), isolated from human DP and kindly provided by R. Gibbons (Forsyth Dental Center, Boston, Mass.), was grown in EX-1 medium supplemented with hemin (15).
Leptotrichia buccalis (LB) was isolated from human DP and grown in EX-1 medium in a CO₂ atmosphere. The organisms were removed from cultures by centrifugation and washed in phosphate-buffered saline, pH 7.2, and the bacteria were frozen at −20°C until used. DP was collected from supra- and subgingival plaque of human subjects, of whom 40% of their teeth were contaminated with blood were discarded. The DP was prepared for sonication by the method of Horton et al. (5).

Pellets of bacteria and plaque were weighed and then suspended in RPMI 1640 (Associated Biomedical Systems, Buffalo, N.Y., containing 100 U of penicillin and 50 μg of streptomycin per ml with 2 mM glutamine) or sterile saline at 10 or 100 mg (wet weight)/ml. Each organism and the pooled DP were sonicated in a Heat Systems Sonifier, model W185 (Branson Ultrasonics, Plainview, Long Island, N.Y.) until >95% of the organisms appeared broken as monitored by phase-contrast microscopy. The antigen suspensions were centrifuged and supernatants were collected. Complete description of the preparation and characterization of the bacterial antigens is given by Reed et al. (18).

Additionally, the nonspecific mitogen phytohemagglutinin (PHA) (Burroughs-Wellcome, Research Triangle Park, N.C.) and the antigen streptokinase-streptodornase (SK-SD) (Lederle, Pearl River, N.Y.) were dissolved in RPMI 1640 at 1 mg/ml and 2,500 SK units/ml, respectively. Lipo polysaccharide (LPS) (extracted from VA and kindly provided by D. Fine, Columbia, University), Escherichia coli O111:B4 LPS, and S. typhimurium LPS (Difco Laboratories, Detroit, Mich.) were suspended in sterile saline at 1 mg/ml. All preparations used for stimulation were stored in frozen aliquots at −20°C. Upon use, an aliquot of each preparation was thawed and diluted appropriately. No material was ever thawed and refrozen.

Selection of subjects. Subjects were selected from the dental patients at the State University of New York at Buffalo School of Dentistry. Each received complete dental examinations including medical history, oral examination, measurement of clinical indexes, and dental radiographs. Subjects with systemic disease or who had had prior periodontal therapy were excluded. Patients were divided according to the Russell periodontal index (RPI) (19) into a normal group (RPI ≤ 0.4), a gingivitis group (RPI = 0.4 to 2.0), and a moderate to severe periodontitis group (RPI > 3.0). An additional group consisted of patients who had been edentulous for at least 5 years and had no visible oral areas of inflammation. Location of an adequate number of subjects for the normal group proved to be surprisingly difficult. After screening of 400 subjects, only two subjects had been found with an RPI ≤ 0.4. Therefore, in order to have a group with significant gingival inflammation, subjects were placed on a stringent oral hygiene regimen, which included frequent scalings and dental prophylaxis. RPI, gingival index (12), and plaque index (21) were measured at regular intervals. After 3 months, these subjects fit the criteria to be included in the normal group (RPI ≤ 0.4). Mononuclear cells from fetal cord blood were used as an additional control to test some antigens.

Lymphocyte blastogenesis. Data presented in this study were accumulated over 2 years. Results obtained by both the initially used lymphocyte blastogenesis macroassay and the later-used microassay are presented. Experiments were conducted to prove the comparability of data obtained by both assays.

Heparinized (20 U/ml) venous blood was obtained from the antecubital fossa of each subject, and mononuclear cells were separated by Ficoll-Hypaque gradient centrifugation (2). The mononuclear cells were washed three times in RPMI 1640, counted, and resuspended at a concentration of 1 × 10⁶ cells per ml in RPMI 1640 with 15 mM HEPES buffer (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (Calbiochem, La Jolla, Calif.) and 20% autologous plasma, which was inactivated by heating at 56°C for 30 min.

For the macroassay, mononuclear cells were cultured in triplicate in 1-ml volumes in Falcon 2000 tubes (Falcon Plastics, Oxnard, Calif.) with 0.1 ml of various dilutions of antigens or PHA. Cultures were incubated at 37°C in 5% CO₂ in humidified air for 96 h. Sixteen hours before terminating the cultures, 1 μCi of [³H]thymidine (specific activity, 2 Ci/mmol) (Amersham/Shearle, Arlington Heights, Ill.) was added to each culture. Pilot experiments showed that maximum [³H]thymidine incorporation occurred at 3 days with PHA and at 5 days with antigens. However, there was significant [³H]thymidine incorporation at 4 days (96 h) with both stimulants. Cultures were terminated by addition of 3% acetic acid and then stored at 4°C until harvested. Upon harvesting, each cell pellet was washed three times in 3% acetic acid, solubilized in 1 ml of NCS (Amersham/Shearle), and transferred to a scintillation vial with 10 ml of a mixture of 60 ml of ScintiPrep II (Fisher Scientific Co., Rochester, N.Y.) and 1 liter of toluene. Samples were counted in a Beckman LS-100C liquid scintillation counter.

To test multiple dilutions of many antigens, a microassay was later used. Two hundred microliters of mononuclear cells (1 × 10⁶ per ml) was added to each well of round-bottom microtiter plates (no. 1-211-24-1, Cooke Laboratory Products, Alexandria, Va.). Twenty microliters of antigen, PHA, LPS, or medium was added in quadruplicate to the cells. In some experiments, 20 μl of both LPS and bacterial antigen was added to each well. The time required for maximal stimulation by antigens and by PHA was determined. Six hours before harvest, 0.2 μCi of [³H]thymidine was added to each well. Samples were harvested on glass fiber filter paper with a Skatron multiple-cell culture harvester (Flow Laboratories, Rockville, Md.). Individual filter disks were placed in 5 ml of scintillation fluid and counted as above. The results are expressed as a stimulation index (SI), which was calculated as follows:

\[ SI = \frac{\text{mean cpm in stimulated cultures}}{\text{mean cpm in control cultures}} \]

Using normal distribution theory and a pooled estimate of variance, SIs exceeding 2.5 were significant at the 95% level. Statistical comparisons among groups were made by using the Mann-Whitney U-
test (22). Dose-response curves were done for each antigen tested; the optimal SIs were used in tabulating the data.

RESULTS

Experiments to determine dose-response relationships of each antigen preparation showed that in the dose range tested, maximal lymphocyte stimulation occurred with 10 to 50 μg of protein per ml of culture. Subjects who were unresponsive to an antigen preparation lacked reactivity at all tested doses.

The SIs of each patient group in response to 5 μg of PHA per ml are depicted in Fig. 1. Lymphocytes were significantly stimulated in all cases, although as a group the fetal cells reacted least strongly. The adult groups did not differ in response to PHA at 5 μg/ml.

In Fig. 2 the responses to the optimal dilution of AV are compared. The fetal cells in five of eight cord bloods were very slightly stimu-

![FIG. 1. Lymphocyte stimulation of subject groups to 5 μg of PHA per ml. Each value is calculated from the mean of triplicate macrocultures.](image_url)

lated by this antigen preparation. In the gingivitis, periodontitis, and edentulous groups, 12 of 13, 11 of 13, and 3 of 9 subjects, respectively, were significantly reactive. This was in sharp contrast to the normals: only three of eight persons in this group were reactive. The periodontitis and gingivitis groups were significantly more reactive than the normal group (P < 0.025). Surprisingly, the edentulous group did not differ statistically from the diseased groups (P < 0.10) in response to the antigens of AV.

The responses of each group to AN are illustrated in Fig. 3. This preparation did not stimulate fetal cells consistently; cells from only one of eight umbilical cords reacted. However, 11 of 13 subjects in the gingivitis group and 10 of 13 in the periodontitis groups had significant SIs. The normal group, with two of eight subjects responding, was significantly less stimulated than either diseased group (P < 0.01). The edentulous group, in which six of nine responded significantly, did not differ statistically from either diseased group (P < 0.15).

In Fig. 4 the responses to the DP antigen preparation are compared. The fetal cells were not significantly stimulated, only one of eight reacting. Subjects stimulated by DP include 4 of 8 subjects in the normal group, 9 of 12 subjects in the gingivitis group, 6 of 13 subjects in the periodontitis group, and 3 of 9 subjects in the edentulous group. The difference between the gingivitis group and the normal group was significant (P < 0.05).

SK-SD, when used at a concentration of 50 SK units/ml, in all cases failed to stimulate fetal cells. However, 30 of 34 adult lymphocyte cultures gave positive reactions, but the groups did not differ significantly.
To test additional antigens, a microassay was next adopted as described above. The first experiments were conducted to determine the time course of stimulation with antigens and PHA in this assay. Using the mitogen PHA, maximal SIs occurred at day 3 or 4, depending on the dose tested. Results with antigens are shown in Fig. 5. Maximal SIs were seen on day 5. Although [3H]thymidine incorporation continued to increase through 7 days of antigen-stimulated cultures, the sharp rise in [3H]thymidine incorporation of control cultures at days 6 and 7 resulted in lowered SIs. Viability of cells at day 5 was >95% by trypan blue dye exclusion. Based upon the results of this and similar experiments, we decided to terminate PHA-stimulated cultures at 96 h and antigen-stimulated cultures at 120 h.

To compare the macro- and microassays, mononuclear cells from several periodontitis patients and fetal cord bloods were tested using both (Table 1). These results indicate quite good agreement between the techniques. Hereafter, the microassay was routinely used in all experiments.

Lymphocytes from several patients of the normal, gingivitis, periodontitis, and edentulous groups were tested by the microassay. The measured responses of the groups to AV, AN, or DP with this technique confirmed the data obtained with the macroassay. The groups again did not differ in magnitude of response to PHA or in the concentration of PHA resulting in maximum lymphocyte stimulation. Sonicates of SS and SM, oral microorganisms found in DP but not thought generally to play a causative role in periodontal disease in man, stimulated very few individuals. LPS, tested at concentrations of 100, 10, and 1 μg/ml, failed to stimulate any subject. When LPS was cocultured with each bacterial sonicate, no increased lymphocyte response above that produced by the sonicate alone was noted. However, significant differences between groups in their blastogenic responses emerged when lymphocytes of patients were cultured with sonicates of *Leptotrichia, Bacteroides*, and *Veillonella*.

In Fig. 6 the response of the tested groups to VA is compared. The periodontitis group (eight of eight subjects reactive) reacted significantly more than the edentulous group (*P* = 0.01) or the normal group (*P* = 0.005). The gingivitis group was also significantly stimulated (five of seven subjects reactive), but did not differ statistically from the other groups in magnitude of response.

In Fig. 7 the response of each group to LB is compared. The responses in the periodontitis group (six of eight subjects reactive) were statistically greater (*P* < 0.025) than those of the edentulous group (one of eight subjects reactive) or those of the normal group (two of seven subjects reactive) (*P* < 0.05). The gingivitis group (three of seven subjects reactive) did not differ from the other groups.

The response of the tested groups to BM is presented in Fig. 8. Unlike the other gram-negative organisms, this antigen preparation only stimulated cells from the periodontitis group (five of eight subjects reactive). All patients of the edentulous and normal groups and all but one patient of the gingivitis group were unreactive. As a unit, the periodontitis group...
showed significantly greater responses than the edentulous (P < 0.025), normal (P < 0.005), and gingivitis groups (P < 0.005). The gingivitis, edentulous, and normal groups did not differ from each other.

DISCUSSION

In the present study, we sought to clarify the relationship between cell-mediated immunity and periodontal disease. Using in vitro stimulation of peripheral blood leukocytes by sonicate antigens from DP bacteria as a correlate of cell-mediated immunity, Baker et al. (1), Ivanyi and Lehner (7, 8), and Horton et al. (5) have demonstrated a direct proportionality between degree of stimulation and severity of periodontal disease. These findings were confirmed by other in vitro tests used to measure cellular immunity, namely leukocyte migration inhibition and cytotoxicity against unrelated target cells (9). More recently, work by Kiger et al. (10) failed to find any correlation between degree of lymphocyte transformation and periodontal disease status.

Such conflicting results could have been the result of differences in the antigen preparations used. None of these studies used well-characterized antigens. Therefore, we attempted to prepare antigens from sonicates of DP bacteria in a reproducible manner. Each batch of antigen was compared to previous batches by immunoelectrophoresis against immune rabbit sera and by determination of protein and sugar content. Information is provided by us in a companion paper (18) and should furnish other investigators with a basis for comparison and standardization of antigens.

In preliminary screening of subjects without definite clinical periodontal disease, the positive responses to AV, AN, and DP of these subjects did not differ from those of the gingivitis and periodontitis groups. The possibility that these positive responses were due to mitogens in the sonicate preparation was investigated. Fetal cord lymphocytes have been shown to be fully responsive to mitogen, but only infrequently responsive to antigen (11). On this basis, we interpret data in Fig. 1 through 4 as showing that sonicate preparations behaved as antigens by comparison to the known mitogen, PHA. This finding confirms those of Baker et al. (1), who found cord lymphocytes unreactive to cell wall preparations of certain oral bacteria. Additional evidence for the lack of mitogenic activity of the bacterial sonicates comes from the observation that not all adult subjects reacted to the preparations, as would be expected for some mitogens such as PHA.

To explain the positive responses obtained, we carefully evaluated the periodontal status of our subjects. Scoring of gingival inflammation using RPI (19) and gingival index (12) proved that those subjects, although previously regarded without frank periodontal disease, actually had some slight gingival inflammation. A continued search for subjects without any gingival inflammation located only 2 such subjects from 400 screened. Therefore, we established a normal group by reduction of gingival inflammation with oral hygiene therapy. When the RPI of these subjects was reduced to 0.4, their lymphocyte responsiveness was significantly lower than that of the gingivitis or periodontitis group. This supports the findings of Ivanyi and Lehner (7, 8), Horton et al. (5), and Baker et al. (1) concerning lack of response in dentally healthy subjects. However, it also places this finding in perspective; that is, the state of gingival health required for consistently negative blastogenic response to plaque and oral Actinomyces antigens is infrequently encountered in the population.

If lymphocyte blastogenesis does correlate with periodontal disease and if plaque is the

Table 1. Comparability of lymphocyte blastogenesis: macroassay versus microassay

<table>
<thead>
<tr>
<th>Stimulant and amt</th>
<th>Cord blood (n = 4)</th>
<th>Periodontitis (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Macro</td>
<td>Micro</td>
</tr>
<tr>
<td>PHA, 5</td>
<td>19.1 ± 2.6</td>
<td>10.3 ± 4.8</td>
</tr>
<tr>
<td>A. viscosus, 57</td>
<td>2.7 ± 0.3</td>
<td>2.1 ± 0.5</td>
</tr>
<tr>
<td>A. viscosus, 11</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>A. naeslundi, 40</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>A. naeslundi, 4</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>SK-SD, 50 U/ml</td>
<td>1.1 ± 0.1</td>
<td>1.2 ± 0.1</td>
</tr>
</tbody>
</table>

a Micrograms of protein per milliliter of culture.
b Stimulation index (SI) ± standard error was calculated as described in Materials and Methods.
c ND, Not done.
immunizing antigen, fully edentulous subjects should be unresponsive. However, edentulous patients did not differ statistically from diseased subjects in reactivity to AV, AN, and DP. When tested with gram-negative organisms such as LB, VA, and BM, edentulous patients were significantly less reactive than periodontitis patients. Furthermore, edentulous patients as a group were unreactive to LB and BM. These differences might be explained by the finding that Actinomyces and Veillonella, but not Leptotrichia and Bacteroides, continue to populate the edentulous mouth (13). Recent bacteriological examination of edentulous mouths revealed an increase in Actinomyces after placement of artificial dentures (14). Thus, Actinomyces organisms harbored on the oral mucosa under artificial dentures may cause continued sensitization of edentulous individuals, resulting in the positive responses seen. The differences in responses between the diseased group and edentulous group might be a result of age differences (23), since the edentulous patients were almost all older than patients of the other groups. This is unlikely, however, since no differences in response were detected with PHA or the non-periodontal antigen SK-SD. We feel it is likely, therefore, that the differences are disease dependent.

It has been recently shown by Gaumer et al. (3) that LPS directly stimulates peripheral blood lymphocytes of patients during experimentally induced gingivitis. No patients in the present study were reactive to LPS, suggesting that spontaneously occurring periodontal disease differs from experimentally induced disease. Additionally, several studies have found a synergistic lymphocyte response when LPS (20) or DP (17) was cocultured with PHA. Although there can be little doubt that LPS is present in our sonicates of gram-negative bacteria or DP, the previously cited cord blood experiments suggest that no mitogenic activity is present in these preparations. Therefore, it is unlikely that such synergy exists in our sonified preparations. Furthermore, no synergism was seen when LPS was cocultured with each bacterial sonicate.

Although we have found normal and edentulous patients to be significantly less reactive than diseased patients, we are unable to demonstrate any correlation between severity of disease and lymphocyte response within diseased groups. Those patients with very mild
LYMPHOCYTE RESPONSE TO ORAL BACTERIAL ANTIGENS

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Only were procedures, only lymphocytes dontal treatment. May prove very patients. Between preparation bacterial, bacteria be elimination. Several observations should be cited agreement with that BM. F. R.

Bacteroides, gingivitis patients as well as (7), report that BM could stimulate gingivitis patients as well as moderate periodontitis patients. Possible differences in bacterial strain, bacterial culture conditions, sonication procedures, or lymphocyte culture conditions between laboratories could account for this difference. However, this finding is in agreement with that of Lang and Smith, who showed that BM stimulated only patients with advanced periodontitis (N. P. Lang, and F. N. Smith. J. Dent. Res. 55:8205, 1976). These observations should be evaluated carefully, since our preparation of BM and those in the other cited studies stimulated periodontitis patients' lymphocytes only weakly. Attempts are now underway to derive an antigen preparation from BM that produces greater stimulation.

Ivanyi et al. (6) reported that patients with severe periodontitis (RPI > 4.0) possess serum inhibitors of lymphocyte transformation. In their study, cells from such patients were uniformly unreactive to DP antigens when cultured in autologous serum. In the present study, all lymphocyte cultures were performed in 20% autologous plasma, and lack of reactivity of the severe periodontitis patients was no greater than that of gingivitis patients. Interestingly, some patients in every group proved to be unresponsive to all antigens derived from oral bacterial, although no difference was observed in PHA or SK-SD responses. The full significance of this finding is unknown, but it suggests that factors other than disease also influence in vitro lymphocyte response to DP antigens.

The role of cell-mediated immunity in periodontal disease remains to be further clarified. The present study confirms that "supernormal" individuals are indeed unreactive by the lymphocyte blastogenesis assay to DP and to antigens from oral Actinomyces. However, since no correlation between severity of disease and reactivity could be demonstrated, and since edentulous subjects maintained reactivity, the clinical usefulness of these antigens in lymphocyte transformation studies in periodontal populations is debatable. However, since the lymphoproliferative response to antigens derived from gram-negative oral bacteria such as Veillonella, Bacteroides, and Leptotrichia is reduced or absent after periodontal disease is eliminated by complete tooth loss, these antigens may prove very useful in studies of periodontal treatment.

We have shown that patients with chronic destructive periodontal disease manifest systemic cell-mediated immunity to prominent members of the periodontal pocket flora such as Actinomyces and some gram-negative organisms. The reactivity to gram-negative organisms is absent in individuals without active destructive periodontal disease. This finding suggests that the gram-negative organisms, and the host response they evoke, are associated with the destruction of alveolar bone and connective tissue seen in advanced periodontitis. On the other hand, the near-ubiquitous systemic cell-mediated immunity observed to oral Actinomyces antigens correlates with the early gingival lesion and persists even after the dentition is lost. Studies are in progress using other major gram-negative periodontal pocket organisms such as spirochetes, Selenomonas, and Campylobacter species to determine whether they elicit cellular immunity during the severe stages of periodontal disease.

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