INFECTION AND IMMUNITY, Jan. 1981, p. 182-192
0019-9567/81/010182-11$02.00/0

Vol. 31, No. 1

Serum Antibodies to Oral *Bacteroides asaccharolyticus* (*Bacteroides gingivalis*): Relationship to Age and Periondontal Disease

CHRISTIAN MOUTON,†* PAULETTE G. HAMMOND, JØRGEN SLOTS, AND ROBERT J. GENCO

Department of Oral Biology and Periodontal Disease Clinical Research Center, State University of New York at Buffalo, Buffalo, New York 14226

An enzyme-linked immunosorbent assay microplate method was used for measuring levels of antibody specific for the oral serotype of *Bacteroides asaccharolyticus* (*Bacteroides gingivalis*) in serum samples obtained from umbilical cords, infants, children, periodontally normal adults, and edentulous adults. Serum from patients with various periodontal diseases, including adult periodontitis, localized juvenile periodontitis, generalized juvenile periodontitis, post-localized juvenile periodontitis, and acute necrotizing ulcerative gingivitis, were also studied. A positive correlation between increase in age and increase in both prevalence and level of specific antibody in the G, A, and M classes of immunoglobulins was observed. This indicates that antibodies reactive with oral *B. asaccharolyticus* found in up to 84% of normal adults are natural antibodies, presumably with a protective role. Among the patient groups, those with adult periodontitis were found to have levels of immunoglobulin G antibodies to oral *B. asaccharolyticus* that were five times higher than the antibody levels found in control subjects. The levels of IgG antibodies to this organism in the other patient groups were comparable to the levels found in the control group. However, 50% of the individuals in the generalized juvenile periodontitis group had high levels of immunoglobulin G antibodies to *B. asaccharolyticus*, suggesting heterogeneity with respect to immune response in these patients. These results indicate that antibodies to oral *B. asaccharolyticus* (*B. gingivalis*) occur at low levels in most normal children and adults and that the rise in titer of the specific antibodies of each major class of immunoglobulins parallels the ontogenic change in serum levels of that isotype. In contrast, there is a marked increase in titer of immunoglobulin G antibodies to oral *B. asaccharolyticus* in the group of patients with adult periodontitis and in patients with the generalized form of juvenile periodontitis.

Recent observations suggest that specificity exists in the bacterial etiology of various forms of periodontal disease (35). *Bacteroides asaccharolyticus* is a predominant isolate from some advancing lesions of adult periodontitis in humans (31, 38, 39) and from experimental periodontitis lesions in monkeys (34; K. S. Kornman, S. C. Holt, and P. B. Robertson, J. Dent. Res., special issue 59A, abstr. no. 483, p. 388, 1980). Localized juvenile periodontitis (LJP; periodontosis) is associated with a predominance of *Actinobacillus actinomycetemcomitans* (26, 30, 39) and *Capnocytophaga* species (25, 30).

Bacterial infections usually are associated with a strong immune response, and diagnosis is often aided by assessing the specific serum antibody response to the candidate pathogens (reviewed in reference 37). A distinction is usually made between hyperimmune antibodies and natural, normal, or background antibacterial antibodies present in most healthy individuals. Natural antibodies occur in small quantities, and their accurate detection often requires the use of sensitive techniques (21).

Little information exists on the human immune response to gram-negative organisms and particularly to *B. asaccharolyticus* in periodontal health and disease. Courant and Gibbons (4) detected antibodies against a mixture of lipopolysaccharides from oral and nonoral strains of saccharolytic and asaccharolytic *Bacteroides melaninogenicus* in each of 16 human serum samples. Hofstad (16) found that most children older than 1 year of age and blood donors have circulating antibodies to the lipopolysaccharide of oral *B. melaninogenicus* (presumably *B. melaninogenicus* subsp. *intermedius*). In a pilot

† Present address: Ecole de Medecine Dentaire, Université Laval, Québec, Canada G1K 7P4.

study [C. Mouton, J. Slots, and R. J. Genco, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, E(H) 8, p. 79], it was shown that immunoglobulin G (IgG) and IgM antibodies reactive with autoclave extracts of oral strains of *B. melaninogenicus* subsp. *intermedius* or *B. asaccharolyticus* could be detected in the sera of periodontally normal children and adults. The levels of antibody in these normal sera reactive with *B. melaninogenicus* subsp. *intermedius* were distinctly higher than those reactive with *B. asaccharolyticus*. In these pilot studies, it was also shown that patients with adult periodontitis had higher levels of antibodies to *B. asaccharolyticus*, but not to *B. melaninogenicus* subsp. *intermedius*, than did age-matched normal adults. In recent studies in our laboratory, we demonstrated that strains of *B. asaccharolyticus* isolated from the oral cavity were serologically distinct from strains isolated at nonoral sites (24, 27). Furthermore, deoxyribonucleic acid composition and homology studies support the separation of the black-pigmented asaccharolytic strains of oral origin from the species *B. asaccharolyticus* and suggest the creation of the new species *Bacteroides gingivOalis* (5).

The present investigation was initiated to study the human immune response to oral *B. asaccharolyticus* (*B. gingivalis*) in various clinical forms of human periodontal disease. Information on the ontogeny of the production of antibodies reactive with oral *B. asaccharolyticus* was also sought. The enzyme-linked immunospecific (immunosorbent) assay (ELISA) was used for this purpose.

MATERIALS AND METHODS

Antigen preparation. Oral *B. asaccharolyticus* strain 381 was grown in 5 liters of diffusate broth medium modified EX-1 (20) for 72 h at 37°C. The cells were harvested by centrifugation at 10,000 × *g* for 20 min at 4°C and washed three times with sterile, phosphate-buffered saline (PBS), pH 7.2 to 7.4. Three grams (wet weight) of washed bacterial cells were resuspended in 35 ml of sterile PBS with 0.005 M ethylene diaminetetraacetic acid, pH 7.2, in a 300-ml Erlenmeyer flask containing 5 ml of sterile glass beads, each with a diameter of 0.13 mm. The mixture was agitated for 48 h at 4°C on a clinical rotator at 160 oscillations per min, and the centrifuged supernatant was collected. The pellet of bacterial cells and glass beads was suspended twice in 5 ml of PBS, blended in a Vortex mixer for 60 s, and centrifuged. All of the supernatants obtained were pooled, centrifuged at 12,000 × *g* for 10 min, dialyzed at 4°C against three changes of distilled water, and lyophilized.

Serum specimens. Serum samples from umbilical cords, infants, and children were obtained from B. H. Park, Children's Hospital, Buffalo, N.Y. Dental students and laboratory personnel with healthy periodontal conditions (no evidence of periodontal disease other than mild gingivitis) provided 56 serum samples constituting the normal-adult group (age range, 21 to 46 years). Serum samples obtained from periodontal patients at the State University of New York at Buffalo School of Dentistry were grouped by clinical condition. An adult periodontitis group ($n = 35$) exhibited generalized alveolar bone loss with multiple vertical osseous defects (age range, 30 to 62 years). An LJP group comprised patients aged 14 to 24 years ($n = 41$) exhibiting vertical alveolar bone resorption localized to the incisors and molars and not more than two additional teeth. The post-LJP group comprised 11 patients who were older than 30 years of age (age range, 31 to 56 years) and exhibited marked alveolar bone loss in the molar or incisor region and had a history of LJP. The generalized juvenile periodontitis (GJP) group ($n = 10$) consisted of patients 14 to 29 years of age with severe bone resorption affecting more than 14 teeth. An acute necrotizing ulcerative gingivitis group comprised 12 patients from whom blood had been collected at the clinically acute phase of the disease (age range, 20 to 32 years). Nine subjects who were edentulous for at least 3 years comprised the edentulous group (age range, 49 to 65 years). All sera were stored at −20 or −70°C until used. Also, selected sera were tested for rheumatoid factors by latex agglutination (Rapi/tex-RF Kit; Calbiochem, La Jolla, Calif.).

ELISA. ELISA described by Engvall and Perlmann (10), modified by Ruitenberg et al. (28), and adapted to microplates by Voller et al. (40) was used with minor modifications. Disposable flat-bottom polystyrene microtiter plates (Dynatech 1-223.29; Dynatech Laboratories, Inc., Alexandria, Va.) were used. The lyophilized antigen mixture was suspended in 0.1 M Na_2CO_3 buffer (pH 9.6) containing 0.02% NaN_3 at a concentration of 50 μg/ml. In preliminary experiments, the antigen concentration for sensitizing the plates found to be the lowest giving optimal results contained 4 μg of protein per ml and 1 μg of carbohydrate per ml, as determined by the method of Lowry et al. (19) with bovine serum albumin as the standard and by the method of DuBois et al. (7) with glucose as the standard, respectively. The wells of the microtiter plate were sensitized with 200 μl of antigen solution at 37°C for 1 h; 100 μl of 2% (wt/vol) bovine serum albumin in 0.1 M Na_2CO_3 buffer containing 0.02% NaN_3 was then added, and the plates were stored at 4°C until used.

Before testing, the wells were washed three times with a solution containing PBS and 0.05% Tween 20. A 200-μl volume of test serum at the appropriate dilution in PBS–0.05% Tween 20 supplemented with 0.5% bovine serum albumin and 0.02% NaN_3 was added, and the plates were incubated for 2 h in a moist chamber at 37°C. After the wells were washed as described above, 200 μl of alkaline phosphatase-conjugated anti-human immunoglobulin diluted in PBS–0.05% Tween 20 supplemented with 0.5% bovine serum albumin and 0.02% NaN_3 was added to each well, and the plates were incubated at 37°C in a moist chamber for 1 h (1.5-h incubation for the anti-IgM conjugate). After two washings as described above and a third washing with the substrate buffer were done, a 200-μl volume of fresh substrate was added to each well. The substrate solution was prepared by dissolving *p*-nitro-

phenylphosphate (type 104; Sigma Chemical Co., St. Louis, Mo.) in 0.05 M Na_2CO_3 buffer (pH 9.8) supplemented with 10^{-3} M $MgCl_2$ at a concentration of 1 mg/ml. After 1 h of incubation at 37°C, the enzymatic reaction was stopped by adding 50 μl of 1 N NaOH, and the absorbance at 400 nm was determined in a custom-made colorimeter (3) through the bottom of each well. The colorimeter was zeroed using a sensitized well incubated with substrate only and 1 N NaOH added (substrate control). Duplicate determinations were obtained for each sample on the same plate. Each assay included (i) a conjugate control, (ii) a substrate control, (iii) a conjugate-substrate reactivity control, and (iv) a serially diluted positive serum control.

Alkaline phosphatase-conjugated anti-human immunoglobulins, produced in swine (Orion Diagnostica, Helsinki, Finland) were purchased from MTC, Hackensack, N. J. The swine antibodies of these conjugates are specific for the γ, α, or μ chain of human immunoglobulins, specificity being achieved by affinity chromatography. The specificity of the anti-human immunoglobulins was tested by reacting the conjugate with chromatographically purified normal IgG, purified myeloma IgA, or myeloma IgM added at various concentrations to the wells of a microplate. These experiments indicated that each conjugate was specific for the immunoglobulin class that it measured and that cross-reactivity was minimal with up to 10 μg of the heterologous immunoglobulin class per ml (data not shown). The optimal working dilution of each conjugate was determined by serial dilutions in preliminary assays. The anti-IgG and the anti-IgA conjugates were used at a dilution of 1:200, and the anti-IgM conjugate was used at a dilution of 1:100.

Immunochemical characterization of the saline-extracted antigen preparation was carried out by comparative immunoelectrophoresis in an agar gel, using an antiserum to homologous whole cells. A precipitating line showing identity with a reference alkali-extracted antigen (K. Okuda, J. Slots, and R. J. Genco, J. Dent. Res., special issue 59A, abstr. no. 475, p. 386, 1980) indicated the presence of a polysaccharide, likely a capsular antigen, in the antigen mixture used for ELISA (Fig. 1). No precipitation line showing identity with a phenol-water-extracted lipopolysaccharide was observed. In a passive hemagglutination test, using an antiserum to homologous whole cells, the ELISA antigen extract failed to sensitize fresh sheep erythrocytes at concentrations of up to 0.1 mg of lyophile per ml of 0.5% erythrocyte suspension. Immunoelectrophoresis and the failure to observe sensitization of erythrocytes suggest that the saline-extracted antigen contained little or no lipopolysaccharide. The other precipitation lines of Fig. 1 likely are the result of slow-migrating protein antigenic structures or aggregates of surface components which account for the 50% of Lowry reactive material in the saline-extracted antigen.

Specificity of ELISA for oral *B. asaccharolyticus* was determined by inhibition of the reaction after absorption with bacterial cells. Washed cells to be used for absorption were suspended in the reference serum pool (diluted 1:50 in PBS) at concentrations of 2×10^9, 2×10^8, and 2×10^7 cells per ml and incubated overnight at 4°C with constant shaking. The suspension was centrifuged, and the supernatant was filtered (0.45 μm; Millipore Corp., Bedford, Mass.). Serial dilutions of the absorbed sera were then tested for IgG class antibodies to the oral *B. asaccharolyticus* antigen.

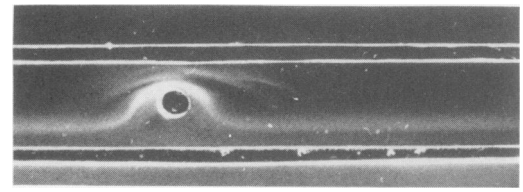

FIG. 1. *Comparative immunoelectrophoretic analysis of the neutral buffer extract of oral B. asaccharolyticus strain 381 at a concentration of 5.0 mg of lyophile material per ml of buffer (well). Electrophoresis was performed at 6 V/cm for 50 min. The upper trough contains an antiserum against whole cells of strain 381. The lower trough contains the capsular polysaccharide extracted from strain 381 with 0.02 M KOH.*

Standardization of the assay. Preliminary checkerboard assays were conducted to identify human sera having a reactivity in the IgG, IgA, and IgM classes of antibody with the oral *B. asaccharolyticus* antigen. Three positive sera were pooled and aliquoted for subsequent use in serial dilutions with each further assay as a reference positive control. A titration curve for determination of the antibodies reactive with oral *B. asaccharolyticus* was thus obtained for each class of immunoglobulin by plotting the mean absorbances obtained for the reference serum pool over the whole experiment against the $-\log_{10}$ of the dilution (Fig. 2). Sigmoid curves were obtained, and values along the rectilinear portion of the dose-response curve were used to fit a linear equation of the regression of absorbance on the $-\log_{10}$ dilution. The computed equation defined each standard curve and was used for all subsequent calculations. The 95% confidence interval on the slope of each standard curve was calculated (Fig. 2).

Deviation from linearity at low response was observed, and the absorbance at which the curve became nonlinear was used as the threshold level of sensitivity. The following cutoff absorbance values at 400 nm were used: 0.11 for IgG, 0.16 for IgA, and 0.18 for IgM. These cutoff absorbance values were 2 standard deviations above the mean background values observed over the whole experiment; no correction for background was introduced. High-response deviation from linearity occurred for absorbance values at 400 nm above 1.0 to 1.1. All of the serum samples yielding absorbance values at 400 nm above 0.9 when screened at a single dilution were subsequently assayed by serial dilution such that the values fell into the rectilinear portion of the dose-response curve.

Results were obtained as ratios of antibody activity by relating the individual sera to the standard curve obtained with the reference serum pool (18). The ratio values thus obtained for determination of IgG antibody activity, multiplied by 100, expressed the level of IgG-specific antibody as ELISA units (EU-G) relative

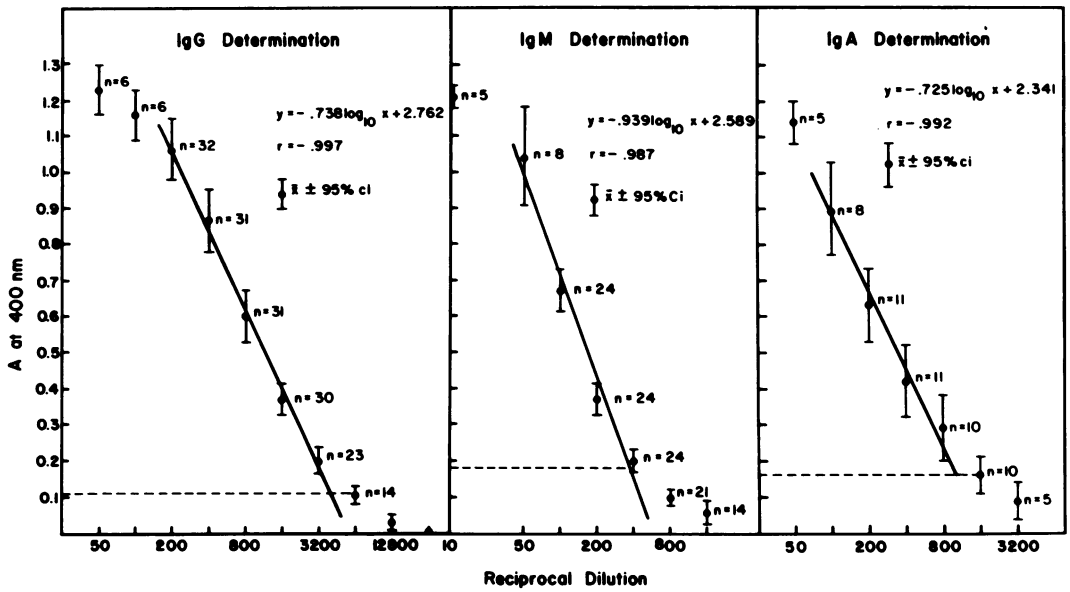

FIG. 2. *Standard curves for determination of class-specific antibody activity to oral B. asaccharolyticus, using the reference serum pool. Each point represents the mean absorbance (A) value observed for n assays. Vertical lines indicate the 95% confidence limits. Absorbance values along the rectilinear position of each dose-response curve were used to define the corresponding regression equation. The indicated equations were used for determination of the relative antibody activity in the test samples (see text). The correlation coefficients (r) are indicated. Horizontal dotted lines indicate the threshold of sensitivity at low response (cutoff absorbance). The 95% confidence interval (Ci) on the slope of each standard curve was calculated: IgG determination, ¯0.639 to ¯0.837; IgM determination, ¯0.473 to ¯1.405; IgA determination, ¯0.562 to ¯0.889.*

to the reference serum given the arbitrary value of 100 EU-G. A coefficient of 10 was used for determination of EU-A and EU-M.

In the first part of the study, or screening experiment, the individual serum samples were routinely tested at a single dilution of 1:200 for IgG and IgA determinations and at 1:100 for the IgM determination. Absorbance values below the cutoff level could correspond to the absence of detectable levels of specific antibodies or to the presence of specific antibodies too dilute for accurate quantitation. Experiments were conducted with serial twofold dilutions from 1:10 or 1:25 on serum samples that had yielded an absorbance value below the cutoff at the screening dilution. For this purpose, the sera most likely to be devoid of specific antibody activity (i.e., all 9 umbilical cord sera and all 12 sera from infants less than 6 months of age) and 30 to 50% of the low-reactive samples of the other groups were tested. Some serum samples, in particular, all 9 umbilical cord sera tested for IgM-specific antibody, yielded absorbance values at all dilutions tested that were below the cutoff absorbance; such sera were considered to contain no detectable specific antibody (EU = 0). Some serum samples yielded absorbance values above the cutoff that permitted construction of a titration curve with a satisfactory slope (slope falling within the limits of the 95% confidence interval of the reference slope). The presence of low amounts of specific antibody, sufficient for quantitation, was therefore confirmed in these samples. The corresponding antibody activities were calculated as EU and used in calculation of the group means. Some serum samples, however, did not allow construction of a titration curve with a satisfactory slope. In such cases, the specific antibody level was calculated from the last dilution yielding an absorbance value above cutoff, and a mean level was obtained. This mean value represented the level of trace amounts of specific antibody, detectable but too low for quantitation. The levels of trace amounts of specific antibody were: 4.0 EU-G, 0.5 EU-A, and 0.6 EU-M. These values were used in calculation of the group means.

Validity of the assay for our population of samples was assessed and confirmed, using the criteria proposed by Hewitt (15). Part of the data appears in Table 1.

RESULTS

Specificity of ELISA developed for oral *B. asaccharolyticus* was verified by whole bacterial cell absorption (Table 2). Absorption of the reference serum pool with oral *B. asaccharolyticus* strain 381 reduced its activity by 91%, whereas absorption with nonoral *B. asaccharolyticus* strain B536, *B. melaninogenicus* subsp. *intermedius* strain 4127, or *A. actinomycetemcomitans* strain Y4 removed little or no antibody activity when antigen of strain 381 was used in the ELISA plate. This indicated that the antibodies detected in the present study were spe-

cific for the oral serotype of *B. asaccharolyticus* (*B. gingivalis*).

Serum antibodies to oral *B. asaccharolyticus* in normal individuals and relation to age. The mean levels of antibodies to oral *B. asaccharolyticus* detected in the various nondiseased groups studied are listed in Table 3. Significant amounts of IgG antibody were distinctly distributed in the various age groups studied. More than 80% of the normal adults had detectable specific IgG antibody activity, with an average of 55.7 EU-G. In contrast, the prevalence of measurable activity ranged from approximately 45% in the samples from umbilical cords (average, 35.2 EU-G) and children 6 to 12 years of age (average, 29.6 EU-G) to 0% in the sera of children less than 6 months of age. The mean IgG antibody level in the umbilical cord sera, only slightly lower than that in the normal-adult group, is consistent with maternofetal transfer.

TABLE 1. *Comparison of the regression functions relating absorbance to dilution of the reference pool and of high-titer serum samples selected from various study groups*[a]

Group	*n*	Slope range	Common[b] slope	*r* range[c]
Reference serum pool	10	−0.683–−1.063	−0.903	0.951–0.999
Normal adult	10	−0.766–−1.036	−0.895	0.971–0.999
Adult periodontitis	10	−0.9–−1.166	−0.997	0.994–0.999
LJP	10	−0.8–−1.166	−0.968	0.956–0.999

[a] The results here are for IgG antibody directed to *B. asaccharolyticus* strain 381 antigens. Testing for the difference between the *n* regression functions was performed by analysis of covariance for comparing more than two slopes (41). In all cases, the null hypothesis of equal slope was not rejected at the confidence level $\alpha = 0.05$. The common regression coefficient was therefore used as an estimate of the slope underlying all samples in the group, and each was compared with that of the reference serum pool to test for the difference between two population slopes (41). Slopes were parallel ($P < 0.05$).

[b] Common, or weighted, regression coefficient.

[c] Range of correlation coefficients obtained for regression of each of the *n* assays; three to four dilutions were used for computation of the regression.

Trace amounts only of specific IgG in individuals 0 to 6 months of age indicated that the IgG antibodies of maternal origin are short lived. An increased, but low, level was observed in the age group 6 months to 6 years (7.9 EU-G), and a marked increase occurred in the age group 6 to 12 years, wherein the mean IgG level of 29.6 EU-G was approximately half that of adults ($P < 0.05$). This pattern of antibody occurrence suggests that active synthesis of IgG antibodies to oral *B. asaccharolyticus* takes place during childhood. The average level of IgG antibodies in the edentulous group was significantly lower than that in the normal-adult group ($P < 0.05$), suggesting that the levels are associated with the presence of dentition.

The changes with age of specific IgM antibody activity followed a pattern distinct from that for IgG. No specific IgM activity was detected in the cord blood samples, however, within 6 months after birth, 25% of the samples had detectable levels which were responsible for a

TABLE 2. *Specificity of ELISA for oral B. asaccharolyticus (B. gingivalis) tested by serum absorption with whole bacterial cells*

Bacterial concn (cells per ml)	Reduction (%) of activity relative to unabsorbed serum[a] for bacterial strain[b]:			
	381	B536	4127	Y4
2×10^9	91	18	5	2
2×10^8	78	8	8	2
2×10^7	61	22	24	2

[a] The absorbed reference serum pool was tested at a dilution of 1:400 with the anti-IgG conjugate. The plates were coated with 50 μg of a lyophilized saline extract of oral *B. asaccharolyticus* strain 381 per ml. Values are expressed as the percent decrease of absorbance readings relative to untreated serum.

[b] Strain 381 is an oral isolate of *B. asaccharolyticus*, strain B536 is a fecal isolate of *B. asaccharolyticus*, strain 4127 is an oral isolate of *B. melaninogenicus* subsp. *intermedius*, and strain Y4 is an oral isolate of *A. actinomycetemcomitans*.

TABLE 3. *Level*[a] *of serum immunoglobulins reactive with oral B. asaccharolyticus in healthy individuals grouped according to age*

Group	Mean EU-G ± SD[b]	Mean EU-M ± SD	Mean EU-A ± SD
Umbilical cord ($n = 9$)	35.2 ± 59.7	0	tr[c]
Infants (<6 mo; $n = 12$)	tr	1.9 ± 2.5	tr
Children: primary dentition (6 mo–6 yr; $n = 30$)	7.9 ± 8.6[d]	2.3 ± 3.2	tr
Children: mixed dentition (6–12 yr; $n = 24$)	29.6 ± 54.2[d]	3.7 ± 4.1	1.5 ± 1.2
Normal adult (21–46 yr; $n = 56$)	55.7 ± 64.6	3.4 ± 3.5	1.8 ± 1.7
Edentulous (49–65 yr; $n = 9$)	14.3 ± 18.6[d]	1.3 ± 1.5	tr

[a] Values are expressed as EU relative to a serum reference.

[b] SD, Standard deviation.

[c] tr, Trace amount.

[d] Significant difference from the normal adult age group ($P < 0.05$); *t* test.

group mean level approximately one-half that of the adult group (1.9 and 3.4 EU-M, respectively). The next years of life were not associated with changes in prevalence or activity levels of specific IgM, but by age 12 it was observed that 50% of the population had reached the level found in normal adults (3.7 EU-M). The mean specific IgM level in the edentulous group was one-third that of the normal-adult group.

The appearance of detectable levels of specific antibodies in the IgA class was observed in sera from subjects 6 to 12 years of age (21% positive; group mean, 1.5 EU-A), whereas only 2 of 30 samples in the group 6 months to 6 years of age had measurable levels, and only trace amounts were found in infants and in umbilical cord sera. In the normal-adult group, 34% of individuals had measurable levels responsible for a group mean of 1.8 EU-A. Only trace amounts of specific IgA were seen in the edentulous group.

The age-related changes in levels of IgG, IgA, and IgM antibodies to oral *B. asaccharolyticus* are graphically depicted in Fig. 3.

Serum antibodies to oral *B. asaccharolyticus* in relation to periodontal disease condition. The levels of specific antibody detected in the various patient groups are listed in Table 4. IgG antibody to oral *B. asaccharolyticus* was found in all groups studied. The mean IgG antibody levels in the LJP (60.5 EU-G) and the acute necrotizing ulcerative gingivitis (46.0 EU-G) groups were similar to that of the normal-adult group (55.7 EU-G). The mean IgG antibody level in the post-LJP group (86.8 EU-G) was slightly elevated; however, it was not significantly different from that in the normal-adult group. In contrast, the mean IgG antibody level was fivefold higher in the adult periodontitis group (257.5 EU-G; $P < 0.05$, Mann-Whitney test) and eightfold higher in the GJP group (397.3 EU-G) than that in the normal-adult group.

The large standard deviations observed reflected the wide range of values among individual sera belonging to the groups studied. This dispersion of individual titers for specific IgG antibody is shown in Fig. 4. A further analysis (chi-square coefficient of dispersion) was conducted to compare the numbers of individuals having an activity level higher than the 95% confidence interval for the mean of the normal-adult group. Fifty percent of the adult periodontitis patients (17 of 34) and of the GJP patients (5 of 10) had an antibody level higher than 73 EU-G, the upper limit of the 95% confidence interval for the mean level of specific IgG in the normal adult group. No other group had a statistically significant number of patients with elevated levels of specific IgG antibodies (Fig. 5).

In contrast to IgG antibody, the adult periodontitis group had a mean specific IgM antibody level (2.9 EU-M) comparable to that in the normal-adult group (3.4 EU-M). The LJP group, on the other hand, showed a mean level (5.7 EU-

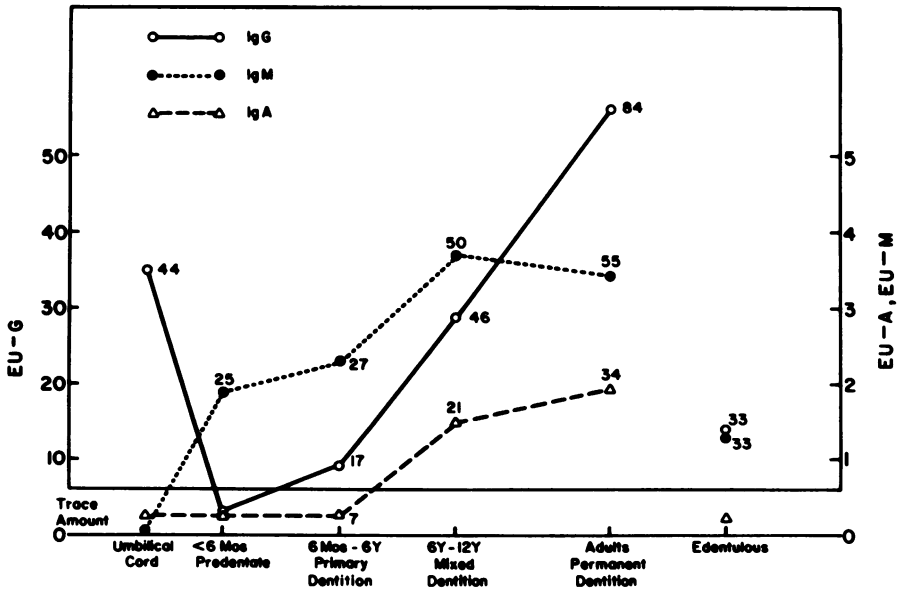

FIG. 3. *Changes with age of serum immunoglobulins reactive with oral B. asaccharolyticus. The curves connect the mean values expressed as EU-G, EU-A, and EU-M obtained for each age group. Numbers adjacent to each point indicate the percentage of positive individuals in each group.*

TABLE 4. *Level[a] of serum antibodies to oral B. asaccharolyticus in groups of patients with various periodontal conditions*

Group	Mean EU-G ± SD[b]	Mean EU-M ± SD	Mean EU-A ± SD
ANUG[c]	46.0 ± 46.7 (n = 12)	2.4 ± 2.5 (n = 12)	2.3 ± 4.7 (n = 12)
Normal adult	55.7 ± 64.6 (n = 56)	3.4 ± 3.5 (n = 55)	1.8 ± 1.7 (n = 56)
LJP	60.5 ± 88.7 (n = 41)	5.7 ± 7.5 (n = 39)	1.3 ± 1.6 (n = 41)
Post-LJP	86.8 ± 52.6 (n = 9)	2.6 ± 4.0 (n = 9)	NT[d] (n = 9)
Adult periodontitis	257.5 ± 468.4[e] (n = 34)	2.9 ± 2.5 (n = 34)	2.3 ± 2.9 (n = 34)
GJP	397.3 ± 515.2 (n = 10)	2.2 ± 2.1 (n = 10)	4.8 ± 7.6 (n = 10)

[a] Values are expressed as EU relative to a serum reference.
[b] SD, Standard deviation.
[c] ANUG, Acute necrotizing ulcerative gingivitis.
[d] NT, Not tested.
[e] Significant difference from the normal adult group ($P < 0.05$) by the Mann-Whitney test.

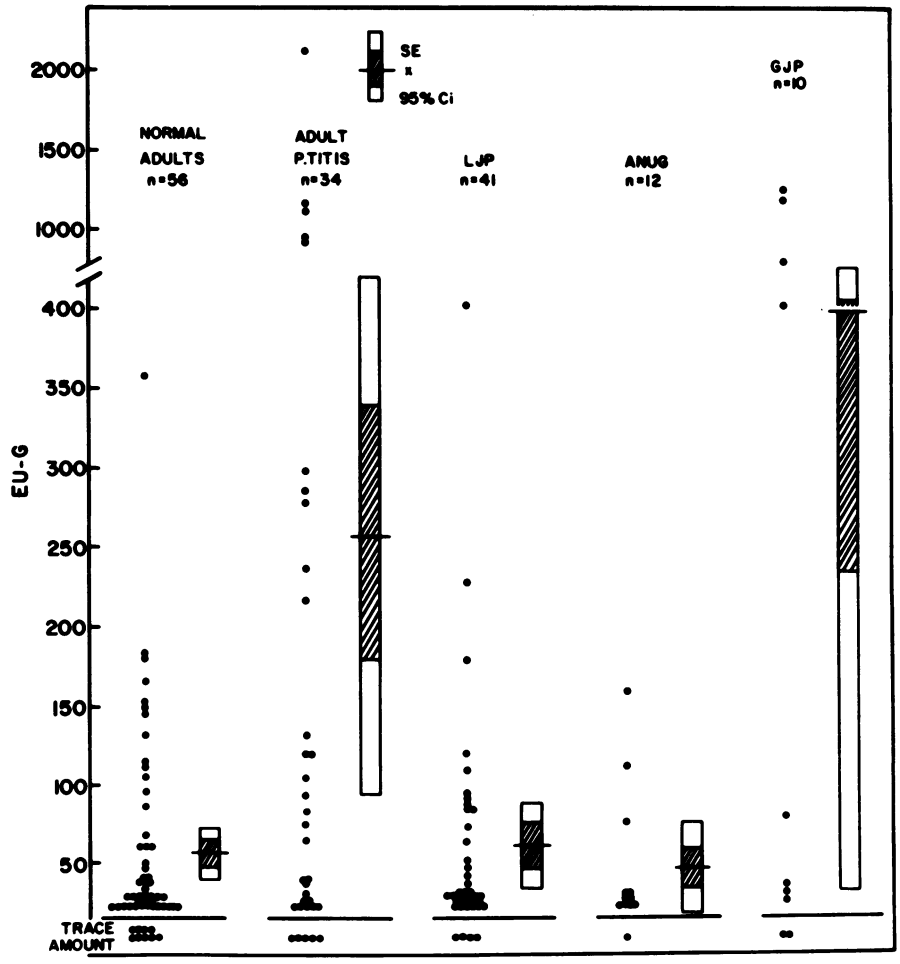

FIG. 4. *Levels of IgG specific for oral B. asaccharolyticus in various periodontal conditions. Each point represents the antibody activity level of an individual serum sample expressed as EU-G. Shown for each group is the mean ± standard error (SE; ▨) and ± the 95% confidence interval (Ci-□). ANUG, Acute necrotizing ulcerative gingivitis; P.titis, periodontitis.*

M) higher than that in the normal-adult group, a difference not statistically significant. However, analysis by the chi-square coefficient of dispersion revealed that the LJP group had a statistically significant number of patients (21 of 39, or 54%) with a level of specific IgM higher

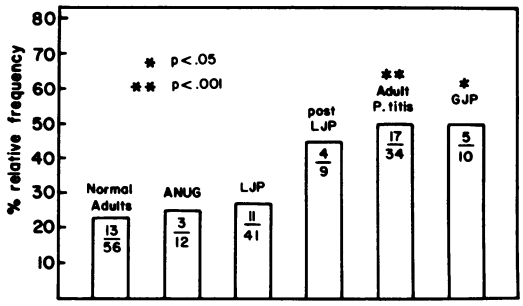

FIG. 5. *Distribution of the specific IgG antibody levels above the 95% confidence interval for the mean of 56 normal adults (upper limit = 73.0 EU-G). Statistical significance of the number of patients with an elevated antibody level was assessed by chi-square analysis with the Yate correction. ANUG, Acute necrotizing ulcerative gingivitis; P. titis, periodontitis.*

than the 95% confidence interval for the mean of the normal-adult group. Of 50 samples that demonstrated IgM activity, only 1 was positive in the latex agglutination test for rheumatoid factors, with an endpoint titer of 1:320. Hence, it is unlikely that the IgM activity detected by ELISA was due to a rheumatoid factor directed to denatured or aggregated IgG molecules in these patients.

IgA antibody specific for oral *B. asaccharolyticus* was the least frequent class detected. The mean quantifiable levels of specific IgA in the adult periodontitis (2.3 EU-A) and LJP (1.3 EU-A) groups were comparable to that in the normal adult group (1.8 EU-A). A twofold-higher level in the GJP group (4.8 EU-A), together with the highest prevalence (60%), was observed.

DISCUSSION

This study has shown that over half of the patients with adult periodontitis and with GJP have elevated levels of serum IgG antibodies specific for oral *B. asaccharolyticus*. It was also shown that specific IgG and IgM antibodies are common but usually at low levels in periodontally healthy individuals. In this respect, our data confirm previous studies with passive hemagglutination (4, 16) in which it was reported that antibodies to *B. melaninogenicus* can be found in most human sera. Taking advantage of the high degree of sensitivity afforded by ELISA (9) and of the potential of detecting immunoglobulin class-specific antibodies, we extended in our study the results previously obtained. However, since IgM and IgG antibodies compete for the same antigenic sites in ELISA, a factor which can make it difficult to demonstrate the total IgM activity in the presence of large amounts of specific IgG (23), the present study may have underestimated the specific IgM levels. Although it has been demonstrated (17) that removal of 94 to 98% of high-avidity antibodies (IgG1 and IgG2) does not result in higher levels of IgA and IgM detected in mouse serum, which implies that competition is not significantly affecting ELISA, a modified ELISA technique (8, 22) might allow the demonstration of higher levels of specific IgM and IgA antibodies in our sera.

The specificity of ELISA for antibody detection can be high and is determined by the quality of the antigen used in the system. The antigen used in our study likely contained mainly outer surface components which are extractable by mild procedures from an oral isolate of *B. asaccharolyticus*. These antigens which are not found in nonoral *B. asaccharolyticus* or other black-pigmented *Bacteroides*, as was verified by inhibition after bacterial absorption, permitted the characterization of antibodies reactive specifically with the oral serotype of *B. asaccharolyticus* (*B. gingivalis*).

Our study of the ontogeny of the production of antibody to oral *B. asaccharolyticus* revealed that adult levels were reached, following patterns different for each immunoglobulin class. Specific IgG activity was found in 44% of the cord blood sera. In contrast, young infants a few weeks to 6 months of age had no detectable IgG to oral *B. asaccharolyticus*, indicating that catabolism of the IgG molecules transferred across the placenta from the positive mother to fetus reduced to nondetectable levels the amount of maternally derived IgG and that these infants produce little or no antibodies of the IgG class to oral *B. asaccharolyticus*. Few individuals from 6 months to 6 years of age demonstrated IgG antibodies reactive with oral *B. asaccharolyticus*. The proportion of reactive children in the group of 6 to 12 years of age was more than doubled, and a threefold increase in the level of specific IgG antibody was observed compared with the findings in the group of 6 months to 6 years of age. These findings suggest that active synthesis of IgG antibody directed to oral *B. asaccharolyticus* starts early in childhood and is strongly enhanced when permanent teeth are erupting.

A positive correlation between increase in age and increase in both prevalence and levels of specific antibody of the M and A classes of immunoglobulins was also seen, but different patterns were observed. A response in the IgM class of specific antibody was found within 6 months of extrauterine life, whereas no detectable level was observed in cord blood sera. Each pattern (Fig. 3) is similar to that observed for the total serum immunoglobulin changes with

age (11, 14), suggesting the development of normal or natural antibodies likely dependent on the presence of environmental antigenic challenge associated with exposure to or presence of bacteria. The finding that each age group which exhibited a distinct antibody activity also was characterized by a distinct dentition status indicates that factors of importance for colonization of oral *B. asaccharolyticus* and consequent stimulation of antibody production include the presence of teeth and gingival crevices. Bacteriological data (13, 33, 36) indicate that the primary habitat of oral *B. asaccharolyticus* is the gingival sulcus. The low antibody levels observed in the edentulous group strengthen this relationship. Our evidence agrees well with the bacteriological findings of Bailit et al. (1), who reported that *B. melaninogenicus* (probably including *B. asaccharolyticus*) is apparently absent before the eruption of the deciduous teeth, is rare in preschool children, becomes increasingly prevalent during the period of mixed dentition, and is almost universally present by adolescence. Collectively, our findings suggest that antibodies in the three immunoglobulin classes G, A, and M reactive with oral *B. asaccharolyticus* contribute to the pool of background, normal, or natural antibodies usually found in humans. Unless they are heteroimmune antibodies stimulated by unidentified cross-reacting antigens, they likely are the result of an antigenic stimulation by oral *B. asaccharolyticus*. Such antigen stimulation may result from respiratory or salivary spread, as suggested by the early IgM response in age groups 0 to 6 months and 6 months to 6 years or from an established bacterial community. The major site of colonization by *B. asaccharolyticus* appears to be the gingival crevice area, although oral *B. asaccharolyticus* also may colonize the tonsils (2).

The observation of antibodies specific for oral *B. asaccharolyticus* in some periodontally healthy individuals suggests the presence of this organism. Most cultural studies, however, have recovered no, or a low proportion of, *B. asaccharolyticus* in periodontal health (6, 29, 32, 38, 39; D. White and D. Mayrand, J. Dent. Res., special issue 59A, abstr. no. 949, p. 506, 1980). The limited number of periodontal sites examined by culture, along with considerable variations in incidence and proportions of *B. asaccharolyticus* from sampling site to sampling site, may explain the low recovery. In addition, the systemic immune response may have been elicited by low levels of organisms or prior infections which would not be detected microbiologically at the time of collection of the serum sample.

Bearing in mind that the syntheses of the M and the G classes of immunoglobulins are sequentially activated, our observation and that of others (J. L. Ebersole, O. E. Frey, M. A. Taubman, D. J. Smith, J. R. Wetherell, and R. J. Genco, J. Dent. Res., special issue 59A, abstr. no. 248, p. 329, 1980) of more frequent and elevated levels of antibody to oral *B. asaccharolyticus* in the G class of immunoglobulin in the adult periodontitis group may result from an intensive, chronic challenge by oral *B. asaccharolyticus* which is consistent with the chronic, long-standing nature of this disease. This immunoserological observation is strongly supportive of several bacteriological studies (31, 38, 39) which have indicated that *B. asaccharolyticus* is a predominant isolate from advancing chronic periodontitis lesions. A similar observation was not made relative to the LJP group, suggesting that oral *B. asaccharolyticus* is not a primary pathogen in the development of typical LJP lesions. A growing body of cultural information suggests, indeed, that this disease is etiologically associated with *A. actinomycetemcomitans* and *Capnocytophaga* species. Companion studies in our laboratory (P. A. Murray and R. J. Genco, J. Dent. Res., special issue 59A, abstr. no. 245, p. 329, 1980) and by others (J. L. Ebersole, O. E. Frey, M. A. Taubman, D. J. Smith, and R. J. Genco, J. Dent. Res., special issue 59A, abstr. no. 249, p. 330, 1980) have revealed that elevated serum IgG antibody levels to *A. actinomycetemcomitans* were common in patients with LJP but not in normal individuals or in patients with other periodontal diseases. A pattern of immunological reactions corresponding well to available bacteriological data on microorganisms etiologically associated with the adult form of periodontitis seems to exist. However, our observation that elevated levels of IgG to oral *B. asaccharolyticus* were significantly more common in GJP might indicate that the spreading of the disease to teeth other than the incisors and molars of the classical juvenile disease would be associated with a *B. asaccharolyticus* pathosis succeeding the initial *A. actinomycetemcomitans* infection. A similar trend was observed in patients with the prolonged type of the disease described as the post-LJP.

The adult periodontitis and the GJP groups had mean specific IgG antibody levels at least five times higher than that of the normal-adult group. However, the individual levels in half of these patients were low (Fig. 4). In our study population composed of patients with severe adult periodontitis, well-established clinical measurements did not allow us to distinguish patients with low levels of specific antibody from patients with high levels of specific antibody.

Our observation of distinct levels of humoral immune response to oral *B. asaccharolyticus* thus indicates that the levels of specific immunoglobulins may vary among patients with the same type of disease. Our finding of low levels of specific antibody in half of the adult periodontitis and GJP patients may be explained in at least three ways: (i) bacteria serologically distinct from oral *B. asaccharolyticus,* such as *B. melaninogenicus* subsp. *intermedius* or members of the genera *Fusobacterium, Capnocytophaga, Selenomonas, Campylobacter, Eubacterium,* and *Propionibacterium,* may predominate in periodontal lesions in these patients, suggesting a periodontopathic potential for organisms other than *B. asaccharolyticus* (34); (ii) a fluctuation in the subgingival microbial composition may result in periods with low numbers of oral *B. asaccharolyticus* and a corresponding low level of specific antibody; and (iii) immunosuppression by infecting microorganisms (12, 23) may induce immunological unresponsiveness to oral *B. asaccharolyticus* in patients infected with this organism. Future studies may help answer this question.

A protective role of antibodies produced against oral *B. asaccharolyticus* may be expected in periodontally healthy individuals via extravasation of these molecules into the gingival fluid, where they provide a local defense mechanism against pathogenic organisms. Antibody-mediated immunopathological mechanisms may also result if these antibodies react with antigens of oral *B. asaccharolyticus* which gain access to periodontal tissues. The observation of an increased immune response to oral *B. asaccharolyticus* in adult periodontitis and GJP contributes additional evidence of the specific role of this organism in the etiology of periodontal disease.

ACKNOWLEDGMENTS

We thank B. H. Park for providing sera, B. C. Nair and K. Okuda for immunochemical characterization of the antigen preparation, and Carole A. Sadowski for assistance.

This work was supported by Public Health Service grants DE 07034 and DE 04898 from the National Institute of Dental Research.

LITERATURE CITED

1. **Bailit, H. L., D. C. Baldwin, and E. E. Hunt, Jr.** 1964. The increasing prevalence of gingival *Bacteroides melaninogenicus* with age in children. Arch. Oral Biol. **9**: 435–438.
2. **Burdon, K. L.** 1928. *Bacterium melaninogenicum* from normal and pathologic tissues. J. Infect. Dis. **42**:161–171.
3. **Clem, T. R., and R. H. Yolken.** 1978. Practical colorimeter for direct measurement of microplates in enzyme immunoassay systems. J. Clin. Microbiol. **7**:55–58.
4. **Courant, P. R., and R. J. Gibbons.** 1967. Biochemical and immunological heterogeneity of *Bacteroides melaninogenicus*. Arch. Oral Biol. **12**:1605–1613.
5. **Coykendall, A. L., F. S. Kaczmarek, and J. Slots.** 1980. Genetic heterogeneity in *Bacteroides assaccharolyticus* (Holdeman and Moore 1970) Finegold and Barnes 1977 (Approved Lists, 1980) and proposal of *Bacteroides gingivalis* sp. nov. and *Bacteroides macacae* (Slots and Genco) comb. nov. Int. J. Syst. Bacteriol. **30**:559–564.
6. **De Araujo, W. C., and J. B. MacDonald.** 1964. The gingival crevice microbiota in five preschool children. Arch. Oral Biol. **9**:227–228.
7. **DuBois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorimetric method for determination of sugar and related substances. Anal. Chem. **28**: 350–356.
8. **Duermeyer, W., and J. van der Veen.** 1978. Specific detection of IgM-antibodies by ELISA, applied in hepatitis-A. Lancet **ii**:684–685.
9. **Engvall, E., and H. E. Carlsson.** 1976. Enzyme-linked immunosorbent assay, ELISA, p. 135–147. *In* G. Feldman, et al. (ed.), First International Symposium on Immunoenzymatic Techniques, INSERM Symposium no. 2. North Holland Publishing Co., Amsterdam.
10. **Engvall, E., and P. Perlmann.** 1972. Enzyme-linked immunosorbent assay, ELISA. III. Quantitation of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen-coated tubes. J. Immunol. **109**:129–135.
11. **Fahey, J. L.** 1965. Antibodies and immunoglobulins. II. Normal development and changes in disease. J. Am. Med. Assoc. **194**:141–144.
12. **Floersheim, G. L.** 1979. Immunosuppressive effects of miscellaneous agents. *In* J. L. Turk and D. Parker (ed.), Drugs and immune responsiveness. University Park Press, Baltimore.
13. **Gibbons, R. J., S. S. Socransky, S. Sawyer, B. Kapsimalis, and J. B. MacDonald.** 1963. The microbiota of the gingival crevice area of man. II. The predominant cultivable organisms. Arch. Oral Biol. **8**:281–289.
14. **Good, R. A., and B. W. Papermaster.** 1964. Ontogeny and phylogeny of adaptive immunity. Adv. Immunol. **4**: 1–115.
15. **Hewitt, W.** 1977. Microbiological assay. An introduction to quantitative principles and evaluation. Academic Press, Inc., New York.
16. **Hofstad, T.** 1974. Antibodies reacting with lipopolysaccharides from *Bacteroides melaninogenicus, Bacteroides fragilis,* and *Fusobacterium nucleatum* in serum from normal human subjects. J. Infect. Dis. **129**:349–352.
17. **Horowitz, S. A., and G. H. Cassell.** 1978. Detection of antibodies to *Mycoplasma pulmonis* by an enzyme-linked immunosorbent assay. Infect. Immun. **22**:161–170.
18. **Leinikki, P., and S. Passila.** 1976. Solid phase antibody assay by means of enzyme conjugated to anti-immunoglobulin. J. Clin. Pathol. **29**:1116–1120.
19. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
20. **Mashimo, P. A., and S. A. Ellison.** 1972. Diffusate media for cultivation of oral anaerobic bacteria. J. Dent. Health **22**:38–45.
21. **Michael, J. G.** 1969. Natural antibodies. Curr. Top. Microbiol. Immunol. **48**:43–62.
22. **Møller, A. M., and L. R. Mathiesen.** 1979. Detection of immunoglobulin M antibodies to hepatitis A virus by enzyme-linked immunosorbent assay. J. Clin. Microbiol. **10**:628–632.
23. **Moller, G., O. Sjoberg, and J. Andersson.** 1973. Immunogenicity, tolerogenicity, and mitogenicity of lipopolysaccharides. *In* E. H. Kass and S. M. Wolff (ed.), Bacterial lipopolysaccharides. The University of Chicago Press, Chicago.

24. **Mouton, G., P. G. Hammond, J. Slots, and R. J. Genco.** 1980. Evaluation of Fluoretec-M for detection of oral strains of *Bacteroides asaccharolyticus* and *Bacteroides melaninogenicus*. J. Clin. Microbiol. **11**: 682-686.
25. **Newman, M. G., and S. S. Socransky.** 1977. Predominant cultivable microbiota in periodontosis. J. Periodontal Res. **12**:120-128.
26. **Newman, M. G., S. S. Socransky, E. D. Savitt, D. A. Propas, and A. Crawford.** 1976. Studies on the microbiology of periodontosis. J. Periodontol. **47**:373-379.
27. **Reed, M. J., J. Slots, C. Mouton, and R. J. Genco.** 1980. Antigenic studies of oral and nonoral black-pigmented *Bacteroides* strains. Infect. Immun. **29**:564-574.
28. **Ruitenberg, E. J., P. A. Steerenberg, B. J. M. Brosi, and J. Buys.** 1976. Reliability of the enzyme-linked immunosorbent assay (ELISA) for the serodiagnosis of *Trichinella spiralis* infections in conventionally raised pigs. J. Immunol. Methods **10**:67-83.
29. **Shah, H. N., R. A. D. Williams, G. H. Bowden, and J. M. Hardie.** 1976. Comparison of the biochemical properties of *Bacteroides melaninogenicus* from human dental plaque and other sites. J. Appl. Bacteriol. **41**: 473-492.
30. **Slots, J.** 1976. The predominant cultivable organisms in juvenile periodontitis. Scand. J. Dent. Res. **84**:1-10.
31. **Slots, J.** 1977. The predominant cultivable microflora of advanced periodontitis. Scand. J. Dent. Res. **85**:114-121.
32. **Slots, J.** 1977. Microflora in the healthy gingival sulcus in man. Scand. J. Dent. Res. **85**:247-254.
33. **Slots, J., and R. J. Gibbons.** 1978. Attachment of *Bacteroides melaninogenicus* subsp. *asaccharolyticus* to oral surfaces and its possible role in colonization of the mouth and of periodontal pockets. Infect. Immun. **19**: 254-264.
34. **Slots, J., and E. Hausmann.** 1979. Longitudinal study of experimentally induced periodontal disease in *Macaca arctoides*: relationship between microflora and alveolar bone loss. Infect. Immun. **23**:260-269.
35. **Socransky, S. S.** 1977. Microbiology and periodontal disease—present status and future considerations. J. Periodontol. **48**:497-504.
36. **Socransky, S. S., R. J. Gibbons, A. C. Dale, L. Bortnick, E. Rosenthal, and J. B. MacDonald.** 1963. The microbiota of the gingival crevice area of man. I. Total microscopic and viable counts and counts of specific organisms. Arch. Oral Biol. **8**:275-280.
37. **Sonnenwirth, A. C.** 1979. Antibody response to anaerobic bacteria. Rev. Infect. Dis. **1**:337-341.
38. **Spiegel, C. A., S. E. Hayduk, G. E. Minah, and G. N. Krywolap.** 1979. Black-pigmented *Bacteroides* from clinically characterized periodontal sites. J. Periodontal Res. **14**:376-382.
39. **Tanner, A. C. R., C. Haffer, G. T. Bratthall, R. A. Visconti, and S. S. Socransky.** 1979. A study of the bacteria associated with advancing periodontitis in man. J. Clin. Periodontol. **6**:278-307.
40. **Voller, A., D. Bidwell, G. Huldt, and E. Engvall.** 1974. A microplate method of enzyme-linked immunosorbent assay and its application to malaria. Bull. W.H.O. **51**: 209-211.
41. **Zar, J. H.** 1974. Biostatistical analysis. Prentice-Hall, Inc., Englewood Cliffs, N.J.