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Functional Properties of Nonhuman Primate Antibody to *Porphyromonas gingivalis*

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The nonhuman primate (NHP) serves as a useful model for examining the host-parasite interactions in *Porphyromonas gingivalis*-associated periodontal disease. This study determined the influence of NHP sera on (i) the direct killing of *P. gingivalis*, (ii) *P. gingivalis*-induced superoxide anion (O_2^-) release from human polymorphonuclear leukocytes (PMNs), and (iii) the ability of PMNs to bind and phagocytize *P. gingivalis*. Three types of NHP sera were utilized: (i) normal or baseline sera; (ii) sera obtained after ligature-induced periodontitis; and (iii) sera obtained following active immunization with formalinized *P. gingivalis*. All assays were performed with or without the addition of human complement. Significantly more ($P < 0.01$) direct killing of *P. gingivalis* occurred with immunized sera and complement than with any of the other treatments. The sera from ligature-induced periodontitis NHPs had significantly less ($P < 0.03$) killing capacity than the baseline sera, which contained natural antibody produced to *P. gingivalis* colonization. Sera from immunized NHPs were used to opsonize *P. gingivalis* and caused significantly greater ($P < 0.01$) levels of O_2^- release from PMNs. Finally, the sera from immunized NHPs significantly enhanced ($P < 0.009$) the uptake of *P. gingivalis* by PMNs, although binding of the bacteria to PMNs was similar among all three serum types. Active immunization of NHPs with *P. gingivalis* elicited a functional antibody that enhanced direct killing, positively influenced the activation of PMNs, and enhanced the ability of PMNs to phagocytize *P. gingivalis*. Moreover, antibody produced as a sequela of progressing periodontitis appeared to lack these functions. A wide variability in functional capacity of the sera from individual NHPs, which may contribute to an individual's susceptibility to *P. gingivalis*-induced disease, was noted. This variability suggested that results from functional tests of serum antibody may aid in predicting host susceptibility to disease and response to therapy.

The complexity of periodontal disease is compounded by the presence of multiple putative pathogens (47, 51) and an intricate balance of host immune responses. The host response is an extremely complex and multidimensional biologic system providing protection for the host. Various studies have revealed that periodontitis patients produce substantial levels of serum antibody, specifically immunoglobulin G (IgG) (13, 30), to putative periodontal pathogens such as *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*. These antibodies are specific for the microorganisms found in the periodontal pocket (10). The role of antibody in host defense of patients with periodontal disease has been speculated upon on the basis of the general properties of antibodies and the potential interactions with host cells (12). These include both protective and potentially destructive responses toward host tissues. Activation of complement by antigen-antibody complexes (7, 20, 39), neutralization of bacterial toxins or tissue-lytic enzymes, and the enhanced opsonization or lysis with complement (1) of plaque bacteria are believed to be protective. However, the in vitro bactericidal activity of serum antibodies in combination with complement appears to be limited against *P. gingivalis* (36). The phagocytosis of antigen-antibody complexes through the interaction with Fc receptors on neutrophils is considered a potentially protective mechanism; however, results of Cutler et al. (7) have suggested that antibodies in sera of periodontitis patients are not particularly effective in opsonizing *P. gingivalis*.

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P. gingivalis appears to be effective in neutralizing humoral elements of host defense, with trypsinlike proteases that degrade IgG, IgM, IgA, IgD, and IgE (16) and complement factors C3, C4, and B (42). There are two major bacterial killing mechanisms associated with neutrophils: oxidative and nonoxidative killing mechanisms. Nonoxidative killing can take place in the absence of oxygen and is mediated by a variety of factors, including lysosomal enzymes, peptides such as defensins, cationic proteins, defensins, and lactoferrin (8). Oxidative killing is dependent on the presence of dioxygen (O_2) and the formation of toxic oxygen metabolites such as H_2O_2, superoxide anion (O_2^-), and possibly hydroxyl radicals (29, 50). *P. gingivalis* appears to be well equipped to disarm the nonoxidative bactericidal mechanisms of the neutrophil by inactivating cathepsin G, elastase, bacterial permeability increasing factor, and defensins (34). *P. gingivalis* may directly inhibit the migration and microbicidal activity of neutrophils and monocytes (14, 24, 33). Not only is this species able to immobilize neutrophils and evade nonoxidative defenses, but it resists oxidative killing through the production of a superoxide dismutase that neutralizes oxygen radicals produced by those cells (2).

The nonhuman primate (NHP) appears to be the best model of human periodontitis available at this time because of its established similarities to the human clinically, microbiologically, and immunologically (37, 48). The humoral immune response has been investigated in this animal model, establishing relationships between specific bacteria, antibody, and clinical responses. Various studies have established an association between increased levels of black-pigmented bacterial species in the gingival crevice and increased levels of serum antibody to this group of bacteria (4, 22). McArthur et al. (28) established

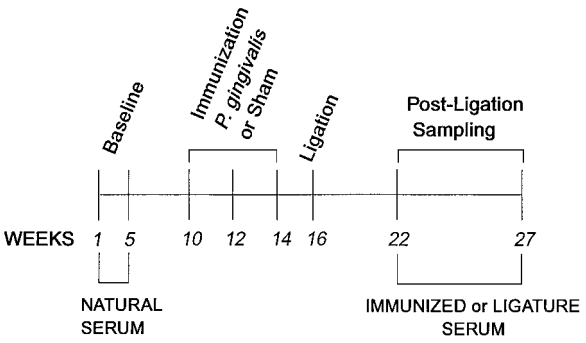

FIG. 1. Schematic of the experimental protocol, using *M. fascicularis* as an animal model of periodontitis. Serum was drawn for the natural group at 1 to 5 weeks, immunized group (10^9 *P. gingivalis* 3079.03) at 22 to 27 weeks, or ligature group at 22 to 27 weeks from sham-immunized animals. Ligature-induced periodontitis was initiated in the mandibular right quadrant at 16 weeks.

a negative correlation between serum anti-*P. gingivalis* IgG levels postimmunization (with *P. gingivalis*) and the black-pigmented bacterial levels in the gingival crevice of squirrel monkeys. A similar study immunized macaque monkeys with *Bacteroides macacae* and showed that immunization increased the serum anti-*B. macacae* IgG level, which correlated with a decreased number of *B. macacae* organisms in the periodontal pocket and decreased bone loss (32). Similar findings were recently reported in the examination of the contribution of *P. gingivalis* to periodontitis in *Macaca nemestrina* (40).

We have previously (15) immunized a group of female macaque monkeys with formalin-killed *P. gingivalis* and *P. intermedia*, resulting in a 2-log increase in IgG, IgM, and IgA levels specific for the homologous bacteria used for immunization. As demonstrated in the above studies, elevated antibodies following immunization appeared to significantly inhibit the emergence of homologous microorganisms in the gingival crevice. The objective of the current study was to characterize the functional activity of NHP antibodies to *P. gingivalis*. These antibodies were assessed for the ability (i) to have a direct killing effect on *P. gingivalis* when combined with complement, (ii) to generate superoxide anion release from human neutrophils in the presence of *P. gingivalis*, and (iii) to increase the ability of human neutrophils to ingest *P. gingivalis*.

MATERIALS AND METHODS

Experimental protocol. Eighteen adult female cynomolgus monkeys (NHP) of the species *Macaca fascicularis* were used. Baseline serum samples for determination of natural antibody levels were drawn at weeks 1 to 5 (Fig. 1). The animals were maintained untreated from weeks 5 to 10, during which time bacterial antigen was prepared and serum IgG antibody levels to *P. gingivalis* were measured (see below). These IgG antibodies were used to stratify the NHPs into two groups, with high, moderate, and low levels of antibody represented in each group. Six animals were immunized with 10^9 formalin-killed *P. gingivalis* 3079.03 organisms (22) emulsified with an equal volume of incomplete Freund's adjuvant (Difco) (9). Immunizations were administered in three separate injections at biweekly intervals, starting at week 10 and ending at week 14. At 16 weeks, a ligature was placed in all animals around three adjacent teeth in the mandibular left quadrant with 3-0 silk sutures (25). The ligature serum samples were collected at 22 to 27 weeks from NHPs that had been sham-immunized. Clinical findings of the sham-immunized, ligated group have been reported previously (9), and they demonstrated a change in plaque index (baseline, 1.7; 22 to 27 weeks postligation, 3.0), bleeding index (baseline, 1.8; 22 to 27 weeks postligation, 2.8), pocket depth (baseline, 2.1; 22 to 27 weeks postligation, 2.8), attachment level (baseline, 3.2; 22 to 27 weeks postligation, 4.1), and bone density measured by CADIA (baseline, 0; 22 to 27 weeks postligation, −4.6 U). Immunized serum samples used in this study were collected at 22 to 27 weeks from those NHPs receiving *P. gingivalis* and subsequently ligated (Fig. 1). All serum samples were heated at 56°C for 30 min to inactivate any residual complement in the NHP serum.

Microorganisms. *P. gingivalis* 3079.03 was grown in an anaerobic atmosphere of 80% N_2–10% CO_2–10% H_2. It was routinely maintained on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) with 5% sheep blood and supplemented with 5 μg of hemin and 1 μg of menadione per ml with reculturing performed at weekly intervals.

For antigen preparation and viability assessment, the organisms were grown in Mycoplasma Broth Base supplemented with hemin (5 μg/ml) and menadione (1 μg/ml) under anaerobic conditions at 37°C. The enriched mycoplasma broth base solution was inoculated with *P. gingivalis* 3079.03 and incubated for 48 h. During late log phase, the bacteria were quantified spectrophotometrically (at 660 nm), with absorbance approaching 1.0 ($\sim 4 \times 10^9$ bacteria per ml).

ELISA for IgG antibody. The level of antibody specific for *P. gingivalis* 3079 in the NHP sera was determined by an enzyme-linked immunosorbent assay (ELISA), using formalin-killed *P. gingivalis* 3079 cells (11). The absorbance values obtained were compared with a reference standard prepared by hyperimmunization of three NHPs with *P. gingivalis* (9). The serum titers were converted to ELISA units by linear regression analysis.

Complement fixation test. An ELISA was performed on potential sources of complement from humans (Complement Sera, 39 50% hemolytic complement units/ml [Sigma]; and two freshly drawn human serum samples from laboratory personnel) or rabbit complement (Pel Freez, Brown Deer, Wis.) according to a standardized ELISA protocol (9) to identify and minimize antibody levels to *P. gingivalis* in these preparations. The complement activity was quantitated by using a complement fixation test (3, 37). One human serum sample (designated DMA) contained the least amount of IgG specific to *P. gingivalis* and could effectively function as a source of complement at a 1:100 dilution, since this serum dilution had a killing activity against *P. gingivalis* similar to that of a bovine serum albumin control.

Direct killing assay. *P. gingivalis* was harvested in late log phase, placed into 1.5-ml plastic microcentrifuge tubes, and centrifuged (13,000 × *g*, 5 min). The supernatant was removed, and 200 μl of a nuclear stain, DAPI (4′,6-diamidino-2-phenylindole · 2HCl [Sigma]; 10 μg/ml in distilled H_2O), was added to approximately 5×10^{10} *P. gingivalis*; the combination was mixed by vortexing and incubated for 10 min at room temperature (7). The suspension was washed with barbital-buffered diluent (0.5 mM $MgCl_2$, 0.16 mM $CaCl_2$, 150 mM NaCl, 5 mM barbital sodium, 0.1% gelatin), resuspended in 1 ml of barbital-buffered diluent, and sonicated on ice at output 3, duty cycle 40%, continuously, for two 5-s periods (Sonifier, Cell Disruptor 200; Branson). The A_{660} of the suspension was again determined to estimate the number of bacteria present after staining with DAPI.

A direct killing assay was performed in a total volume of 1 ml. To accomplish this, 500 μl of DAPI-stained *P. gingivalis* (5×10^9) suspension was added to either 10 μl of the NHP sera, 10 μl of NHP sera plus 10 μl of human complement, or 10 μl of 5% bovine serum albumin (control), and the total volume was brought to 1 ml with barbital-buffered diluent. The suspension was sonicated for two 5-s periods, incubated on a tilting table at 37°C for 30 min, and sonicated again following this incubation for two 5-s periods. To stop the reaction, the sample was diluted 50% by combining 500 μl of chilled barbital-buffered diluent with 500 μl of test solution. Finally, 5 μl of propidium iodide (500 μg/ml in phosphate-buffered saline [PBS]) was added. The samples were stored in an ice bath until read.

Three slides for each sample were created by placing 3.5 μl of the suspension on a freshly cleaned glass slide and allowing the suspension to settle under a coverslip for 2 min. Ten consecutive fields for each slide were counted. The samples were read at ×1,000 on a Vanox T (Olympus) microscope equipped with fluorescence. To enumerate DAPI-stained (vital) cells, a U filter was used, and for propidium iodide-stained (nonvital) cells, a G filter was used. All samples were run against a control of 5% bovine serum albumin (Sigma).

Preparation of human neutrophils. Neutrophils were isolated from peripheral venous blood of healthy adult donors by dextran sedimentation and Ficoll-Paque density centrifugation, as previously described (33). The isolated neutrophils were suspended in PBS (135 mM NaCl, 5 mM KCl, 10 mM sodium phosphate buffer [pH 7.4]) at 5×10^7 cells ml^{-1} and kept on ice. All assays were performed in Kreb's ringer phosphate (PBS plus 1.0 mM $CaCl_2$ and 1.3 mM $MgSO_4$ [pH 7.4]) in the presence of 5 mM glucose.

Superoxide anion assay. Continuous measurements of superoxide anion (O_2^-) production were performed in a Perkin-Elmer Lambda 6 dual-beam spectrophotometer at 37°C, as previously described (5, 31). The sample cuvette contained ferricytochrome *c* (100 nmol), polymorphonuclear leukocytes (PMNs; 10^6), and *P. gingivalis* 3079 in a final volume of 1 ml of Krebs ringer phosphate with 5 mM glucose with (i) NHP serum (1:100 dilution), (ii) human serum containing complement (1:100 dilution) with no detectable antibody to *P. gingivalis*, (iii) NHP serum with the complement source, or (iv) buffer only. The reference cuvette contained superoxide dismutase (20 μg) in addition to the above. The ratio of PMNs to *P. gingivalis* was approximately 1:400. All assays were run in the presence of cytochalasin B (5 μg) to ensure maximal superoxide release, which was monitored continuously as the superoxide dismutase-inhibitable reduction of ferricytochrome *c* at 550 nm. The rate of O_2^- release was calculated by using the molar extinction coefficient for ferricytochrome *c* reduction of $2.11 \times 10^4\ M^{-1}\ cm^{-1}$ (29). All assays of O_2^- release were carried out on at least three different occasions, using different healthy blood donors.

TABLE 1. IgG anti-*P. gingivalis* antibody in NHP sera

Serum group	*n*	ELISA units				*P*
		Mean	Median	SD	Minimum/ maximum	
Natural	6	11.38	10.0	8.29	3.02/23.84	
Immunized	6	556.81	457.6	388.12	234/1306	<0.005
Ligature	6	22.83	15.0	2.62	3.38/60.9	

Attachment and ingestion assay. The attachment and ingestion assay quantitated the number of DAPI-stained *P. gingivalis* organisms that were either attached to or ingested by PMNs in the presence or absence of immune and nonimmune sera, with or without the addition of complement. PMNs (10^6) were incubated for 15 min in a 37°C water bath with *P. gingivalis* (10^8) and stained with DAPI as previously described for the assessment of direct killing, and the reaction was stopped at 4°C.

The following procedure was used to preserve the specimens for future analysis. Each plastic retainer in a Cytospin 2 centrifuge (Shandon) was prepared with 300 μl of PBS. Twenty microliters of chilled test solution (PMNs and *P. gingivalis*) was gently vortexed, added to a retainer, and spun at 850 rpm for 3 min. The slides with deposited cells were removed and allowed to air dry. Finally, after the slides were completely dry, 1 drop of cyanoacrylate adhesive (adhesive cyanoacrylate, MIL-A-46050C, type II, CL. 2 Three Bond of America) was placed over the concentrated area of cells and a coverslip was added. The adhesive was allowed to set completely at room temperature before specimens were stored at −20°C in the dark.

Analysis of each condition was performed with PMNs from three separate donors on three different days. Each of the 18 NHP serum samples was run once each day with and without human serum containing complement. The sequence of testing the NHP sera was systematically changed each day to control for any decrease in PMN activity over time. All slides were coded to blind the examiner during analysis. Twenty fields on each slide were evaluated, using a Vanox T microscope (Olympus) equipped with fluorescence excitation and emission filters. At a magnification of ×1,000, the following information was obtained from each field: (i) the number of PMNs present; (ii) the number of PMNs that had *P. gingivalis* either in contact or inside the periphery of the cell; (iii) the number of *P. gingivalis* bacteria in contact with the periphery of the PMN; and (iv) the number of *P. gingivalis* bacteria inside the periphery of the PMN. Treatment of the PMNs with *N*-ethylmaleimide (10 mM) was used to discriminate ingested from adherent bacteria (27). *N*-Ethylmaleimide physiologically shuts down the phagocytosing abilities of PMNs while leaving the normal number of surface receptors. Thus, the *P. gingivalis* organisms observed within the periphery of the PMNs in the presence of *N*-ethylmaleimide were treated as noningested and used to adjust the estimate of PMN-ingested *P. gingivalis*.

Statistical analyses. Statistical tests were carried out with a microcomputer program, CSS:Statistica (StatSoft), on an IBM personal computer. Differences between groups based on antibody type were evaluated by using a Mann-Whitney-Wilcoxon rank test for the direct killing, superoxide anion, and ingestion or attachment data. Antibody levels in ELISA units and superoxide release data were subjected to Spearman rank correlation analyses.

RESULTS

IgG anti-*P. gingivalis* in NHP sera. Natural serum samples (baseline) were drawn prior to any experimentation; ligature serum samples were drawn 6 to 10 weeks following ligation of three posterior teeth, inducing periodontitis. The immunized sera were collected after three immunizations of 10^9 formalin-killed *P. gingivalis* and ligation-induced periodontitis. IgG antibody levels were significantly greater in the immunized group than in the natural or ligature-treated group (Table 1). There were no differences in antibody levels between the NHP serum samples obtained at baseline (i.e., natural) and those obtained following shamimmunization and ligation (i.e., ligature).

Antibody- and complement-mediated killing of *P. gingivalis*. In initial experimental trials, *P. gingivalis* was opsonized with NHP serum for 30 mins; this was followed by a 30-minute incubation period with complement, which resulted in minimal killing activity above control values (Fig. 2). A second protocol which utilized a 30-min incubation period was then developed, with both the NHP serum and complement being added simultaneously at the beginning of the assay. This procedure resulted in direct killing of *P. gingivalis*, which was especially evident in sera from the immunized group (Fig. 2). Significantly more direct killing occurred with the sera from the immunized group compared with sera from either the natural or the ligature group; however, there was no significant difference between the activities of the serum samples from the natural and ligature groups in this assay system (Fig. 2).

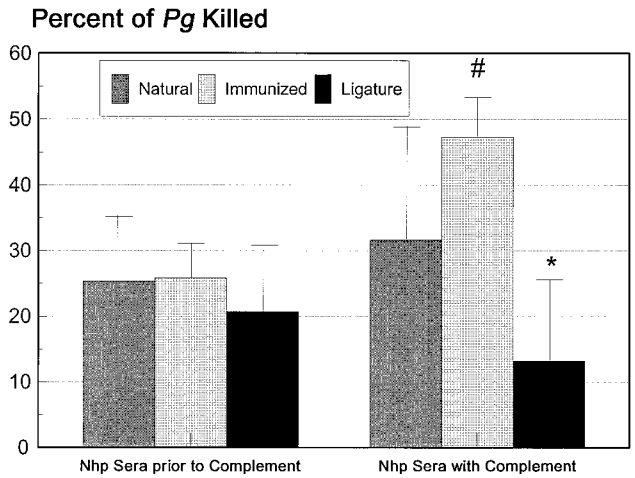

FIG. 2. Direct killing of *P. gingivalis* (Pg) by NHP sera plus complement, using two incubation protocols. Bars denote the mean level of killing from triplicate determinations of six serum samples in each group, and the brackets enclose 1 standard deviation. In all trials a 1:100 dilution of 5% bovine serum albumin was included as a control, with consistent killing of <10%. This percentage was subtracted from the values presented. Each assessment was based on the mean of triplicate determinations for each serum sample, and at least 200 bacteria were counted for each assessment. *, significantly less than the natural ($P < 0.008$) group; #, significantly greater than the natural ($P < 0.04$) and ligature ($P < 0.005$) groups.

NHP antibody and complement effects on PMN superoxide production. Superoxide production by PMNs in response to *P. gingivalis* was followed by a change in absorbance of cytochrome *c* at 550 nm. *P. gingivalis* opsonized with NHP serum from the immunized group, in the presence or absence of complement, routinely demonstrated the greatest change in absorbance, suggesting that this combination caused the most profound activation of the neutrophil microbicidal respiratory burst (Fig. 3). Sera from the natural and ligature groups, with or without complement, routinely demonstrated less change in absorbance than the immune sera. In the absence of complement, no statistical differences in the activation of the respiratory burst with sera from the different groups were noted (Fig. 3). However, in the presence of complement, significantly more superoxide was produced by the immune sera than by sera from the natural group ($P < 0.008$), the ligature group ($P < 0.04$), or the controls ($P < 0.02$).

NHP antibody effects on *P. gingivalis* attachment to and ingestion by PMNs. PMNs were incubated with *P. gingivalis* and labeled with the fluorescent dye DAPI for 15 min in combination with NHP serum alone or NHP serum plus complement. An analysis of the total number of opsonized *P. gingivalis* organisms associated with each PMN showed no significant differences among the three groups of sera in the presence or absence of added complement. However, it was clear from the data that the immunized sera had increased the numbers of *P. gingivalis* associated with PMNs, and when this increased association was broken down into *P. gingivalis* attached versus organisms that had been ingested (Fig. 4), it became clear that there was a significant increase in the numbers of ingested *P. gingivalis* when the bacteria had been opsonized either with immunized serum alone or immunized

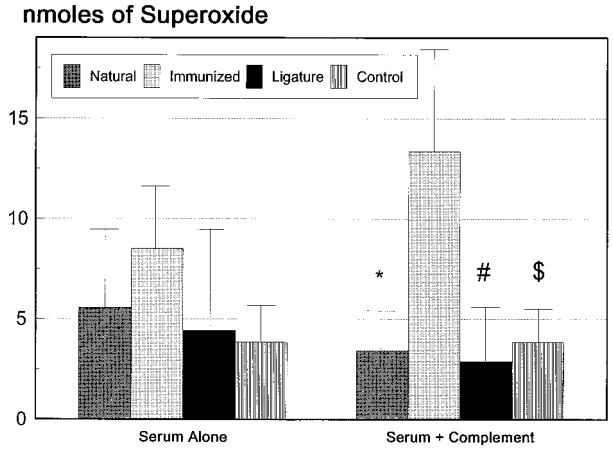

FIG. 3. Neutrophil superoxide assay. Bars denote the mean superoxide released from six serum samples alone (left) or serum samples with the addition of complement (right) in each group, and assays were run in triplicate. The brackets denote 1 standard deviation. A significantly increased release of superoxide by immunized versus natural sera (*, $P < 0.008$), ligature sera (#, $P < 0.04$), and controls ($, $P < 0.02$) was noted.

serum plus additional complement ($P > 0.0005$) (Fig. 4). The levels of phagocytosis were therefore higher in the presence of immune serum, and this elevated activity appeared to be independent of additional complement.

A comparison of the number of *P. gingivalis* bacteria attached and the number ingested per PMN (with and without complement) indicated that the number of attached bacteria remained fairly constant, but the number ingested varied depending on the serum type being tested. When the total numbers of *P. gingivalis* bacteria both attached and ingested per active PMN were compared among serum types and conditions, the ligature group sera contributed less to this activity than the immunized sera under all conditions. The natural group sera were found to be less effective than the immunized

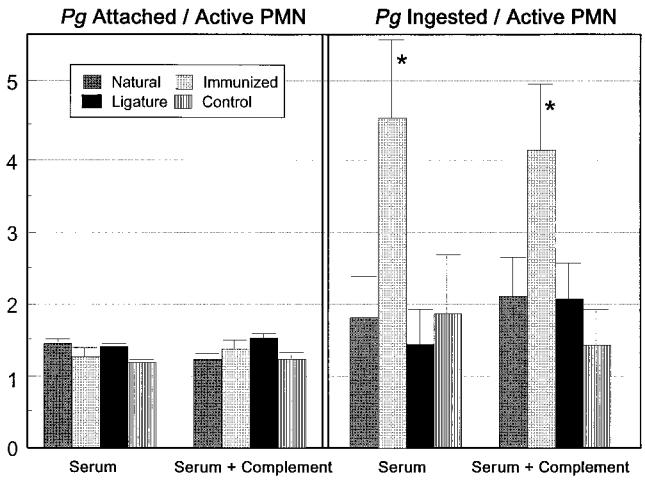

FIG. 4. Interactions between *P. gingivalis* (Pg) and PMNs, with *P. gingivalis* attached per active PMN and *P. gingivalis* ingested per active PMN shown. Bars denote the values of six serum samples from each group performed in triplicate, and the brackets enclose 1 standard deviation. The number of *P. gingivalis* bacteria ingested has been corrected to compensate for experimental error by subtracting the number of *N*-ethylmaleimide-treated control *P. gingivalis* bacteria that were determined to be found within the periphery of the PMNs. *, serum alone and serum plus complement ingested significantly more ($P < 0.005$) bacteria that the other comparable treatment groups.

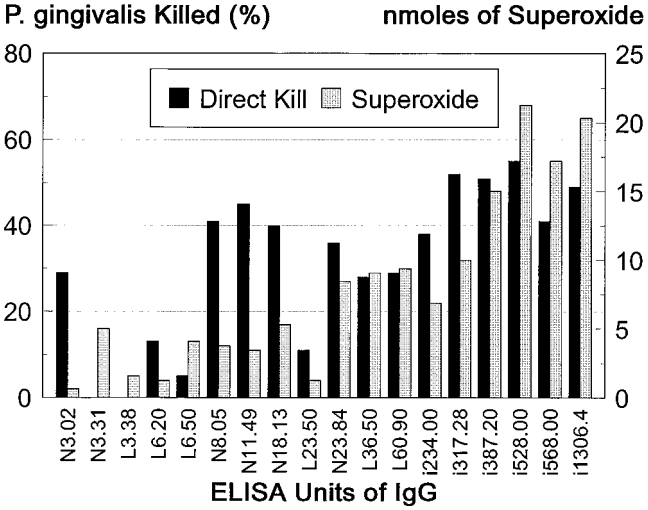

FIG. 5. Correlation of superoxide production and direct killing of *P. gingivalis*. Bars denote the percentage of *P. gingivalis* killed and superoxide released by individual serum samples plus complement. The 18 individual serum samples are presented on the abscissa by the amount of IgG antibody specific for *P. gingivalis* in ascending levels and are denoted as N (=natural), L (=ligated), and i (=immunized).

group sera when serum plus complement was used and more reactive than the ligature group sera when serum alone or complement plus serum was tested. The ligature group sera were minimally effective, and in most cases values were comparable to the control values (*P. gingivalis* plus PMNs) in these tests.

Correlations of NHP antibody functions. Figure 5 presents the percentage of direct killing of *P. gingivalis* and the nanomoles of superoxide released by PMNs as related to the titer of IgG specific for *P. gingivalis*. The immunized sera produced a higher level of both functional activities; however, these two tests were not parallel in their levels of activity across the full spectrum of IgG titers. In particular, there was a disparity among the three sera at N8.05, N11.49, and N18.13 (N denotes natural), where the level of direct killing activity was quite high but the stimulation of production of superoxide anion was low. Figure 6 compares the percentage of direct killing of *P. gingivalis* with the total number of *P. gingivalis* bacteria attached plus ingested per active PMN. A trend reflecting the mean data was seen, in that the immunized sera had higher levels of both activities. However, when the results for the natural IgG antibody were considered, the observation was made that while certain sera acted effectively in direct killing (i.e., N8.05 and N11.49), they did not enhance the attachment or ingestion of *P. gingivalis*. Finally, Fig. 7 compares the nanomoles of superoxide produced and the number of *P. gingivalis* bacteria attached or ingested per active PMN. In this comparison, a near-parallel response curve is seen between these two tests. This suggests that there may be some relationship between the type of antibody that affects the amount of superoxide anion produced and the number of *P. gingivalis* organisms attached or ingested per active PMN.

DISCUSSION

The interactions between the host and pathogens are complex in periodontal disease, involving a great number of variables for disease initiation, progression, and maintenance. The balance and absolute number of bacterial species vary from

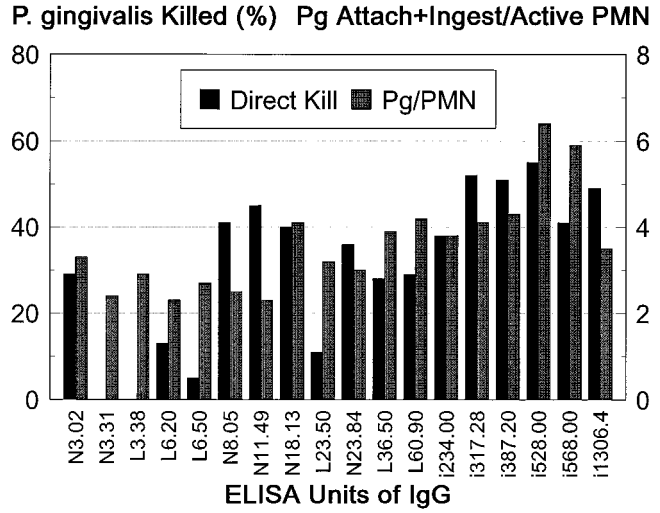

FIG. 6. Correlation of *P. gingivalis* (Pg) attached and ingested and direct killing of *P. gingivalis*. Bars denote the percentage of *P. gingivalis* killed and the number of *P. gingivalis* organisms attached plus ingested per active PMN by individual serum samples plus complement. The 18 individual serum samples are presented on the abscissa by the amount of IgG antibody specific for *P. gingivalis* in ascending levels and are denoted as N (=natural), L (=ligated), and i (=immunized).

host to host, as does the pathogenicity of the microorganisms (18). Likewise, resistance to disease varies between patients and may depend on a number of immunological factors. The NHP appears to provide a good model for studying periodontal disease, especially as it relates to the pathogen *P. gingivalis* (48). Exploring the functional capabilities of the immune system, specifically the immunoglobulins, present in whole serum should better define the role of the immune system in host defense against this disease.

Bacterial resistance to the cidal activity of serum is believed to be associated with the virulence of the bacteria, since some

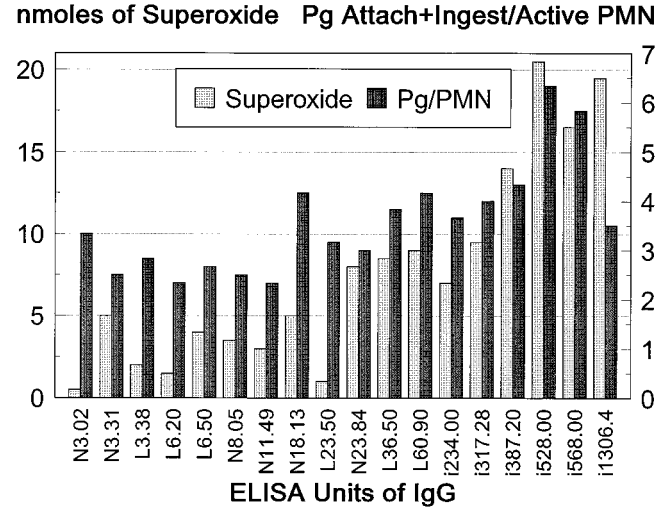

FIG. 7. Correlation of *P. gingivalis* (Pg) attached and ingested and superoxide production by PMNs. Bars denote the number of *P. gingivalis* bacteria attached plus ingested per active PMN and superoxide released by individual serum samples plus complement. The 18 individual serum samples are presented on the abscissa by the amount of IgG antibody specific for *P. gingivalis* in ascending levels and are denoted as N (=natural), L (=ligated), and i (=immunized).

of the more virulent species appear to resist serum killing better than nonvirulent ones (21). A functional test assessing the ability of serum to directly kill bacteria evaluates two parameters: the level of host defense and the resistance of the bacteria. The results from this study revealed a plateau of around 50% killing for some individual serum samples in both the high- and low-titer IgG groups. Various possibilities may explain why those sera achieved no greater than 50% killing in the presence of complement. The first was that the serum samples with low IgG titers may have been performing at their maximum levels in the direct killing of bacteria and would have achieved no greater killing under altered conditions. This may have been related to the ratio of IgG subclasses present, since IgG1 and IgG3 are well known to have strong complement-activating capacity; IgG2 and IgG4, however, are generally considered to have a low capacity for this functional property but would be included in the titer of IgG present (19). One study (49) suggested that the presence of IgG4 acts to protect bacteria from the biological effects of the IgG subclasses which fix complement; therefore, the ratio of IgG subclasses may be important in defining direct killing activity. Giardino (15), in examining NHP serum responses to *P. gingivalis*, found an increase in the level of IgG4 postimmunization and a drop in IgG4 from baseline in a group of periodontally ligated animals. A second explanation for the plateau of direct killing included the presence of IgA in the immunized group sera: we (15) found a rise in IgA postimmunization. The level of IgA becomes important because *P. gingivalis* has been shown to possess IgA proteases capable of cleaving IgA (17). This cleavage results not only in the inactivation of IgA but also in the presence of an IgA Fabα component which has been shown to have a protective function by coating the bacteria. This fragment appears to compete for the same antigen site used by the IgG molecule but without any biologic function of its own. This effectively blocks other immune effector systems such as complement activation (41).

The presence of antibody, as well as an adequate level of complement, has been shown to be important in a direct killing assay. Numerous studies (42, 43) have shown that certain strains of *P. gingivalis* have the ability to degrade complement proteins, disrupting the mechanism of complement activation. It has also been shown that *P. gingivalis* has the ability to deplete complement from its cell surface by activating C3 in the environment through the expression of a complement factor D-like activity which in turn provides protection to the cell (43). This appears to be an important virulence factor associated with this bacterium and may be important in avoiding host immune defenses. Studies (16) have now shown that the trypsinlike protease produced by *P. gingivalis* is active against all four subclasses of IgG, IgA1, IgA2, IgM, IgD, and IgE. The results of the present study reinforce the conclusions of the studies noted above, since opsonization with serum containing antibody for 30 min prior to the addition of complement resulted in no increase in direct killing activity over that of normal serum. However, when serum and complement were added simultaneously, a significantly greater amount of direct killing occurred, which is consistent with activation of the classical complement cascade before the antibodies were inactivated by *P. gingivalis* proteases.

Overall, the sera containing higher levels of IgG outperformed the lower-titer sera; however, the association was not linear, perhaps for some of the reasons given above. With the natural and ligature sera, some samples performed nearly as well as the immunized serum group for direct killing, suggesting a heterogeneity of function despite similar IgG titers. One early study tested (46) pooled human serum against *Bacte-*

roides melaninogenicus subsp. *melaninogenicus* ATCC 15930 and ATCC 25845. The authors were not able to show any correlation between IgG titer and the bactericidal effectiveness of the serum. They speculated on the differences in the resistance to killing between strains of bacteria and concluded that they could be due to differences in the bacterial cell wall. Okuda and Kato (36) compared sera from healthy adults and adult periodontitis patients in a bactericidal assay against *P. gingivalis* 381. The healthy adult serum showed little killing activity, and the periodontitis patient serum reduced the bacterial counts by 99%. No correlation was found between the titer of immunoglobulin and the activity of the serum against the bacteria. The data from this study agree with these earlier studies in that no linear correlation existed between IgG antibody titer and direct killing; however, the immunized sera, while containing a variable amount of IgG, did consistently outperform the natural and ligature sera. Another important result of this study is that the ligature group sera showed less direct killing activity than the natural group sera. While it is tempting to attribute a loss of functional ability to the induction of periodontitis, the sera were not selected longitudinally from the same animals; therefore, it is not known if the ligature sera originally had a higher level of activity prior to disease progression. A conclusion from these results was that increased levels of serum IgG antibody were more effective in direct killing and thus may be more protective by killing this pathogen.

An experimental protocol was designed to test the influence of whole NHP serum on the interaction between a specific bacterium, *P. gingivalis*, and human neutrophils. In the presence of oxygen, neutrophils, monocytes, macrophages, and eosinophils (6) have the ability to produce superoxide, which at neutral pH results in the production of H_2O_2. One advantage of this method was that the protective or virulence factors of the bacteria were left intact, providing a more natural interaction with the neutrophil. In this study the total amount of superoxide produced under each set of conditions was interpreted as an indirect measurement of the interaction between the bacteria and the neutrophil. Complement and antibody were both required for optimal neutrophil activation, with little activity occurring in the absence of complement. As was noted in the direct killing assay, a great deal of variability was also found in the individual serum responses contributing to the production of superoxide. It is interesting to note that some of the nonimmunized animals that responded with a high percentage of direct killing were low responders in superoxide production; likewise, some of the low responders in direct killing induced high amounts of superoxide. The reason for this phenomenon may be attributed to the presence of different subsets of antibody or antigen specificities, each with associated functions, excelling in different areas of host defense.

The ability of host defense cells to attach to the target organism is believed to be the first step in the defensive mechanisms of the neutrophil; this is followed by ingestion and, finally, killing of the target organism within the phagolysosome (45). Attachment of bacteria to the surface of the neutrophil can occur under two conditions, with or without the participation of an opsonin. The interaction between neutrophils and bacteria occurring in the absence of opsonins appears to be through a mechanism involving carbohydrate-binding proteins, collectively known as lectins (27, 35). There are two main types of opsonins which can participate in this reaction: antimicrobial antibodies, usually IgG, which bind to the target cell and allow binding of the Fc portion of the antibody molecule to the Fc surface receptor on the PMN; and complement fragments such as C3b and C3bi, which bind to the target bacteria and form a complex with the CR1 or CR3 receptors on the PMN. The third level of binding in the presence of opsonins occurs when antibody and complement fragments combine on the surface of the target cell, with a subsequent interaction with both Fc and CR receptors on the neutrophil. This binding is much stronger than that with either the antibody or complement fragments alone (1). In this study the number of *P. gingivalis* bacteria attached to the surface of the neutrophil remained fairly constant throughout all experimental conditions (no serum, serum alone, and serum plus complement), with no significant differences noted. The number of neutrophils participating also remained fairly constant throughout the same set of conditions, with approximately 65% having some interaction with *P. gingivalis*. These results suggest that there may be some opsonin-independent mechanisms which allow the binding of *P. gingivalis* to the neutrophil surface. Katancik et al. (23) evaluated the interaction of *P. gingivalis* with neutrophils with and without anti-*P. gingivalis* IgG-positive human serum. Their results were similar to those of this study in that the presence of the antibody-positive serum did not alter the number of bacteria attached to the neutrophils. Cutler et al. (7) performed a series of experiments that used fluorescent microscopy to enumerate the attached, ingested, and killed *P. gingivalis* bacteria per PMN and reported up to 100% participation of the neutrophils in the presence of serum and complement depending on the strain of *P. gingivalis* used.

In this study a significantly greater number of *P. gingivalis* bacteria were ingested in the presence of the immunized sera compared with sera from the other two groups. The results of a kinetics assay (data not shown) demonstrated that one immunized serum sample continued to cause an increase in the number of *P. gingivalis* bacteria phagocytosed for up to 60 min compared with the natural and ligature group sera. The presence of complement did not seem to have any influence on the number of bacteria phagocytosed. Nisengard et al. (32) found the same trend in testing the opsonic activity of rabbit anti-*P. gingivalis* serum (whole cell or cell fractions). They found that the antisera to the cell fractions had opsonic properties for neutrophil phagocytosis and that complement did not increase the number of bacteria ingested. Van Dyke et al. (50) looked at the number of *P. gingivalis* organisms ingested by neutrophils with the addition of human sera from both healthy and adult periodontitis patients. They found only 7% of the neutrophils participating under the influence of the adult periodontitis serum and around 1% percent participating with the healthy serum. The bacteria/neutrophil ratio in the adult periodontitis serum was only 0.074. In the study by Cutler et al. (7), the number of *P. gingivalis* bacteria ingested per neutrophil ranged from two to eight depending on experimental conditions. This range is similar to that achieved in this study. It appears that IgG-specific antibody plays a role in host defense by increasing the interaction between neutrophils and pathogenic bacteria through a mechanism specifically involving the Fc receptor.

Numerous studies have attempted unsuccessfully to correlate the titer of antibody with either the functional activity of the serum or the clinical parameters of periodontal disease (26). The only relationship that consistently appeared was that found by Nakagawa et al. (30), who showed that a significantly elevated level of anti-*P. gingivalis* IgG antibody in the serum correlated with elevated levels of *P. gingivalis* in the periodontal pocket. This would suggest that the antibody response is not protective in periodontal disease patients, possibly for a number of reasons, such as inaccessibility to the infection, lack of antibody specificity, inadequate PMN function, or a combination of these (7). It may be that the elevated antibody re-

sponses actually provide a protective mechanism to the pathogen, depending on the specific isotype and subclass of antibody being produced by the host. It was interesting to note that a comparison of direct killing and superoxide production suggested a low relationship in individual serum samples as did a comparison of direct killing and attachment or ingestion. However, when superoxide production and attachment or ingestion assays were compared, a positive relationship was revealed, with somewhat of a parallel response curve of functionality, suggesting that the subset of antibody responsible for superoxide production may be the same subset active in the ingestion of *P. gingivalis*.

The functional tests presented in this study demonstrate that immunization with a formalin-killed strain of *P. gingivalis* can produce a serum with increased antibody levels which exhibits greater immunologic functions than sera containing antibody from *P. gingivalis* colonization (i.e., natural) or sera obtained following ligature-induced periodontitis. The variability in the serum functions in this study may actually prove to be the most important finding, because this may allow an evaluation which correlates the specific functional test with the clinical response of the animal. In order to clarify the importance of the host antibody levels, the functional ability of the antibodies must be defined (52). There are a variety of functions proposed for IgG which may play a role in host defense and/or host tissue destruction. The present study reveals a great deal of variability between subjects for these three tests, with some sera having very effective antibody for some functional tests and almost no activity in other tests. A critical assessment of this concept will require longitudinal evaluation of matched serum samples derived from the same NHP throughout the experimental protocol. The overall results show a close correlation between the production of superoxide and ingestion of *P. gingivalis* but few parallels between these two tests and direct killing by serum. This suggests that there may be at least two separate subsets of antibody operating in the host. The functional tests reported in this study may help to form a firm foundation for future studies analyzing the complicated immune response system and its significance in periodontitis.

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