

Humoral Immune Response to Oral Microorganisms in Periodontitis

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Serum antibody titers from patients with periodontitis were compared with those from periodontally healthy subjects. With the micro-enzyme-linked immunosorbent assay, immunoglobulin G (IgG), IgA, and IgM antibody titers to isolates of *Streptococcus sanguis*, *Actinomyces viscosus*, *Bacteroides gingivalis*, *Bacteroides melaninogenicus* subsp. *intermedius*, *Bacteroides ochraceus*, and *Fusobacterium nucleatum* were determined. Antibody titers of the IgG and IgA classes to *B. melaninogenicus*, *B. ochraceus*, *F. nucleatum*, and *S. sanguis* were found to be significantly higher in the controls than in the patients. No correlations were found with serum IgM titers. These findings indicate that periodontitis may be associated with depressed antibacterial serum antibody titers of the IgG and IgA classes.

The destructive manifestations of the immunological response to oral microorganisms has been proposed to play a role in the development and chronic nature of inflammatory periodontal disease (1, 7, 8, 13, 22, 25, 37). Activation of cell-mediated immunity by such microbes and their products has been documented by many investigators (12, 14-16). The nature of the humoral response to these substances is not well defined. Early reports (4, 9, 18, 19, 21, 24, 26) reveal the ubiquitous nature of antibodies to oral bacteria in both healthy and periodontally diseased individuals. Whether the presence of antibodies in serum actually reflects plaque ecology and oral health is unclear. Many investigators have reported a lack of correlation of humoral antibodies with severity of periodontal disease (19, 28), whereas in other studies a correlation between specific antibacterial titers and periodontal disease has been made (4, 9, 23). Others have reported an absence of elevated titers to specific oral microorganisms in advanced disease, most notably associated with antibodies to oral spirochetes (36), to anaerobic gram-negative, surface translocating filamentous organisms, and to *Actinobacillus actinomycetemcomitans* (27). We report the use of the micro-enzyme-linked immunosorbent assay to measure serum antibody titers of the three major immunoglobulin heavy-chain classes to a panel of selected periodontal disease-associated microorganisms to assess differences between individuals with either healthy or diseased periodontal tissues.

MATERIALS AND METHODS

Experimental subjects. Heparinized plasma from patients having periodontitis and from healthy volunteers was evaluated for the presence of antibacterial antibodies. Twenty-four patients (ages, 24 to 60 years) having one or more periodontal pockets extending 4 mm or more apically to the cementum-enamel junction and at least 20 treatable teeth were evaluated. Patients with poor health or in need of extensive restorative dentistry were not included in this study. Ten subjects recruited from the university community (ages, 21 to 60 years) with good oral hygiene were assessed for plaque on all four tooth surfaces with the plaque index system (34) and for gingival health by the criteria of the periodontal disease index (29, 30). The level of periodontal attachment in relation to the cementum-enamel junction (26) and pocket depth was also measured. These measurements are shown in Table 1.

Bacterial antigens. Isolates of the following oral microorganisms were used as sources of antigens in this study: *Streptococcus sanguis* ATCC 10558, *Actinomyces viscosus* GA, *Bacteroides gingivalis* W, *Bacteroides melaninogenicus* subsp. *intermedius* 155.6, *Bacteroides (Capnocytophaga) ochraceus* 374B, and *Fusobacterium nucleatum* 191F. Unless otherwise noted, these isolates were obtained from naturally occurring gingivitis or periodontitis sites during prior clinical studies. The nonoral microorganisms *Escherichia coli* and *Staphylococcus epidermidis* served as sources of ubiquitous, non-periodontal disease-associated antigens. Culture of these microorganisms has been previously described (20). The cell cultures were harvested by centrifugation at $12,000 \times g$ for 30 min. The cell pellets were washed extensively in sterile phosphate-buffered saline (0.05 M PO_4 , 0.15 M NaCl, pH 7.4; PBS) and suspended in sterile distilled water to give a concentration of 20 mg (wet weight) per ml.

TABLE 1. Gingivitis index, plaque index, and distribution of pocket depths in control and experimental groups before treatment

Group	Mean plaque index ^a ± SD	Mean gingivitis ^b index ± SD	Attachment loss as % of sites ^c				Pocket depth as % of sites ^d		
			0.0 mm	1-3 mm	4-6 mm	≥7 mm	1-3 mm	4-6 mm	≥7 mm
Control (n = 10)	0.55 ± 0.30	0.32 ± 0.16	78	22			96	4	
Experimental (n = 25)	1.75 ± 0.46	1.80 ± 0.20	8	63	26	3	60	36	4

^a Plaque index system (31).

^b Periodontal disease index (27).

^c Level of periodontal attachment in relation to the cementum-enamel junction at five aspects of each tooth examined.

^d Level of periodontal attachment and pocket depth measured with a calibrated M1 probe with a point diameter of 0.4 mm. Measurements rounded to nearest millimeter. For control group n = 1,780; for experimental group, n = 2,950.

The cell harvest was approximately 100 mg (dry weight) per 1,000 ml of culture medium. The washed cells were subjected to a total of 40 min of ultrasonic disruption (model W185 D, 85 W; Heat Systems-Ultrasonics, Inc., Plainview, N.Y.) delivered in 5-min intervals with alternating periods of cooling in an ice bath. The ultrasonic extract was centrifuged at 12,000 × g for 30 min, and the supernatant was retained. After dialysis in distilled water at 4°C to remove low-molecular-weight material, the supernatant fraction was lyophilized.

Total immunoglobulin levels in patient and control subjects. The single radial immunodiffusion method of Fahey and McKelvey (5) was used to assess levels of immunoglobulin A (IgA), IgG, and IgM in the plasma of patients and control subjects. AccraAssay plates (Miles Laboratories, Inc., Elkhart, Ind.) containing precalibrated amounts of heavy-chain-specific anti-human immunoglobulin were employed. Briefly, 10-μl samples of each plasma specimen were added to separate wells. Reference sera, previously calibrated against primary standards obtained from the World Health Organization, were used to generate standard curves for each heavy-chain class. After incubation for 24 h at 4°C (IgA and IgG) or 48 h at room temperature (IgM), the ring diameters were measured. Calibration curves were prepared by plotting the logarithm of the concentration versus the ring diameter (linear coordinate).

Determination of plasma antibody titers. A micro-enzyme-linked immunosorbent assay was used to measure antibacterial antibody titers in plasma samples (3). The previously described ultrasonic extracts of oral microorganisms were diluted (5 μg/ml) in 0.05 M sodium carbonate coating buffer (pH 9.6, containing 0.02% NaN₃), and then 0.20 ml of the dilution was added to each well of a round-bottom polystyrene substrate plate (Immulon; Dynateck Laboratories, Inc., Alexandria, Va.). This concentration had been previously shown to result in optimal sensitization for the microorganisms tested. The plates were sealed with cellophane tape and incubated at room temperature (22 to 24°C) overnight (16 h) in a closed chamber with high humidity. The following morning the plates were washed five times with PBS containing Tween 20 (PBS-T; Matheson, Coleman, and Bell, Norwood, Ohio). Plasma samples were serially diluted in PBS-T, and 0.20 ml of each dilution was added to sensitized wells. After a 3 h incubation at room temperature, the

plates were washed five times with PBS-T. Alkaline phosphatase (calf intestine, type VII; Sigma Chemical Co., St. Louis, Mo.) was conjugated to heavy-chain-specific rabbit anti-human immunoglobulin (Bio-Rad Laboratories, Richmond, Calif.) by glutaraldehyde treatment (3). The conjugates were diluted in PBS-T, and 0.2 ml was added to each well. After overnight incubation at room temperature, the plates were washed with PBS-T. Alkaline phosphatase substrate (Sigma 104; Sigma Chemical Co., 1 mg/ml in 0.05 M sodium carbonate [pH 9.8]-1mM MgCl₂) was added to each well. After 30 min at room temperature, the hydrolysis reaction was stopped by the addition of 0.05 ml of 3 N NaOH. The absorbance at 405 nm was measured spectrophotometrically (Multiskan; Flow Laboratories, Inc., McLean, Va.), and the titer was defined as the reciprocal of the last dilution resulting in an absorbance which was greater than 0.01.

Nonparametric statistical analysis (Mann-Whitney [33]) was used to establish significant differences between patient and control titers for each antigen. The Student *t* test was not used because of the unequal variances of the two groups.

RESULTS

Total immunoglobulin levels in patient and control subject plasmas. Since differences in specific antibody titers may reflect gross differences in total immunoglobulin, especially under circumstances in which patient and control groups cannot be totally age matched, total immunoglobulin levels were determined for each major heavy-chain class (Table 2). There was no significant difference between total immunoglobulin levels of any major class (IgG, IgM, or IgA) when patient and control subject plasmas were compared. In a separate comparison, there was no correlation between age and immunoglobulin level in either the patient or the control group (data not shown).

Antibacterial antibodies (oral microorganisms). The serum IgG titers to selected oral microorganisms are shown in Fig. 1. There was no significant difference between healthy and diseased subjects when the titers to *B. gingivalis* and *A. viscosus* were compared. For the remaining

TABLE 2. Plasma immunoglobulin levels in patient and control groups

Group	Immunoglobulin level (µg/dl)					
	IgA		IgG		IgM	
	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range
Patients	207 ± 73	84-307	1,167 ± 290	623-1,473	130 ± 37	58-175
Controls	198 ± 64	92-271	1,120 ± 364	598-1,536	141 ± 42	49-186

microorganisms (*B. melaninogenicus*, $P < 0.0005$; *B. ochraceus*, $P < 0.001$; *F. nucleatum*, $P < 0.0005$; and *S. sanguis*, $P < 0.01$) healthy subjects possessed higher titers than did diseased individuals.

Serum IgA (Fig. 2) titers were lower when compared with IgG titers. As shown for IgG antibodies, there was no difference in titer to *B. gingivalis* or *A. viscosus* when healthy and diseased subjects were compared. Significantly elevated titers in healthy subjects were measured to the remaining four microorganisms (*B. melaninogenicus*, $P < 0.01$; *B. ochraceus*, $P < 0.005$; *F. nucleatum*, $P < 0.01$; and *S. sanguis*, $P < 0.001$), as previously shown for IgG.

Serum IgM titers (Fig. 3) to the same oral microorganisms were also quite low in all cases

compared with the serum IgG levels. In no case was there a significant difference between patient and control values ($P > 0.05$).

Antibacterial antibodies (nonoral microorganisms). Two nonoral microorganisms were used to determine whether the trends shown above were limited to oral microorganisms associated with periodontal disease. *E. coli* and *S. epidermidis* were chosen since they are ubiquitous nonoral bacteria. IgG ($P < 0.005$) and IgA ($P < 0.01$) titers to *S. epidermidis* were higher in the control compared with the patient plasmas (Fig. 4). As shown with oral bacteria, there was no difference in the IgM titers. In contrast, with *E. coli*, there was no difference in any immunoglobulin class when patient and control subject plasmas were compared.

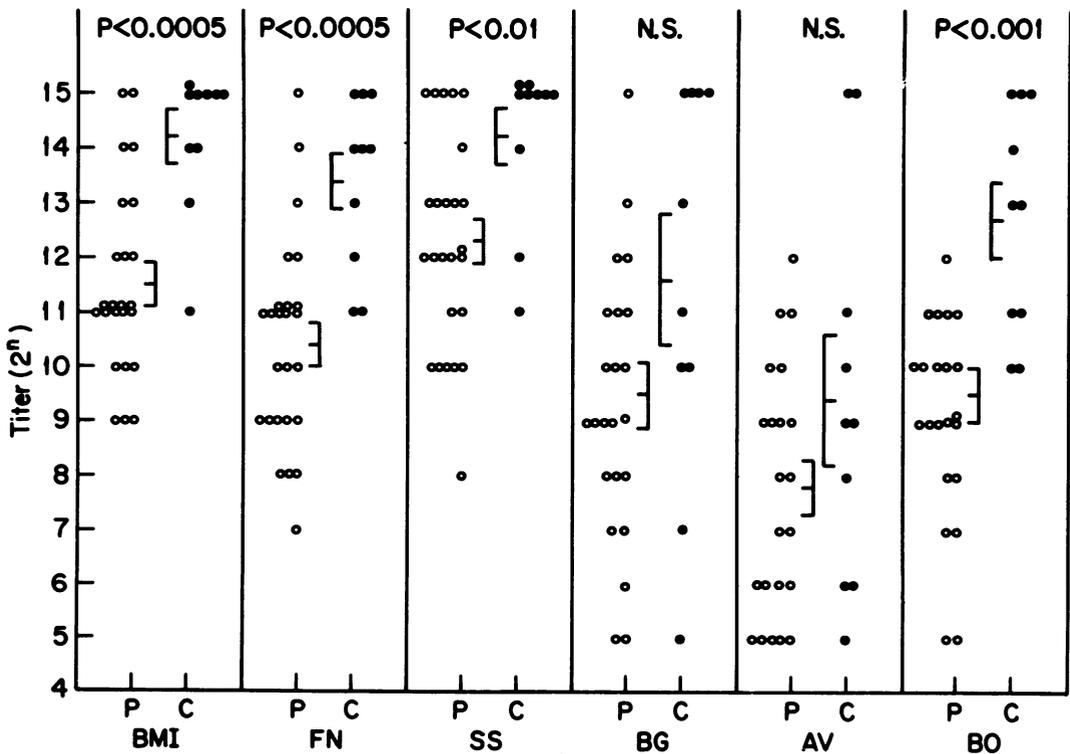


FIG. 1. IgG antibody titers to ultrasonicated oral microorganisms, expressed as \log_2 . Mean titers are shown \pm standard error of the mean. Significant differences between patients (P) and controls (C) are indicated. Symbols: O, patients; ●, controls. N.S., Not significant; BMI, *B. melaninogenicus*; FN, *F. nucleatum*; SS, *S. sanguis*; BG, *B. gingivalis*; AV, *A. viscosus*; BO, *B. ochraceus*.

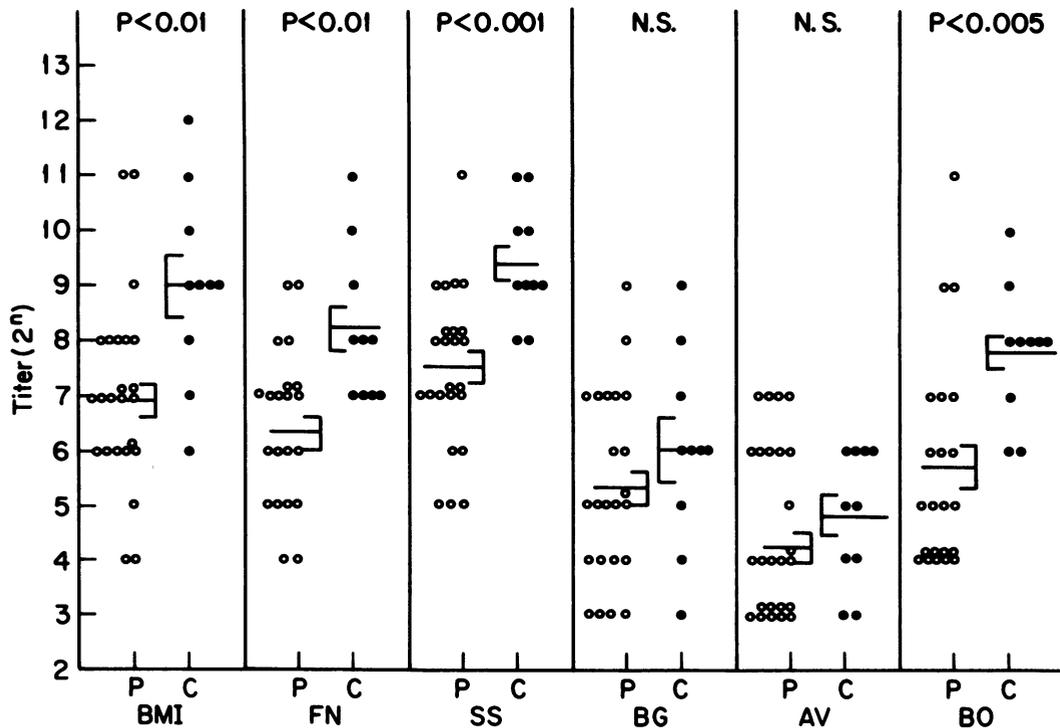


FIG. 2. IgA antibody titers to ultrasonicated oral microorganisms, expressed as log₂. Mean titers are shown ± standard error of the mean. Significant differences between patients (P) and controls (C) are indicated. Symbols: ○, patients; ●, controls. See legend to Fig. 1 for abbreviations.

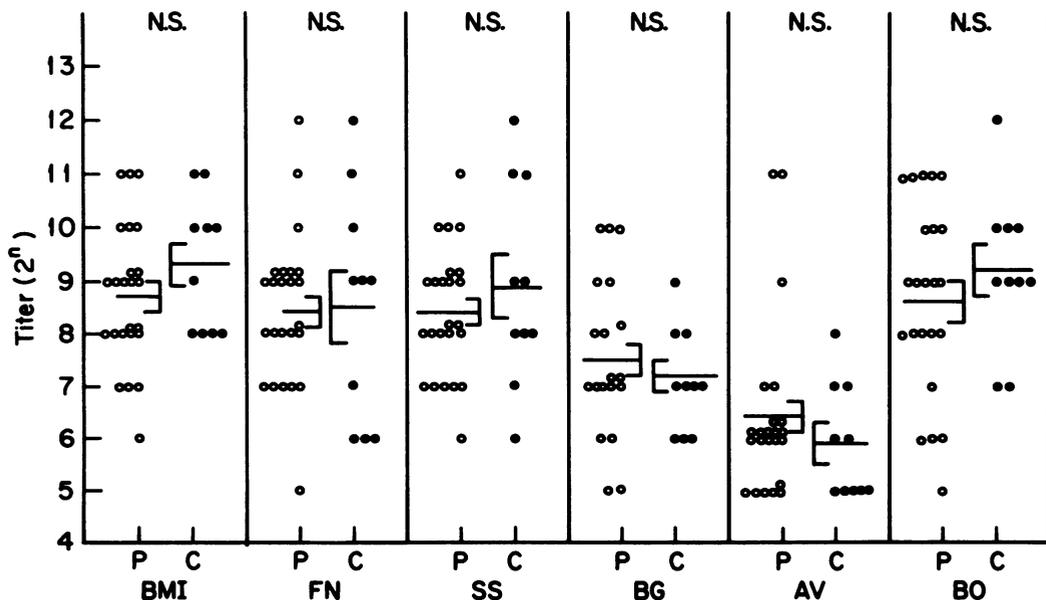


FIG. 3. IgM antibody titers to ultrasonicated oral microorganisms, expressed as log₂. Mean titers are shown ± standard error of the mean. Significant differences between patients (P) and controls (C) are indicated. Symbols: ○, patients; ●, controls. See legend to Fig. 1 for abbreviations.

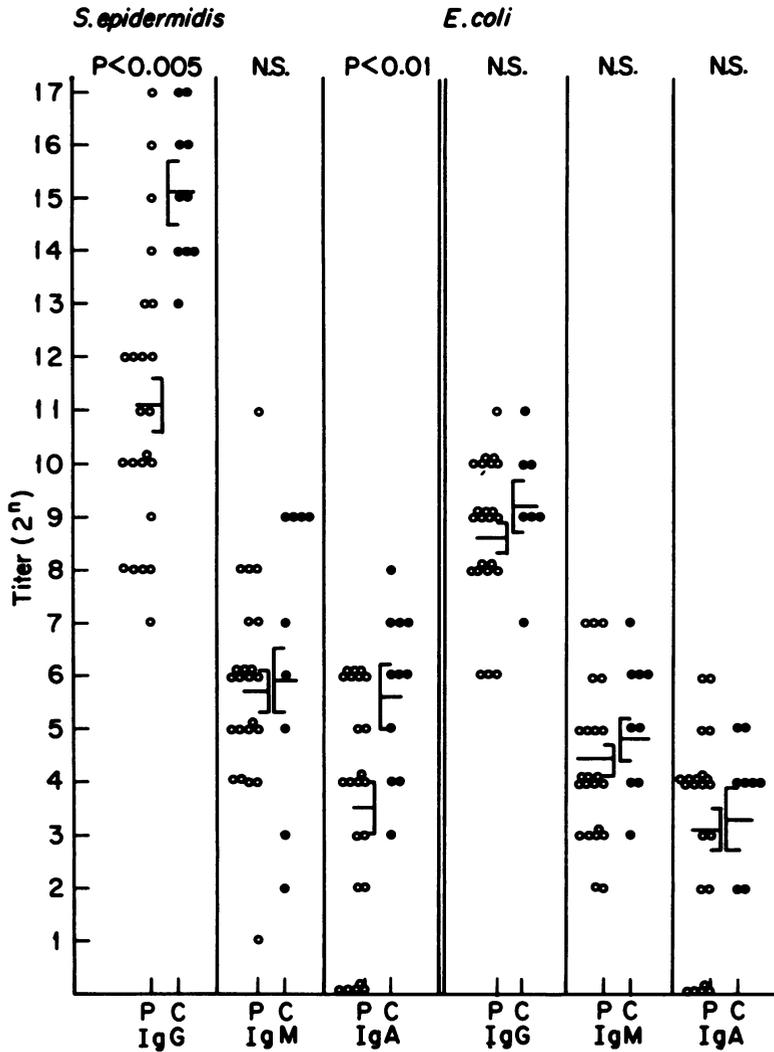


FIG. 4. Antibody titers to ultrasonicated nonoral microorganisms, expressed as \log_2 . Mean titers are shown \pm standard error of the mean. Significant differences between patients (P) and controls (C) are indicated. Symbols: \circ , patients; \bullet , controls. N.S., Not significant.

Relationship of age and antibacterial antibody titers in the control population. To determine whether there was a relationship between age and antibody titer to a specific microorganism, regression analysis was performed for each microorganism with both the control and the patient groups (data not shown). In no instance was a correlation between age and antibody titer found.

DISCUSSION

The results of this study indicated that IgA and IgG antibody titers to certain periodontal disease-associated microorganisms are depressed in the sera of periodontitis patients

when compared with those of healthy controls. Patient serum IgM titers, however, were not significantly different than those of controls. These results are not dissimilar from the findings of other laboratories (27, 36) which reported that humoral antibody titers decrease in advanced or chronic periodontitis.

In contrast, others (4, 9, 19, 23, 24, 36) have found either no correlation or elevated antibody titers in diseased subjects. Discrepancies between our findings and these reports are difficult to resolve. There are numerous variables which preclude meaningful inter-laboratory comparisons, including inconsistent clinical assessment of disease severity and differences in source,

mode of culture, and terminal processing of bacterial isolates. In addition, several of the immunological assays employed have not generally differentiated among the various major immunoglobulin classes. Thus, the titers reflect an admixture of various antibody classes or, more significantly, a bias for one class. For example, studies reporting no difference in serum antibody titers between patients and controls (19, 36) may be reflecting the bias of the hemagglutination assays for IgM antibodies, with other classes not even detected.

Depression of the humoral response may result from a variety of causes. (i) The depressed response to specific types of bacteria may be innate; that is, periodontitis patients have a general predisposition to develop lower antibody titers. This may compromise immunity to certain species residing in the marginal plaque. However, independent evaluation of the control subject data revealed no evidence of an age-associated antibody or total immunoglobulin level depression. Significantly, we did not observe depressed antibody levels to all bacteria. These findings tend to dispute gross, age-dependent reductions in immune competency in our experimental groups. (ii) The depression of antibody titers may reflect the absence or low levels of specific microorganisms in the gingival sulcus during this stage of the disease. Although the crevicular flora of our patients was not specifically assessed, comparable individuals have been evaluated within associated studies (S. Syed, personal communication, as well as by others, 35). These studies indicate that periodontal disease-associated microorganisms are present in substantial numbers at this level of disease severity. (iii) Antibody may be consumed as these microorganisms interact with sera (i.e., *in vivo* immunoadsorption), thus lowering titers in patients. Although this may occur at low levels, it is likely that the levels of bacteremias or septicemias (10) necessary to consume significant levels of antibody would result in other systemic disorders. In addition, such consumption of antibody is argued against by the fact that antibodies of the IgM class were not depressed. The data suggest that a class-specific regulatory mechanism, rather than simple immunoadsorption, is responsible for controlling antibody levels. (iv) The presence of locally high concentrations of specific microbial antigens may inhibit the immune response. Immune paralysis, or inhibition of humoral immunity, has been shown to result after the administration of supraoptimal antigen doses (6, 17). Current findings regarding the relationship between the humoral and secretory immune responses may more simply explain the specific inhibition of humoral immunity. Oral immuniza-

tion has been shown by several investigators to result in a depressed antigen-specific humoral response (2, 31, 38). Self-immunization of the gut-associated lymphoid tissue via swallowing or local sensitization with periodontally associated antigens may be expected to depress the antigen-specific humoral response. The absence of an effect on the IgM class cannot, however, be explained.

Data on antibody titers to the nonoral antigens are difficult to interpret. Although there was no difference between patient and control titers to *E. coli*, as might be predicted, the IgG and IgA titers to *S. epidermidis* were lower in patients. These findings may indicate a degree of cross-reactivity between the ubiquitous nonoral antigens and the oral flora.

Clearly, when evaluating the sera of individuals for reactivity to specific microbial isolates, or even to specific antigenic determinants, the true immunogen can never be positively identified. It was the intent of this study to assess gross reactivity of patient and control sera to a variety of periodontal disease-associated antigens. Immunoabsorptive procedures were not used to remove common antigens since any adsorbed reactivity might represent major antigens in the disease process. In diseases associated with either protective or destructive antibodies, one cannot discount the influence of cross-reactive or common antibodies, since it is the total immune reactivity which affects the disease process. With such an approach, trends and significant differences in immune reactivity might then identify representative microorganisms meriting further in-depth evaluation.

Although it is generally held that the secretory immune system is responsible for protection of the mucosal surfaces, there is evidence that significant levels of humoral antibody enter the oral cavity via exudates or transudates in the crevicular areas of the gingiva (11, 32). Also, antibodies of the IgG and IgM classes reactive with plaque microorganisms have been shown in gingival biopsies, suggesting a humoral source (8). As a result, humoral antibodies may be more important to the crevicular areas than are the products of the secretory immune system; therefore, modulation of humoral antibody levels may play a significant role in the immunological aspects of inflammatory periodontal disease.

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