Opsonic Antibody Activity against *Actinobacillus actinomycetemcomitans* in Patients with Rapidly Progressive Periodontitis

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*Actinobacillus actinomycetemcomitans* has been closely associated with early-onset, severe periodontitis, and such patients often have serum immunoglobulin G (IgG) antibodies reactive with antigens of this gram-negative pathogen. We examined the functionality and potential importance of these antibodies. The opsonic activity against *A. actinomycetemcomitans* of sera from 30 patients with rapidly progressive periodontitis (RPP) and from 28 periodontally normal subjects was tested by using polymorphonuclear leukocyte (PMN) chemiluminescence and bactericidal assays. Peak chemiluminescence values correlated strongly with killing observed in the PMN-dependent bactericidal assay (r = 0.88; P < 0.001). Neither the mean IgG titer nor the mean peak chemiluminescence differed significantly between the two groups. However, when the relationship between chemiluminescence and titer was examined, regression analysis showed that antibodies present in low-titer normal sera were significantly more effective at opsonizing *A. actinomycetemcomitans* than antibodies present in low-titer RPP patient sera (P = 0.04). Thus, periodontally normal individuals may be better able than RPP patients to clear *A. actinomycetemcomitans* in early stages of colonization, and anti-*A. actinomycetemcomitans* antibodies in RPP patients may be relatively ineffective in preventing infection by this organism.

*Actinobacillus actinomycetemcomitans* is a facultative gram-negative cocobacillus which has been implicated in the etiology of early-onset, severe forms of periodontitis (27). Many studies have documented the presence of elevated titers of antibody to *A. actinomycetemcomitans* in some patients with early-onset periodontitis (5, 7, 17, 22, 26) and the frequent presence of the organism in the subgingival microflora of such patients (5, 19). *A. actinomycetemcomitans* is resistant to complement-mediated killing either in normal serum or in the presence of immune immunoglobulin G (IgG) (25). However, normal polymorphonuclear leukocytes (PMN) are able to kill *A. actinomycetemcomitans* (13). This killing by normal PMN is enhanced after opsonization of *A. actinomycetemcomitans* by sera from some patients with localized juvenile periodontitis (LJP) (1), one form of early-onset disease which affects predominantly first molars and central incisors. Rapidly progressive periodontitis (RPP) is an aggressive form of early-onset periodontitis affecting many teeth of the permanent dentition (14). Some RPP patients have high titers of serum IgG antibody to the antigens of *A. actinomycetemcomitans*, while others have minimal antibody response. Elevated titers of antibodies to antigens of *A. actinomycetemcomitans* are also seen in some periodontally normal individuals. Whether these antibodies are protective, destructive, or irrelevant to the progress of disease remains unknown. In this study we sought to determine if functional differences existed between anti-*A. actinomycetemcomitans* antibodies present in RPP patient sera and normal sera at either high or low titers.

We used opsonization of *A. actinomycetemcomitans* for phagocytosis and killing by human PMN from periodontally normal donors as a measure of antibody function in sera from 30 RPP patients and 28 periodontally normal individuals. The degree of opsonization was assessed by two different procedures. First, we measured the chemiluminescent (CL) response of PMN during phagocytosis (20) of *A. actinomycetemcomitans*. Sera from the RPP patients and normal controls were used with and without complement to opsonize the bacteria. Second, a subset of 21 serum samples (16 RPP and 5 normal, selected to represent the range of peak CL values) was tested in a PMN-mediated bacterial killing assay under conditions similar to those for the CL assay. The two measures of opsonization we employed had a high positive correlation coefficient (r = 0.88; P < 0.001), indicating that the CL assay is a valid and rapid measurement of antibody function in our system. We found differences in the relationship of titer to opsonic ability between RPP patient sera and normal control sera which indicate a less functional antibody response to *A. actinomycetemcomitans* in some RPP patients. The lower opsonic function of some RPP sera may be especially relevant during the early stage of the disease.

**MATERIALS AND METHODS**

**Subject sera.** Informed consent was obtained from all study participants, following a protocol approved by the University of Washington Human Subjects Division of the Office of Research. Venous blood was collected from 30 patients diagnosed as having RPP according to published criteria (3, 14). There were 17 male and 13 female patients, with a mean age of 32.97 ± 4.77 years (range, 25 to 40 years). One patient was Asian, one was African-American, and the remainder were Caucasian. The patients had radiographic evidence of bone loss (16) prior to age 35; they had 20 or more teeth with at least 7 teeth other than first molars and central incisors having a minimum of 5 mm of attachment.

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loss and 4 mm or greater probing depths. The mean interproximal pocket depth (IPPD) was 4.79 ± 0.93 mm (range, 3.6 to 8.3 mm). Bone loss was 26.48% ± 7.82% (range, 11.9 to 48.5%). The 28 control subjects had no evidence of periodontal disease on clinical exam, had a mean interproximal pocket depth of <3 mm, and were matched to patients by age, race, and gender. The two missing controls were Caucasian, a 31-year-old male and a 25-year-old female. Sera from four additional periodontally normal individuals were collected for use as normal pooled serum. Sera obtained after clotting and centrifugation were stored in aliquots at −30°C until used. Sera were heated at 56°C for 30 min to inactivate serum complement prior to CL or bacterial killing assays and diluted in gelatin–Veronal-buffered saline, pH 7.5, supplemented with 5% glucose (GGVB++) (6). Hyperimmune rabbit serum against *A. actinomycetemcomitans* Y4 was raised by immunizing New Zealand White rabbits with lyophilized bacteria four times at 2-week intervals. Five hundred micrograms (dry weight) of antigen in 1 ml of Dulbecco’s phosphate-buffered saline (PBS) with SAF-1 adjuvant (Syntex, Palo Alto, Calif.) was used per rabbit per immunization (8). Peripheral blood, collected 10 days after the final immunization by bleeding from an ear vein, was allowed to clot, and the serum was collected, sterilized by filtration, and stored at −30°C.

**Complement source.** A C5-depleted human serum preparation (Quidel Laboratories, San Diego, Calif.) was used as the complement source in these experiments. One hundred microliter aliquots of undiluted complement preparation were stored at −70°C until immediately before the assay, when the required amount was thawed, diluted in GGVB++, and supplemented to final concentrations of 2 mM Mg²⁺ and 5 mM Ca²⁺.

**Preparation and identification of bacteria.** *A. actinomycetemcomitans* ATCC 43718 (serotype b; strain Y4) was obtained from the American Type Culture Collection. Low-passage aliquots, frozen in horse serum, were thawed and plated on tryptic soy-serum-bacitracin-vancomycin (TSVB) selective medium (18). Colonies isolated from the TSVB medium fit the biochemical profile of *A. actinomycetemcomitans* (23), had the “star-positive” morphology (15), and were used within two or three passages for experiments. Clones were maintained on plates made from brain heart infusion (Difco, Detroit, Mich.) (40 g/liter) supplemented with 0.1% yeast extract (Difco) (BHY broth) and 1% agar (Difco) (BHY plates) until the day before an experiment. At that time, colonies were suspended in BHY broth to a Klett-Summern photoelectric colorimeter (Klett Manufacturing Co., Inc., New York, N.Y.) reading of 80, using a red filter (approximately 10² CFU/ml). This bacterial suspension was inoculated at 1:1,000 into BHY broth and grown in a reduced O₂ atmosphere (candle jar) for 8 to 10 h. Late-log-phase bacteria were centrifuged at 10,000 × g for 20 min, washed once with GGVB++, and suspended in the same buffer to a volume equivalent to 1/10 of the culture volume. The suspension contained 3 × 10⁷ to 1 × 10⁸ CFU of viable *A. actinomycetemcomitans* per ml and was used at this concentration in CL assays. A 1:20 dilution of this suspension, which had a Klett reading of 60 U and contained about 3 × 10⁶ CFU/ml, was used in bacterial killing assays. The presence of *A. actinomycetemcomitans* in plaque samples was determined by culture (18) and by biochemical tests (23).

**Isolation and viability of PMN.** Thirty milliliters of venous blood, obtained from one of two healthy periodontally normal adult male donors on the day of assay, was drawn into a Vacutainer tube containing 200 U of heparin per ml, layered on Mono-Poly Resolving Medium (MPRM; Flow Laboratories) at a ratio of 5 ml of blood per 4 ml of MPRM, and centrifuged at 300 × g for 45 min. The PMN band was removed, taking care to avoid erythrocyte contamination, washed with PBS, and suspended in 10 ml of phenol-red-free RPMI 1640 medium ( Gibco, Grand Island, N.Y.), which was supplemented with 5% gelatin. PMN isolated by this method had purity and viability (assessed by trypan blue exclusion) of greater than 98%. To assess viability over time, PMN were held at room temperature in RPMI 1640 for up to 4 h and in GGVB++ at 37°C for 3 h. Aliquots were periodically withdrawn, and viability was assessed by trypan blue exclusion. PMN were also incubated with *A. actinomycetemcomitans* at ratios of 1:1 and 1:10 (PMN to bacteria), and viability was measured at the end of 2 h at 37°C. PMN maintained >95% viability under all conditions tested.

**CL assay.** The PMN CL assay was a modification of the luminol-dependent assay reported by Eastmond et al. (4). A 0.02 M stock solution of 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol; Sigma Chemical Co., St. Louis, Mo.) was made in dimethyl sulfoxide (J. T. Baker, Phillipsburg, N.J.) and stored at 4°C. A working dilution of 1:100 in GGVB++ was made on the day of the assay. Measurements were taken on a Bioscan 1251 chemiluminometer (Phar- macia/LKB Nuclear, Gaithersburg, Md.), with output read as millivolts. Each open polystyrene luminometer tube (Pharma- cia/LKB Nuclear) contained one or more of the following constituents added in the order listed: 100 μl of serum diluted in GGVB++ (for most assays 1:5 or 1:10), 100 μl of *A. actinomycetemcomitans* suspension containing approximately 3 × 10⁷ bacteria per ml, 100 μl of complement (diluted 1:50 in GGVB++, 600 μl of working luminol solution, and finally, 100 μl of PMN diluted in GGVB++ to a concentration of 3 × 10⁶ cells per ml. Equivalent amounts of GGVB++ were substituted for any omitted constituents in order to bring the total volume in each tube to 1 ml. With each assay, a tube containing hyperimmune rabbit serum (final dilution, 1:1,000) served as the positive experimental control. Additional assay controls included PMN alone, PMN with bacteria, and PMN with bacteria and complement. Up to 25 tubes were assayed for 90 to 120 min in each experiment. All assays were run at 37°C with constant mixing. Mean peak CL for each serum was calculated from three separate experiments.

**Bacterial killing assay.** Sera were chosen to represent the range of peak CL values. Components described above for the CL assay were added in the same order to 1.5-ml polypropylene microcentrifuge tubes. However, the concentration of *A. actinomycetemcomitans* was 3 × 10⁶ CFU/ml to yield the recommended ratio of 1:1 (bacteria to PMN) (11) and GGVB++ was substituted for the working luminol solution. Sample tubes were incubated at 37°C in a sample holder which rotated end over end at 10 rpm. Aliquots were withdrawn at 60 and/or 120 min and placed on ice to halt phagocytosis. Duplicate samples were serially diluted in GGVB++, spread on BHY agar plates, and incubated for 48 h at 37°C. Percent killing was calculated as [CFU/ml (T₀) − CFU/ml (T₅₀) or T₁₀₀]/CFU/ml (T₀) × 100 to indicate the reduction in bacterial population over time. Mean killing with each serum was calculated from results of three experiments. Experimental controls as described above were included in each assay.

**Antibody titer.** The titers of serum IgG antibody against *A. actinomycetemcomitans* for the RPP patients and normal subjects were measured in an enzyme-linked immunosorb-
bent assay (ELISA) as previously described (24). A whole-cell homogenate of A. actinomycetemcomitans (1 μg [dry weight] per well) was used as the antigen. A human serum with known high titer against A. actinomycetemcomitans was used as a control. Alkaline phosphatase-conjugated goat anti-human IgG (A-5403; Sigma) was used to quantitate IgG. ELISA units were calculated by the method of Butler (2).

Statistical calculations. The t test was used to compare the RPP patient and normal peak CL and titer values. Peak CL and titer values were analyzed on the log_{10} scale. Mean values were converted back to the original scale. Pearson's correlation coefficient was used to assess the relationship between killing and peak CL. Least squares regression analysis was used to assess the relationships between mean peak CL, titer, and disease states. Regression lines were plotted from these analyses. All tests were two-sided.

RESULTS

Titers of serum IgG antibody to antigens of A. actinomycetemcomitans. There was no statistical difference between the mean IgG titer of the RPP patient sera (29.92 ± 2.51 ELISA units [EU]) and the mean titer of the normal sera (20.31 ± 4.70 EU; P = 0.25). The range and distribution of titer values in the two groups were similar (Fig. 1).

Presence of A. actinomycetemcomitans in RPP patients. A. actinomycetemcomitans was detected in the subgingival plaque of 40% (12 of 30) of the RPP patients at the time of their initial examination. There was no correlation between the presence or absence of detectable A. actinomycetemcomitans and either serum IgG antibody titers or CL values.

Optimization of the CL assay. We performed preliminary experiments to determine the optimum assay parameters for our system, including bacterial and PMN cell density, serum dilutions, and complement dilutions (data not shown). The sensitivity of the CL assay was substantially enhanced by the inclusion of complement. When complement at a dilution of 1:500 was included with serum in the assay, the CL values produced were greatly increased over those observed with serum alone. The peak CL value for a high-titer RPP serum which elicited a high CL response was decreased by 80% when complement was inactivated (Fig. 2A). Hyperimmune rabbit serum, the assay positive control, was also dependent upon complement for enhancement of CL (Fig. 2B). A twofold dilution of complement in the presence of rabbit serum decreased the peak CL value by 50%, while inactivation of complement reduced CL by 90%. Peak CL values produced by PMN alone and by PMN plus serum and/or complement but without A. actinomycetemcomitans were the same as baseline measurements (GGVB^{*+}, serum, and complement). CL values generated by PMN incubated with bacteria and complement alone were close to the baseline (data not shown). Preimmune rabbit serum did not enhance baseline CL (data not shown).

Effect of serum dilution on CL. Peak CL decreased when sera were diluted (Fig. 3). CL values were halved when high-CL sera were diluted twofold from 1:50 to 1:100 and reduced approximately another 10-fold when sera were diluted 1:1,000. Low-CL sera showed little decrease in CL values when diluted more than 1:100. Hyperimmune rabbit serum showed a constant threefold decrease in peak CL for each 10-fold dilution from 1:1,000 to 1:100,000 (data not shown).
Opsonic ability of serum antibodies against *A. actinomyctemcomitans* as measured by PMN CL. Samples containing serum generally had higher peak CL values than the controls without serum (PMN plus bacteria or PMN plus bacteria plus complement), indicating that antibody-dependent phagocytosis was occurring. Opsonic function, as determined by peak CL, was consistent for most of the sera, with values varying less than ±10% from experiment to experiment. The distributions of CL values of the two groups appeared to differ in range and variability (Fig. 4). Ninety percent of the normal sera had CL values greater than the RPP serum median, and several of the RPP sera produced values lower than complement controls. Although the mean peak CL value for the RPP group (68.68 ± 3.15 mV) did not differ significantly from the normal mean (109.38 ± 1.98 mV) ($P = 0.068$), there was a strong trend toward a greater functional response in the normal group.

Comparison of PMN-mediated bacterial killing and CL assays. Since preliminary experiments indicated that a 2-h incubation period could distinguish between PMN-mediated killing seen with hyperimmune rabbit serum and that seen with normal pooled serum, we tested 21 serum samples (16 RPP and 5 normal, selected to represent the overall range of CL responses) for their ability to enhance PMN killing over a 2-h period. Representative samples were monitored at 60 and 120 min (Fig. 5). In order to determine if there was a correlation between the CL response and the bactericidal assay, we measured bacterial killing at 2 h for the entire subset and compared the results with CL values for the same sera. Those sera which were able to opsonize *A. actinomycetemcomitans* for efficient killing by PMN were also those which stimulated high CL values (Fig. 6). The peak CL value evoked by a serum was an excellent predictor of the PMN killing seen in the presence of the same serum, with a correlation of 0.88 ($P < 0.001$).

**FIG. 3.** Effect of serum dilution on peak CL response for selected sera. For high-CL sera (peak CL > 100 at a 1:50 dilution), a 10-fold dilution resulted in approximately a 10-fold loss of peak CL response. CL values for low-CL sera (peak CL < 100 at a 1:50 dilution) were less affected by dilution. Broken lines represent RPP sera, and solid lines represent normal sera.

**FIG. 4.** Distribution by percentiles of peak CL values for RPP patient sera and normal sera. RPP sera: median = 55.8 mV; range = 9.3 to 677.0 mV; mean = 68.68 ± 3.15 mV. Normal sera: median = 89.0 mV; range = 27.0 to 848.5 mV; mean = 109.38 ± 1.98 mV. The peak CL means for the two groups are not statistically different ($r$ test = 1.86, $df = 56; P = 0.68$).

**FIG. 5.** Killing of *A. actinomycetemcomitans* by PMN in the presence of selected RPP patient sera (broken lines) and normal sera (solid lines) at 60 and 120 min. Serum dilution = 1:50; complement dilution = 1:500. Bacterium/PMN ratio = 1:1. Sera with higher CL values (included in parentheses) showed greater killing. Control (*) = serum plus complement without PMN.
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FIG. 6. Comparison of peak CL values with percent killing by PMN after 2 h of incubation for 21 serum samples (16 RPP, 5 normal). Sera were selected to represent the range of CL responses. Sera with high CL values were significantly better at enhancing PMN killing than were sera with low CL values (Pearson's correlation coefficient \( r = 0.88; P < 0.001\)). The least squares regression line is displayed.

0.04) (Fig. 7). The opsonic capacity of RPP patient sera did not reach that of normal sera until the titers of RPP serum IgG against A. actinomycetemcomitans were threefold greater than the normal median.

DISCUSSION

A. actinomycetemcomitans is believed to be a pathogen in patients with severe forms of periodontitis. A. actinomycetemcomitans has been isolated from the supragingival microflora of many of these patients, and many have elevated titers of serum antibody to antigens of A. actinomycetemcomitans (14, 22, 26, 28). Little is known, however, about the role of these antibodies in the pathogenesis of periodontal disease. Phagocytic cells, particularly PMN, are thought to play an important role in the prevention of bacterial infection. Antibody and complement can amplify the efficiency of PMN-mediated killing by opsonizing the invading organism. However, antibodies which are not protective may contribute to the disease process by exacerbating inflammation. We investigated the role of antibodies made against A. actinomycetemcomitans in RPP by testing sera from 30 RPP patients and 28 periodontally normal subjects for the ability to opsonize A. actinomycetemcomitans in the presence of complement for killing by normal human PMN. We correlated opsonic function with serum IgG titer to see if there was a difference in antibody function against A. actinomycetemcomitans between normal subjects and RPP patients.

To compare this large number of sera for opsonic ability, we used the generation of luminol-enhanced CL by PMN during phagocytosis and killing as a rapid means of assessment. The use of CL to measure opsonization and phagocytic function has been widely accepted (see reference 9 for a review), but there were no previous reports of the use of the assay in our system. We verified that PMN CL correlates strongly with bactericidal activity of PMN against A. actinomycetemcomitans. These results are consistent with previous studies in other systems (4, 9, 20). No bacterial killing was seen in the presence of serum and/or complement without PMN, supporting previous reports (1, 25) that sera from some LJP patients had opsonic but not bactericidal antibodies against A. actinomycetemcomitans.

In our study, there was no significant difference in the mean titers of IgG antibody to A. actinomycetemcomitans between RPP patient sera and normal sera, although other studies have found significantly elevated titers of serum antibody to A. actinomycetemcomitans in RPP patients compared with those in normal controls (26). It is unlikely that the lack of a significantly higher antibody response in our RPP patient group was due to lack of exposure to A. actinomycetemcomitans, as plaque samples from nearly half of the RPP patients were positive for A. actinomycetemcomitans and the presence of detectable A. actinomycetemcomitans was unrelated to antibody titer. Also, several of our periodontally normal subjects had high titers of antibody to A. actinomycetemcomitans, which raised the normal mean. That some normal subjects have antibodies reactive with A. actinomycetemcomitans may be a consequence of subclinical or transient infection with A. actinomycetemcomitans or with bacteria which are closely related antigenically.

Since PMN function in some patients with early-onset periodontitis appears to be compromised (10, 21), neutrophils from periodontally normal individuals were used in order to concentrate exclusively upon antibody function. A. actinomycetemcomitans serotype b (strain Y4) was the target organism in this study because of the association of
serotype b with human periodontal diseases (27). PMN viability was unaffected by the presence of this nominally leukotoxic strain of *A. actinomycetemcomitans* under our experimental conditions. The most star-positive morphology (15) was maintained in our *A. actinomycetemcomitans* cultures, and the bacterial preparations had consistently strong reactions with the hyperimmune rabbit serum (positive assay control). Therefore, we feel that the bacterial cultures were antigenically relevant to the periodontally pathogenic Y4 strain.

The ability of both RPP sera and normal sera to enhance PMN CL and killing of *A. actinomycetemcomitans* was dependent upon the presence of complement. Complement was an especially effective opsonin in combination with high-CL sera, as the removal of the complement source greatly diminished the CL response. Sera which elicited low CL responses were less affected by removal of complement. We used a complement preparation which had been depleted of the C5 component in order to eliminate a possible source of nonspecific CL: a CL response can be elicited from PMN in the absence of phagocytosis by the anaphylatoxin C5a (which is released after the enzymatic cleavage of C5) (12). The relatively low levels of CL and killing in the presence of PMN and complement without serum indicates that the alternate pathway was not playing a significant role in our assay system.

The amount of anti-*A. actinomycetemcomitans* antibody present in a serum was directly related to the ability of that serum to enhance phagocytosis. There was greater variability in the relationship between titer and function in the RPP patient group than in the normal control group, most likely a reflection of heterogeneity in disease status among the patients. For both RPP and normal sera, dilution of the serum resulted in a reduction of PMN CL. These results agree with those of Baker and Wilson (1), who found a correlation between titers of serum IgG to *A. actinomycetemcomitans* and opsonic ability in four LJP patients. Work done in our laboratory to investigate the opsonic ability of LJP sera against *A. actinomycetemcomitans* also showed a statistically significant positive correlation between antibody titer and CL values (unpublished data). Kalmar et al. (10) found no difference in opsonic ability between normal serum and LJP serum, but antibody titers were not reported.

Although both RPP patient sera and normal sera were able to opsonize *A. actinomycetemcomitans* in our in vitro system, there was not a high functional response in RPP sera. Analysis showed a strong trend toward higher opsonic function, as measured by the CL assay, in normal sera compared with RPP patient sera. However, the greatest difference in opsonic function between the RPP patient serum group and the normal serum group occurred when the titer of antibody to *A. actinomycetemcomitans* was low. The functional response of RPP patient sera became progressively poorer than the normal response as titers of IgG to *A. actinomycetemcomitans* dropped. Low-titer normal sera were significantly more effective at promoting phagocytosis and killing of *A. actinomycetemcomitans* than were low-titer RPP patient sera. While the molecular basis for these observations is presently unknown, we believe that the biological implications are important. RPP patients may be less able to clear *A. actinomycetemcomitans* in the early stages of colonization and thereby are at greater risk for periodontal infection. In addition, the presence of nonprotective antibodies may contribute to the gingival inflammation found in RPP patients. Even antibodies which are nominally opsonic may not be protective once disease is established, as *A. actinomycetemcomitans* was detected in plaque samples from some RPP patients with highly functional sera. The capacity to mount an early protective antibody response may be a major determinant in host resistance and susceptibility to periodontal disease. Antibody function in RPP patients combined with possibly compromised PMN function may result in clinical disease.

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