Serum antibody reactive with predominant organisms in the subgingival flora of young adults with generalized severe periodontitis.

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Serum Antibody Reactive with Predominant Organisms in the Subgingival Flora of Young adults with Generalized Severe Periodontitis

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In the present study we sought to determine whether serum antibody was present against microorganisms which predominate in the subgingival flora of young adults with generalized severe periodontitis (SP). Subjects with SP were often seropositive for Eubacterium brachy, Fusobacterium nucleatum E3C22, and Peptostreptococcus micros, whereas subjects with juvenile periodontitis (JP) and subjects with healthy periodontium (HP) were not. Both SP and JP subjects were more frequently seropositive for Bacteroides gingivalis, F. nucleatum D52816, and F. nucleatum E1D1 than were HP subjects. The data were most striking for B. gingivalis, for which both the incidence and the magnitude of specific antibody was clearly elevated for SP and JP subject groups. However, SP subjects generally had either a high antibody titer or no detectable titer. In contrast, JP and HP subjects generally had at least very small amounts of antibody. Except at very low levels of antibody, neither SP nor JP groups differed significantly from the HP group for antibody to Eubacterium nodatum, Bacteroides intermedius (homology group 4197 or 8944), or Lactobacillus minutus antibody. There was a high frequency of antibody to E. nodatum, with very high titers in all groups despite the fact that this organism is rarely found in HP subjects. For Eubacterium timidum, the JP group was clearly more frequently seropositive than the HP group. Despite high levels of L. minutus in subgingival flora, none of the 50 SP subjects had a detectable antibody titer, and only four of the HP and JP subjects had detectable antibody. These results indicate that many organisms in the subgingival flora elicit antibody responses. B. gingivalis is probably the best example among the species tested. However, some organisms that are present in high concentration, e.g., L. minutus, apparently fail to induce significant antibody responses.

Several recent reports indicate that many individuals with periodontitis are seropositive for certain microorganisms present in their gingival plaque (1, 4-8, 10, 13, 16, 17). However, the relationship between the predominant periodontal flora of different clinically characterized patient groups and serum antibodies reactive with individual species in the predominant flora has not been clearly established. Recently the microbial flora of subgingival plaque of young adults under 30 years of age with generalized severe periodontitis (SP) has been described (15). The objective of the present study was to determine whether subjects with SP have an increased incidence or elevated titer of serum antibody reactive with the organisms that predominate in their subgingival flora as compared with young adults with healthy periodontium (HP) or patients with juvenile periodontitis (JP).

A sensitive radioimmunoassay (RIA) was utilized to detect and quantify specific antibody to nine bacterial species that make up over 38% (see Table 2) of the cultivable flora of subjects with SP (15). Antibody titers in the serum against these organisms were determined in a large group of over 150 clinically characterized young adults. The results support the view that, with some exceptions, a general relationship exists between the organisms present in the subgingival flora of patients with periodontitis and the presence of specific antibody in the serum.

MATERIALS AND METHODS

Human subjects. Sera for analysis of antibodies were obtained by venipuncture from 52 young adults with SP, 47 with localized JP, and 52 subjects with no periodontitis (HP), defined as absence of probeable depth in excess of 3 mm and absence of periodontal attachment loss. Specific clinical criteria for the JP and SP categories were as previously described (2). An examination to document periodontal status (2) was performed at the time of venipuncture. Age ranges were 12 to 31 years for the SP group, 13 to 28 years for the JP group, and 21 to 29 years for the HP group. Mean ages and clinical findings are shown in Table 1.

Bacteria and antigen preparations. The bacteria and their

| TABLE 1. Ages and clinical measures of study populationsa |
|------------------|------------------|------------------|------------------|
| Population      | Age (yr)         | PIb              | GIc              | No. of teeth withd |
| SP               | 25 ± 0.6         | 1.41 ± 0.07      | 1.28 ± 0.06      | 15 ± 0.7 24 ± 0.6 |
| JP               | 19 ± 0.6         | 0.97 ± 0.07      | 0.97 ± 0.05      | 3 ± 0.4   9 ± 0.8  |
| HP               | 25 ± 0.3         | 0.30 ± 0.24      | 0.48 ± 0.11      | 0         0         |

a Results are given as means ± standard errors of the means.

b See Silness and Löe (20).

c See Löe and Silness (11).

d 5 mm LA, Teeth present and having a measurement from the cemento-enamel junction to the point of maximum probeable depth (loss of attachment) equaling or exceeding 5 mm on at least one surface; 2 mm LA, teeth having ≥2 mm loss of attachment on at least one surface.

* Corresponding author.
proportions in the subgingival flora of SP subjects were recently reported by Moore et al. (15). Comparative data for the JP flora are as described elsewhere (14a). All strains were isolated by W. E. C. Moore and L. V. Holdeman of Virginia Polytechnic Institute and State University from samples of subgingival floras. The bacterial species and their detected proportions in the subgingival floras of SP, JP, and HP populations are listed in Table 2. Because of the genetic heterogeneity of Fusobacterium nucleatum, strains representing three DNA homology subgroups were used. The two

TABLE 2. Bacteria used for antigen preparations and the proportions of each species in the subgingival floras of the clinical groups

<table>
<thead>
<tr>
<th>Species</th>
<th>Strains used</th>
<th>% of isolates (% positive samples)*</th>
<th>SP</th>
<th>JP</th>
<th>HP</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. brachy</td>
<td>D6B23</td>
<td>1.5 (24) 1.1 (19) 0 (0)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. timidum</td>
<td>D84B2</td>
<td>6.2 (70) 2.7 (56) 0.9 (14)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. nodatum</td>
<td>D40D21</td>
<td>8.3 (50) 5.6 (38) 0 (0)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F. nucleatum</td>
<td>E3C22, D52B16, E1D1</td>
<td>7.6 (85) 7.2 (66) 4.3 (48)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Data for the SP group are from reference 15, based on 51 samples and 1623 total isolates; data for the JP group are from Moore et al. (14a), based on 32 samples and 1,064 total isolates; data for the HP group are from reference 14, based on 22 samples and 679 total isolates.

FIG. 1. Relationship between radioactivity specifically bound and concentration of serum from a subject with a substantial antibody titer. The linear portion of the dose-response curve is indicated by the heavy dotted line. The cutoff for seropositivity, which was chosen to be well within the linear portion of the curves of the 5% of the sera (seven to eight individuals) which contained the most antibody to the given test species, is indicated by the light (horizontal) dotted line.

TABLE 3. Cutoff for minimum seropositivity for each organism tested

<table>
<thead>
<tr>
<th>Strain</th>
<th>Assigned cutoff (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F. nucleatum E1D1</td>
<td>1,400</td>
</tr>
<tr>
<td>F. nucleatum E3C22</td>
<td>500</td>
</tr>
<tr>
<td>F. nucleatum D52B16</td>
<td>800</td>
</tr>
<tr>
<td>E. timidum D84B2</td>
<td>600</td>
</tr>
<tr>
<td>E. nodatum D40D21</td>
<td>1,600</td>
</tr>
<tr>
<td>E. brachy D6B23</td>
<td>500</td>
</tr>
<tr>
<td>L. minutus D48C21</td>
<td>500</td>
</tr>
<tr>
<td>P. micros D31C23</td>
<td>600</td>
</tr>
<tr>
<td>B. intermedius 4197 (D10D1)</td>
<td>500</td>
</tr>
<tr>
<td>B. intermedius 8944 (D25B1)</td>
<td>500</td>
</tr>
<tr>
<td>B. ginvialis (D43B4)</td>
<td>500</td>
</tr>
</tbody>
</table>

* No serum contained sufficient antibody to define an RIA curve and allow selection of a cutoff point.
(ii) Analysis. An entire serum dilution series was run for each patient with two antigen-coated wells and one naked well per dilution. Results from naked wells showed a reproducible difference in the background counts of different individuals and of different clinical groups. The SP group had significantly higher background counts than the HP or JP groups over a wide range of the dilutions (P < 0.01). Because of this, the background counts (counts per minute) from the naked wells were subtracted from the counts per minute in antigen-coated wells at each dilution. This difference in counts per minute (ΔC.P.M.) at each dilution was used as the basic data unit. A plot of ΔC.P.M. versus dilution (log transformation) was produced by the computer. All computer-generated curves were inspected for deletion of occasional outlying data points. A prototypical curve based on one of the most reactive sera is shown in Fig. 1. In this example, the 64,000 and 4,000 dilutions would delimit the beginning and ending of the linear portion of the curve. A minimum (cutoff) difference in counts per minute, representing a positive response for each species, was assigned within the linear portion of curves. The cutoff point for a positive antibody response to each bacterial species is shown in Table 3. These were arrived at by examination of the RIA curves for the 5% of sera which contained the most antibody for a given species. The cutoff point was selected to be in the lower portion of the linear portion and above the inflexion point of each of these curves (seven to eight individual curves). If the serum of a subject produced no values above the cutoff, the response was deemed negative. Figure 2A shows results from higher reactive sera with four or more points above the cutoff, three points above the cutoff (Fig. 2B), and results from the least reactive sera that

Fig. 2. Sample RIA curves illustrating the several criteria for seropositivity used in this study. Panels A, B, and C respectively illustrate curves with more than four points, three points, and two points above the cutoff. Within panels A, B, and C, three different patterns of binding (illustrative sera) with the same antigen preparation are shown, each of which would meet the stated criterion. Meeting one of these criteria is the basis for inclusion of subjects as positive responders in the histograms of Fig. 3 through 6, whereas the magnitudes of responses depicted in those figures are derived from the serum dilution (x axis) at the end of the linear portion of the curve.
FIG. 3. Frequency and magnitude of serum antibody reactive with *E. nodatum*, *E. timidum*, and *E. brachy* in HP, JP, and SP groups. The percent seropositive in each clinical group is indicated by the bars. The magnitude of the response of each seropositive subject, in RIA units per milliliter, is indicated by a dot. Statistical differences at a minimum of *P* < 0.01 are indicated by an asterisk when the JP or SP group was different from HP and by a plus sign to indicate that the JP group was different from the SP group.
FIG. 4. Frequency and magnitude of serum antibody reactive with three strains representing three DNA homology subgroups of *F. nucleatum*. An explanation of the symbols is given in the legend to Fig. 3.
produced two points above the cutoff (Fig. 2C). Titer, in RIA units, was defined as the inverse of the serum dilution at the intersection of the linear portion of the curve and the cutoff (e.g., the titer in Fig. 1 would be approximately 60,000 RIA units per ml.)

The HP, SP, and JP groups were compared at three levels of sensitivity in which positive was defined as four or more counts-per-minute values above the cutoff, three or more points above cutoff, or two or more points above cutoff by logistic regression at $P < 0.01$. A one-way analysis of variance was used to test comparisons of antibody titer. Tukey's honestly significant difference test was used to test these comparisons if the F test was found to be significant ($P < 0.01$). We required $P < 0.01$ because of the danger of excess false-positives at higher $P$ values when multiple comparisons are made. The analyses were performed on the log-transformed values, but inverse-transformed mean values are reported here for ease of interpretation.

(iii) Estimation of RIA sensitivity. IgG was isolated from a patient's serum containing over 500,000 RIA units per ml against *Actinobacillus actinomycetemcomitans* Y4. The purification steps included ammonium sulfate precipitation (3) followed by chromatography on a Sephacryl S-200 superfine column (bed height, 80 cm; flow rate, 10.0 ml/cm² per h). The protein in the IgG peak was determined by the method of Lowry et al. (12), and a small amount of the IgG was iodinated to 330 µCi/µg of IgG and diluted in 50% newborn calf serum. Doubling dilutions of both iodinated and non-iodinated IgG were placed in *A. actinomycetemcomitans* Y4-coated wells and naked wells and incubated for 16 h at 5°C as for the serum samples. All wells were washed 20 times in tap water as in the standard assay, and the wells with cold IgG were then incubated with the radiolabeled goat anti-IgG, washed, and counted. The counts-per-minute curve versus IgG dilution was plotted from wells with cold human IgG, and the cutoff was established from the curve. From the counts per minute of the radiolabeled human IgG bound to each assay well and the known specific activity of the labeled human IgG, we determined how much human IgG was attached to each well in the dilution series. The well which most nearly corresponded to the cutoff contained 250 pg of specific human IgG. Since the minimum amount of human IgG required to attain the cutoff value is defined as 1 unit, we estimated that 1 RIA unit was approximately 250 pg of IgG.

**RESULTS**

The frequency and magnitude of antibody to *Eubacterium nodatum*, *Eubacterium timidum*, and *Eubacterium brachy* in the sera of the three groups are recorded in Fig. 3. *E. nodatum* is the most numerous organism in the subgingival flora of SP subjects. Together, *E. brachy*, *E. timidum*, and *E. nodatum* make up 16% of the cultivable subgingival flora of SP subjects but less than 1% of the flora of HP subjects. Nearly 25% of the SP subjects were seropositive for *E. brachy* when seropositivity was defined as a minimum of four points in sequence above the cutoff. In contrast, none of the JP subjects were seropositive, and only one HP subject was seropositive. SP subjects had detectable antibody titers in serum more frequently than did HP or JP subjects, regardless of whether four or more, three or more, or only two or more points were required to consider an individual seropositive ($P < 0.01$). At three or more points above the cutoff, the differential between SP subjects and HP or JP subjects was greatest; over 50% of the SP subjects were seropositive, as compared to with about 15% of the HP or JP subjects. The magnitude of the antibody titers of subjects seropositive for *E. brachy* was similar in all groups, although

**FIG. 5.** Frequency and magnitude of serum antibody reactive with *B. gingivalis* and two strains representing the two genospecies of *B. intermedus*. An explanation of the symbols is given in the legend to Fig. 3.
those of the SP groups were higher than those of the JP group ($P < 0.01$) when seropositivity required only two points in sequence above the cutoff. The difference in magnitude between the two groups, however, was only about twofold.

SP subjects responded more frequently to *E. timidum* than did HP subjects, but the difference was not significant ($P > 0.01$). The frequency in the JP group was higher than that in the HP group when three or more points above the cutoff were required for seropositivity. The magnitude of the responses of seropositive individuals in the JP group was higher than that of seropositive HP subjects. Similarly, seropositive SP subjects usually had higher antibody titers than seropositive HP subjects. Thus, although the SP group failed to show a significantly greater frequency of response, individuals that responded showed higher levels of antibody than HP subjects.

Despite the fact that *E. nodatum* has not been detected in the periodontal flora of HP subjects, most HP subjects were seropositive by all three standards used, and the antibody titers were very high. Some SP and JP subjects had higher titers than any HP subject, but as a group they were not significantly different. The only indication of between group differences was the response level of two or more points above cutoff, where the SP subjects responded more frequently than did HP or JP subjects.

The frequency and magnitude of serum antibody titers to *Florobacterium nucleatum* is shown in Fig. 4. Strains of three different genetic subgroups of *F. nucleatum* were used in this study. This species represents over 7% of the subgingival flora of SP subjects (15). SP subjects tended to be more frequently seropositive than HP subjects, but the magnitude of the responses was low. Maximum titers for all three groups were in the range of 4,000 to 5,000 units per ml (estimated to be about 1 to 2 μg of specific anti-*F. nucleatum* antibody per ml of serum).

The frequency and magnitude of antibody to *Bacteroides gingivalis* or *Bacteroides intermedius* genospecies 4197 and 8944 are shown in Fig. 5. These species represent about 4% of the cultivable subgingival flora of the SP group. Only one HP subject was seropositive for *B. gingivalis* when the criterion of a minimum of four points in sequence above the cutoff was used. In contrast, about 30% of the SP subjects were seropositive and 50% of the JP subjects were seropositive. Curiously, at two points above the cutoff, the frequency of seropositive SP subjects was suppressed compared with that of HP or JP subjects. Of the SP subjects, 49% did not have any indication of antibody to *B. gingivalis*. In contrast, only 20% of HP subjects and 14% of JP subjects failed to have at least one point above the cutoff (data not shown). Thus, most SP subjects had high antibody or no titers. The magnitude of the average HP response at a minimum of two points above the cutoff was 1,600 units per ml, compared with 3,800 units per ml for JP subjects and 4,900 units per ml for SP subjects.

The antibody responses to *B. intermedius* were less striking. Only at two points above the cutoff was the frequency of antibody to genospecies 4197 significantly higher in the SP group. The frequency of antibody to genospecies 8944 was even lower, although antibody titer in most seropositive individuals was high (over 20,000 units per ml).

The frequency and magnitude of the antibody titer to *Peptostreptococcus micros* are shown in Fig. 6. This species constitutes between 4 and 5% of the subgingival flora of the SP group (Table 2). At high levels, antibody to *P. micros* was significantly more frequent in the SP group than in either the HP or JP groups, but the magnitude of the antibody titers in seropositive individuals did not differ among groups.

Although *Lactobacillus minitus* represents over 5% of the subgingival flora of SP subjects (Table 2), the frequency of seropositivity was negligible. None of the SP subjects and only two of the HP subjects and two of the JP subjects were seropositive by our most lenient criterion (a minimum of two points in sequence above the cutoff). This was the only nonspirochetal species we have tested in this and other studies to which the frequency of response was so low.

**Discussion**

The results support the view that, with some exceptions, a general relationship exists between the subgingival flora of patients with periodontitis and the incidence of specific antibody to predominant members of this flora. The most striking exception was *L. minitus*, which constitutes 5% of the flora in SP subjects (15), but no SP subject was seropositive in our study. This result suggests that *L. minitus* is poorly immunogenic or, possibly, can selectively suppress an immune response. Other periodontal species, including several spirochetes, have been reported to be immunosuppressive (18, 19).

The *B. gingivalis* data (seropositivity at four or more points above cutoff) are similar to those reported by others (1, 7), except that the proportion of seropositive subjects in our SP and JP groups is reversed in comparison with earlier reports. The response frequency in HP subjects was 2% in this study and was reported to be similarly low (6%) in a previous study (7). We found, however, that 50% of the JP subjects and 28% of the SP subjects were seropositive, which is reversed from the values of 19 and 26% for JP (LJP) subjects and 50 and 58% for SP (ADP, RP) subjects reported by others (1, 7). It is possible that the unexpected, statistically higher level of background counts on naked wells relates to the difference in our results. If we had not subtracted background but worked only with counts per minute bound to antigen-coated wells, significantly more SP subjects would have appeared positive. The reasons for the increased background counts in the SP group are not clear. Although more SP subjects have elevated serum IgG than do HP subjects (17), this does not seem to be a total explanation. There were sera used in the present study which had high IgG levels but gave low background counts, and vice versa.

The findings in this study are not likely to be attributable to nonspecific binding, such as to the Fc region of IgG, because of the differences found between subject groups. Nonspecific reasons for binding could be expected to produce similar results among subject populations. The results with respect to *E. nodatum* may cause some concern in this regard, as a high percentage of HP subjects exhibited a titer, even though this species is rarely, if ever, found in such individuals (14). Absorption of sera with *E. nodatum*, however, was found to remove almost all binding of IgG to *E. nodatum*, whereas absorption with this species did not reduce binding to other species tested (data not shown).

About half of the SP subjects had no detectable titer of antibody to *B. gingivalis*, although those that were positive had very high titers. In contrast, most JP and HP subjects had low levels of antibody. The dichotomy in the SP group is not understood but could relate to a fundamental difference in the immune response to *B. gingivalis* in these two groups of SP subjects. In one group, a marked productive specific antibody response existed, and in the other, there appeared to be a profound specific suppression.
None of the individual species elicited responses that would enable the differentiation of SP or JP as currently defined. Combinations of organisms, however, might provide useful differentiation. Theoretically, coating an RIA strip with a cocktail of antigens consisting of F. nucleatum E3C22, E. brachy, and P. micros would present an antigen profile in which most subjects clinically characterized as SP would react, as compared with very few HP or JP subjects. Responses to combinations of organisms has been reported to correlate with clinical status in previous reports (7), and the possibility exists that such antibody data may be useful in helping to define clinical groups. Disease progression or remaining quiescence could possibly correlate with the presence or absence of certain antibodies.

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LITERATURE CITED