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Immunoglobulin G Response to Subgingival Gram-Negative Bacteria in Human Subjects

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Serum and gingival crevicular fluid from normal healthy adults and patients with periodontitis were screened for immunoglobulin G antibodies to antigens from *Bacteroides gingivalis* 381, *Bacteroides intermedius* 24, *Bacteroides loescheii* ATCC 15930, *Fusobacterium nucleatum* ATCC 25586, *Eikenella corrodens* 1073, *Actinobacillus actinomycetemcomitans* ATCC 29522, and *Capnocytophaga* sp. strain M-12. Immunoglobulin G antibody titers to the antigens were measured by an enzyme-linked immunosorbent assay. The antibody levels to *B. gingivalis* in serum and gingival crevicular fluid were significantly higher in the samples from patients with periodontitis than in samples from healthy individuals. Although there were individual differences within patient groups, a positive correlation ($P < 0.01$) was found between the serum immunoglobulin G levels to *B. gingivalis* and the development of periodontitis. The antibodies to *F. nucleatum* ($P < 0.05$), *E. corrodens* ($P < 0.05$), and *A. actinomycetemcomitans* were slightly higher in patients with periodontitis than in normal subjects. There were no remarkable differences between the two groups in titers to *B. intermedius*, *B. loescheii*, and *Capnocytophaga* sp.

Several studies have indicated that the proportion of gram-negative anaerobic bacteria increases in the subgingival microflora as periodontal inflammation develops (10, 12, 15). These findings opened a new approach for clarification of the etiology and pathogenesis of periodontal disease. Recently, it has become widely accepted that specific bacteria are involved in the pathogenesis of periodontitis. To further evaluate this concept, many studies have been directed toward an accurate characterization of the immune response to these pathogens. Elevation of cell-mediated and humoral immune response to *Bacteroides gingivalis* in subjects with periodontitis has been reported (7, 8). A study of the local immune response to *B. gingivalis* in the gingival tissue also indicated that a greater immune response was exhibited in the more advanced stages than in the earlier forms of periodontal disease (4). On the other hand, Doty et al. (1) reported that serum antibody to oral microorganisms, including *B. gingivalis*, in patients with periodontitis was significantly lower than in the serum from periodontally healthy subjects. A lack of positive correlation of humoral antibodies with severity of periodontal disease has also been reported (6). Thus, the nature of the humoral response to this periodontal pathogen is not certain.

The present report deals with the determination of the serum and gingival crevicular fluid (GCF) immunoglobulin G (IgG) antibody levels to seven gram-negative periodontopathic bacteria, in patients with periodontitis, using the micro-enzyme-linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

Bacterial strains. *B. gingivalis* 381, *Eikenella corrodens* 1073 (kindly supplied by S. S. Socransky, Forsyth Dental Center, Boston, Mass.), *Bacteroides loescheii* ATCC 15930, *Fusobacterium nucleatum* ATCC 25586, *Actinobacillus actinomycetemcomitans* ATCC 29522, *Bacteroides intermedius* 24, and *Capnocytophaga* sp. strain M-12 isolates from our laboratory were used.

Antigens. For cultivation, several diffusate broth media were prepared. Trypticase peptone (BBL Microbiology Systems, Cockeysville, Md.), 34 g; Phytone peptone (BBL), 6 g; and yeast extract (Difco Laboratories, Detroit, Mich.), 6 g were dissolved in 150 ml of distilled water and dialyzed against 1 liter of water at 4°C for 48 h. To this diffusate NaCl, 5 g; K_2HPO_4, 2.5 g; glucose, 2.5 g; cysteine-hydrochloride, 0.2 g; L-asparagine, 0.2 g; L-tryptophan, 0.2 g were added per liter of broth, and agents essential to the respective species were supplemented. Hemin at 5 μg/ml and menadione at 0.5 μg/ml, hemin at 5 μg/ml and KNO_3 at 0.1%, and hemin at 5 μg/ml were added for *Bacteroides* spp., *E. corrodens*, and *A. actinomycetemcomitans*, respectively, per liter of broth. Bacterial cells were cultured in 2 liters of the broth for 48 to 72 h in an anaerobic glove box. Grown cells were harvested by cold centrifugation at 12,000 × *g* for 20 min at 4°C and washed three times with sterile phosphate-buffered saline (PBS; pH 7.2). Washed cells were suspended in PBS to give a concentration of 200 mg (wet weight) per ml and were subjected to ultrasonic disruption (model 5213, 300W; Ohtake, Tokyo, Japan) in an ice bath. The cell disruption was monitored by phase-contrast microscopy. The sonicates were centrifuged at 12,000 × *g* for 20 min at 4°C. The supernatants were dialyzed against distilled water at 4°C, lyophilized, and stored at −20°C until use.

Serum and GCF samples. Serum samples from periodontal patients were obtained from the Department of Periodontics of Tokyo Dental College and Tokyo Medical and Dental University. A total of 42 patients (19 males and 23 females; aged 23 to 54 years) who were clinically diagnosed as suffering from advanced periodontitis were tested. Patients were graded into P1, P2, and P3 groups by the degree of bone loss and pocket depth, with the number increasing as the disease advanced and extended. Group P1 (incipient or mild periodontitis, $n = 17$) exhibited generalized alveolar bone loss, but the value measured by the method of Schei et al. (9) was under 20%. Group P2 (moderate periodontitis, $n = 14$) comprised patients aged 23 to 54 years exhibiting marked alveolar bone loss (20 to 40%) and deepended pocket depths (2 to 7 mm). Group P3 (advanced periodontitis, $n = 11$) consisted of patients 30 to 53 years of age with considerable alveolar bone loss (40% or greater). Patients with systemic disease were not included in this study. A total of 17 serum

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samples (13 males and 4 females) were obtained from dental students and laboratory staff members aged 23 to 41 years with no evidence of periodontal disease. These samples were used as references. All sera were stored at −70°C until use.

GCF was collected by the method described by Löe and Holm-Pederson (5). Samples from six patients with periodontitis, and from six periodontally healthy subjects were collected with paper strips from the periodontal pockets or gingival crevices of six incisors. The age, grade of the disease and sex of the subjects are noted in Table 3. After removal of plaque at the gingival margin, the area was isolated by cotton rolls and dried by a stream of air, and the strips were placed into the crevices or pockets for 5 min. Soaked strips were immediately weighed to measure the GCF quantity and then put into PBS in a small vial. The vial was vigorously shaken for 30 s. After the strips were removed, the GCF concentration was adjusted with PBS, and the samples were stored at −70°C until use.

Rabbit antiserum. Cells grown in Trypticase broth were harvested and washed thoroughly with sterilized PBS. The final bacterial concentration of immunogen was adjusted to 10 mg (wet weight) per ml in PBS. A 1-ml amount of the whole cell suspension was injected into the marginal ear vein of young female New Zealand white rabbits. Increasing doses of the immunogen were given every 2 or 3 days for 3 weeks. The animals were bled 1 week after the last booster injection, and separated sera were stored at −70°C.

Serological characterization of extracted antigen. To check the specificity of ultrasonicated supernatants, we carried out an inhibition assay after adsorption with intact cells of the strain. A mixed serum of seven rabbit antisera against tested strains was used for the assay. Washed cells to be used for adsorption were suspended in diluted serum at 10 mg/ml and kept at 4°C overnight with constant shaking. The suspension was then centrifuged, and the supernatant was filtered (0.22-μm filter; Millipore Corp., Bedford, Mass.). A serial dilution of the adsorbed serum was prepared and tested by ELISA for the corresponding antigen.

Antibody measurement. The IgG antibody titer of samples was determined by ELISA. The antigen was suspended in a 0.1 M carbonate buffer (pH 9.6) containing 0.02% NaN_3 to give a concentration of 4 μg of protein per ml and added to a microtiter plate (Immulon; Dynatech Laboratories Inc., Alexandria, Va.). This concentration had been previously found to result in optimal sensitization of wells by antigens of the microorganism. The plates were sealed and incubated at 37°C for 2 h. A 100-μl amount of 2% bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) in 0.1 M carbonate buffer was added, and the plates were stored at 4°C until use.

Before testing, the wells were washed three times with 0.005% Tween 20-supplemented PBS. A diluted test sample (200 μl) in PBS (pH 7.2) containing 0.05% Tween 20, 0.5% bovine serum albumin, and 0.02% NaN_3 was added to each well, and the plates were incubated at 37°C for 2 h. After the wells were washed thoroughly, 200 μl of alkaline phosphatase-conjugated anti-human IgG (gamma-chain specific; Sigma Chemical Co.) diluted in PBS buffer was added to each well, and the plates were incubated at 37°C in a moist chamber for 1 h. The wells were again washed three times with the same buffer, and 100 μl of the substrate was added to each well. The alkaline phosphatase substrate solution was prepared by Kit reagent (Sanko Co., Tokyo, Japan), including phenylphosphate disodium in 0.1 M carbonate buffer (pH 9.6). After 1 h of incubation at 37°C, the enzymatic reaction was stopped by the addition of 100 μl of the reagent (Sanko Co.), 4-aminoantipyrine diluted in NaOH solution with $KClO_4$.

The absorbance at 490 nm was determined in micro-ELISA minireader (MR590; Dynatech Laboratories, Inc.) through the bottom of each well. All measurements were performed in duplicate.

Determination of EU. The antibody levels obtained by ELISA were expressed as ELISA units (EU). As a preliminary experiment, checkerboard assays were conducted to identify human sera having a reactivity in the IgG classes of antibody with *B. gingivalis* antigen. Three positive sera against *B. gingivalis* were pooled and tested by ELISA in serial dilutions (1:50 to 1:12,800) as a reference. A standard titration curve was obtained by plotting the mean absorbance of the reference serum, and a straight-line equation of the regression ($y = -1.01 \log_{10} x + 3.89$, $\gamma = 0.999$) along the curve was drawn. EU of all samples were calculated by relating optical density values from each experimental sample to the reference serum, which was assigned a value of 100 EU (optical density at 490 nm = 1.60 at a 1:200 dilution). EU of the antibodies against the other antigens were measured by methods similar to that described above, except that the antibody was diluted 1:400, to which a value of 100 EU was assigned.

In the first part of the assay, the individual serum samples were tested at a single dilution of 1:200 or 1:400, and the GCF samples were diluted to 1:400. All of the serum samples and GCF samples yielding absorbance values at 490 nm above 1.30 when screened were diluted such that the values fell into the rectilinear portion of the dose-response curve. A negative sample reaction was determined as that value (0.2) for sera tested at which further dilution of the samples resulted in no change in the final optical density reading.

RESULTS

Specificity of the antigen. The results of the inhibition assay are summarized in Table 1. Absorbed with 10 mg of intact *B. gingivalis* 381 cells, the ELISA titer was reduced 70%, whereas a reduction of the ELISA titer for heterogeneous strains were not observed. Similar inhibition was found by the adsorption assay with *B. intermedius*, *B. loescheii*, *F. nucleatum*, *A. actinomycetemcomitans*, *E. corrodens*, and *Capnocytophaga* sp. These findings indicated that the antibodies detected in the present study were specific for the respective microorganisms. Cross-absorption experiments of the pool of sera from three patients were also carried out. The results were similar to those exhibited in rabbit antisera.

Serum IgG antibodies. In the early stage of the study to measure EU, the ultrasonicated supernatant from *B. gingivalis* 381 was compared with the surface antigen extracted by the method of Mouton et al. (7). No significant difference in titer was noted between the two antigens. The serum IgG titers to ultrasonicated supernatant of *B. gingivalis* are shown in Table 2. The periodontitis group had more specific IgG antibodies than the healthy group, although a considerable variation was observed in samples from patients with periodontitis. The EU levels in the periodontitis groups were higher than those in the healthy group (P1, P2, P3; $P < 0.001$). The difference between the healthy group and periodontitis groups was statistically significant. Furthermore, the severity of periodontitis was correlated with the elevation of antibody titer to the ultrasonicated supernatant of *B. gingivalis* (P1, P2; $P < 0.01$; P1, P3: $P < 0.01$). As to the serum IgG levels against *B. intermedius*, there were no remarkable differences between the healthy and periodonti-

TABLE 1. Specificity of tested antigens of gram-negative bacteria by an absorption test in an ELISA system

Antigen	Inhibition (%) of activity[a]						
	B. gingivalis 381	*B. intermedius* 24	*B. loescheii* ATCC 15930	*F. nucleatum* ATCC 25586	*A. actinomycetemcomitans* ATCC 29522	*E. corrodens* 1073	*Capnocytophaga* sp. strain M-12
B. gingivalis 381	70	0	0	0	0	0	0
B. intermedius 24	0	89	0	0	0	0	0
B. loescheii ATCC 15930	0	0	58	0	0	0	0
F. nucleatum ATCC 25586	0	0	0	58	0	0	0
A. actinomycetemcomitans ATCC 29522	0	0	0	0	72	0	0
E. corrodens 1073	0	0	0	0	0	48	0
Capnocytophaga sp. strain M-12	0	0	0	0	0	0	70

[a] The absorbed anti-rabbit sera were treated at a dilution of 1:1000 with the anti-rabbit IgG conjugate. Values are expressed as the percent decrease of absorbance readings relative to untreated anti-rabbit sera. Absorbed with 10 mg (wet weight) of cells per ml in serum.

tis groups. The serum IgG level to *B. loescheii* ATCC 15930 was almost the same as that for *B. intermedius*. For mean values, the highest EU levels were observed in the P2 group. No statistically significant differences were found between the healthy group and periodontitis groups in antibody titers to either *B. loescheii* or *B. intermedius*.

The serum EU to *F. nucleatum* strain ATCC 25586 in the periodontitis groups were higher than that of the healthy group (Table 2). The P2 and P3 groups had much higher titers than the healthy group ($P < 0.05$).

The antibody titers to *A. actinomycetemcomitans* ATCC 29522 varied among the tested sera. Sera from six patients and one healthy subject exhibited more than 200 EU to this organism.

The serum titers to *E. corrodens* 1073 in the P2 group were higher than those in the healthy group ($P < 0.05$). The mean EU level of *E. corrodens* in the P2 group was the highest titer among three periodontitis groups. This is due to extremely high titers of two serum samples from P2 patients.

There was no marked difference in the mean antibody levels against *Capnocytophaga* sp. strain M-12 between healthy and periodontitis groups.

Comparison of serum IgG with GCF IgG antibodies. Samples of GCF from six patients with periodontitis and six healthy individuals were used to measure specific antibody to *B. gingivalis*, *B. intermedius*, and *F. nucleatum*. The results are summarized in Table 3 together with those of serum. The antibody to *B. gingivalis* in GCF was found in five of six patients and one individual in the healthy group. A parallelism of titer between GCF and serum taken from individuals was also apparent. The average EU in GCF of six patients was 142 ± 76 EU and that of healthy group was 19 ± 19 EU.

The GCF antibodies to *B. intermedius* and *F. nucleatum* in periodontitis patients were lower than those of serum in most cases. In the healthy group, as was observed with *B. gingivalis*, antibodies to *B. intermedius* and *F. nucleatum* were not detected, with a few exceptions.

DISCUSSION

Recent studies on humoral immune responses to subgingival oral microorganisms examined IgG antibody levels in terms of periodontal inflammation (1–3, 6, 7, 13, 14). Our major objective was to elucidate the difference in immune response between the healthy group and periodontitis groups. This subject has been studied recently by many investigators using ELISA with IgG. We limited our study to the measurement of IgG antibody so that comparison with other findings would be possible.

In the present study, the specific IgG antibody levels to *B. gingivalis*, *F. nucleatum*, *E. corrodens*, and *A. actinomycetemcomitans* in the sera of patients with periodontitis were higher than those in healthy individuals. This was especially true for *B. gingivalis*. Severity of periodontitis closely correlated with the elevation of the antibody level to this species. Antibodies were detected to the other species, especially to *B. intermedius* and *B. loescheii*. On the other hand, the levels were lower in the P3 group than in the P2 group. The mechanism of this phenomenon might be due to immune depression or antigenic competition. Further study on the

TABLE 2. Level of IgG reactive with bacterial antigens in healthy individuals and periodontitis patients

Antigen	IgG level[a] in the following group:			
	Healthy (n = 17)	P1 (n = 17)	P2 (n = 14)	P3 (n = 11)
B. gingivalis 381	10.0 ± 3.0	102.6 ± 17.8[b]	280.1 ± 67.4[b]	321.4 ± 85.8[b]
B. intermedius 24	56.4 ± 11.2	69.4 ± 11.9	111.0 ± 26.9	86.1 ± 15.8
B. loescheii ATCC 15930	41.1 ± 7.7	70.1 ± 11.2	94.0 ± 13.0	70.1 ± 12.6
F. nucleatum ATCC 25586	44.8 ± 9.5	75.9 ± 29.4	138.9 ± 39.0[c]	127.3 ± 46.4[c]
A. actinomycetemcomitans ATCC 29522	68.2 ± 29.3	92.3 ± 22.2	150.5 ± 52.2	167.3 ± 69.5
E. corrodens 1073	30.4 ± 4.9	35.7 ± 6.5	74.4 ± 21.9[c]	46.4 ± 11.9
Capnocytophaga sp. strain M-12	60.5 ± 13.0	72.3 ± 15.8	102.2 ± 32.4	86.0 ± 17.7

[a] Values are expressed as EU relative to a serum reference ± the standard error.
[b] Significant difference from the healthy adult group ($P < 0.001$) by Student's t test.
[c] Significant difference from the healthy adult group ($P < 0.05$) by Student's t test.

TABLE 3. IgG antibody levels[a] in serum and GCF of periodontitis patients and healthy individuals to *B. gingivalis*, *B. intermedius*, and *F. nucleatum*

Group and subject	Age (yr)	Sex	IgG antibody levels to:					
			B. gingivalis 381		*B. intermedius* 24		*F. nucleatum* ATCC 25586	
			Serum	GCF	Serum	GCF	Serum	GCF
Periodontitis								
A	28	M	206	507	93	89	39	ND
B	38	M	54	ND	53	ND	36	ND
C	38	F	121	158	87	32	53	22
D	28	F	208	76	312	42	179	32
E	50	M	34	54	22	8	30	33
F	45	M	53	55	82	25	45	ND
Mean ± SE			113 ± 32	142 ± 76	108 ± 42	33 ± 13	64 ± 23	15 ± 7
Healthy								
G	24	M	8	ND	87	ND	46	ND
H	30	M	56	116	89	ND	34	ND
I	40	M	31	ND	84	ND	54	ND
J	25	M	12	ND	17	ND	17	ND
K	27	F	6	ND	36	119	22	ND
L	27	M	7	ND	27	ND	58	66
Mean ± SE			20 ± 8	19 ± 19	57 ± 13	20 ± 20	39 ± 7	11 ± 11

[a] IgG antibody titer to *B. gingivalis*, *B. intermedius*, *F. nucleatum* was expressed as EU.
[b] ND, Not detected in 200-fold-diluted samples.

changes in immuneresponse during inflammation is needed.

The specific antibodies to *B. gingivalis*, *B. intermedius*, and *F. nucleatum* in GCF of the patient were found in the present study. In a few periodontitis patient samples of GCF, the specific IgG levels to *B. gingivalis* were higher than those in the serum samples. The findings imply that local antibody accumulation or synthesis at a higher level occurred in those cases.

Local antibody accumulation to *B. gingivalis* has been suggested by animal experiments (11) and cellular response in periodontitis lesion (4). The possible function of the antibodies observed in this study is still debatable. They might play a role in protection or destruction of periodontal tissue as has been discussed by many investigators (1, 4). Further longitudinal studies may help to answer this important question.

Mean antibody levels to *B. gingivalis* of the P2 and P3 groups were from 20 to 30 times higher than that of the healthy group. However, the individual level of the specific antibody varied among the patients. It is assumed that the patients who had low levels of antibody to *B. gingivalis* were infected with different organisms, such as *B. intermedius*, *B. loescheii*, *E. corrodens*, *F. nucleatum*, *A. actinomycetemcomitans*, and *Capnocytophaga* sp. We are planning to examine the relation between the specific antibody levels and predominant organisms in periodontal lesions of these patients.

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