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Lymphoproliferative Responses to Oral Bacteria in Humans with Varying Severities of Periodontal Disease

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We assessed in vitro the lymphocyte blastogenic responses of peripheral blood lymphocytes to antigen extracts of a large battery of oral microorganisms in a population of humans with varying severities of periodontal disease. When the magnitudes and frequencies of statistically positive blastogenic responses to various oral microorganisms were compared, three general patterns emerged. The *Actinomyces* species proved to be potent stimulators of lymphocyte blastogenesis in most subjects tested, whereas *Streptococcus sanguis*, *Campylobacter*, and *Eikenella corrodens* stimulated few individuals. The response to these organisms correlated poorly with the severity of periodontal disease in the tested patients. However, several gram-negative anaerobic organisms, including *Bacteroides asaccharolyticus* and *Treponema denticola*, elicited statistically more frequent positive responses in subjects with destructive periodontitis compared with patients with gingivitis. These results, taken together with recent microbiological findings, suggest that the specificity of the lymphocyte blastogenic response to antigens of oral bacteria correlates with the presence of these organisms in the subgingival microflora during various periodontal disease states.

Research into the etiology and pathogenesis of inflammatory periodontal disease has led to several important conclusions. It is clear that periodontal diseases are infectious diseases of bacterial origin and that these disease do not occur in the absence of bacteria (12, 33). Since direct tissue penetration of microorganisms occurs in only the most severe periodontal lesions (7), it can be inferred that tissue destruction results from the effects of bacterial products. The available evidence indicates that the host response to these bacterial products may be very important in the pathogenesis of periodontal disease (18).

A major advance in the study of the pathogenesis of periodontal disease has come from the recent finding that there is some specificity of the microorganisms comprising subgingival plaque as related to type and severity of periodontal lesions (17, 25-27, 29, 31). Therefore, if host response to bacterial antigens is important in the pathogenesis of periodontal disease, it seems likely that such a response should be elicited specifically toward the inciting bacterial antigens. To test this hypothesis, we assessed lymphocyte blastogenic responses to antigen preparations derived from a large and representative battery of oral microorganisms in a population of humans with varying states of periodontal health and disease. Specifically, we sought to determine (i) the general relationship of blastogenic responsiveness to periodontal disease state, (ii) whether the blastogenic response to a particular organism or group of organisms was characteristic of a given disease type or severity, and (iii) whether blastogenic responsiveness measured present or past antigenic stimulation, as assessed by evaluating edentulous subjects with a history of tooth loss due to periodontitis.

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MATERIALS AND METHODS

Selection of subjects. The subjects studied were selected from dental patients at the State University of New York at Buffalo School of Dentistry. Each received a complete dental examination, including medical history, oral examination, measurement of clinical indexes, and dental radiographs. Subjects with systemic disease, those taking medications, those with abnormal blood chemistry or hematological findings, or those who had had prior periodontal therapy were excluded. Patients were divided by the Russell periodontal index (24) into a normal group (Russell periodontal index, <0.5), a gingivitis group (Russell periodontal index, 0.5 to 2.0, and having no clinical or radiographic evidence of alveolar bone loss), a mild-to-moderate periodontitis group (Russell periodontal index, 2.0 to 4.0), and a severe periodontitis group (Russell periodontal index, >5.0). Patients who had been edentulous for at least 5 years and had no obvious oral areas of inflammation were also studied. Histories

of tooth loss due to periodontitis were obtained from these subjects. For the purposes of statistical comparison, the mild-to-moderate periodontitis and severe periodontitis groups were pooled into a group designated the combined periodontitis group. Likewise, the gingivitis, mild-to-moderate periodontitis, and severe periodontitis groups were pooled and designated the combined diseased group, and the normal and edentulous groups were pooled and designated the combined nondiseased group.

Preparation of antigens. *Actinomyces viscosus* ATCC 19246, *Actinomyces naeslundii* ATCC 12104, and *Streptococcus sanguis* ATCC 10556 were grown in Trypticase soy broth for 48 h.

Leptotrichia buccalis ATCC 19616 was grown for 72 h in EX-1 medium (16) in a CO_2 atmosphere. *Fusobacterium nucleatum* ATCC 25586 was grown in EX-1 medium for 72 h. *Selenomonas sputigena* isolate GB1 and *Capnocytophaga* strain S-10 were isolated by using the anaerobic procedures of Newman and Socransky (17) from a deep infrabony lesion of a patient with juvenile periodontitis. These last two organisms were grown anaerobically in EX-1 medium for 72 and 120 h, respectively. All bacteria were cultured at 37°C. All organisms were removed from cultures by centrifugation, washed three times in phosphate-buffered saline (0.02 M phosphate; pH 7.2), and frozen at −20°C until used.

In addition, frozen bacteria of the following types were kindly provided by S. Socransky, Forsyth Dental Center, Boston, Mass.: *Treponema denticola* strain K-1, *Bacteroides asaccharolyticus* strains 381 and 382, *Eikenella corrodens* strains 373 and 374, *Selenomonas sputigena* isolate 289, and a strain of *Campylobacter* (*Vibrio* Δ29). All cultures were checked for purity by morphology by phase-contrast microscopy, Gram staining, and subculture on appropriate solid media.

Sonication procedure. The bacteria were suspended in pyrogen-free saline, placed in a rosette sonication vessel immersed in ice water, and disrupted by a Heat Systems Sonifier (model W185; Branson Ultrasonics, Plainview, N.Y.) at approximately 95 to 100 W with an appropriate probe. The temperature of the solution in the vessel ranged from 0 to 10°C during sonication. The sonicated suspensions contained 10 to 100 mg (wet weight) of bacteria per ml. The suspensions were sonicated until more than 95% breakage of microorganisms had occurred, as determined by phase-contrast microscopy (23).

The sonic extracts were centrifuged at 12,000 × *g* for 30 min at 4°C, and the clear supernatants were removed. The concentrations of protein (13) (using bovine serum albumin as a standard), neutral sugar (5) (using glucose as a standard), and methylpentose (4) (using fucose as a standard) were determined for on each sonic extract supernatant. These measured concentrations were useful in determining whether new preparations of sonic extract were identical to previous preparations. The supernatants were divided into small samples and stored at −20°C until used.

In addition, the nonoral antigens streptokinase-streptodornase (Lederle Laboratories, Pearl River, N.Y.) and streptolysin O (Difco Laboratories, Detroit, Mich.) were prepared at concentrations of 50 U of streptokinase per ml and 50 μl of streptolysin O per ml of culture.

Lymphocyte blastogenesis. Lymphocyte blastogenesis was assessed by a microassay, as previously described (19). Briefly, heparinized peripheral blood was obtained from each donor, and the mononuclear cells were isolated on a Ficoll-Hypaque gradient (2). These cells were washed, counted, diluted to 10^6 cells per ml, and cultured in RPMI 1640 medium in 200-μl volumes with 20% autologous plasma (heated at 56°C for 30 min) in a microtiter plate. Quadruplicate cultures were incubated with and without (bacterial) preparations for 5 days, which in pilot experiments was found to give optimal stimulation. The last 6 h of culture was in the presence of 0.2 μCi of tritiated thymidine (specific activity, 2.0 Ci/mmol; Amersham/Searle). The cells were then harvested with an automated multiple-sample harvestor (Flow Laboratories), and tritiated thymidine incorporation was determined by liquid scintillation spectrometry with a Beckman model LS 3133T scintillation counter. Lymphocyte blastogenesis was quantitated by calculating a stimulation index, which was determined by taking the ratio of the geometric means calculated from the logarithms of the counts per minute in stimulated versus unstimulated cultures.

Dose-response curves were calculated for each individual for each antigen tested; the optimal stimulation index was used in tabulating the data. By using normal distribution theory and a pooled estimate of variance calculated from a large set of replicates, it was determined that stimulation indexes exceeding 2.5 indicated significant lymphocyte stimulation at the 95% confidence level. Statistical differences between groups were determined by a 2 × 2 Fisher exact test.

RESULTS

Characterization of bacterial antigens. The results of the chemical assays on each bacterial sonic extract are shown in Table 1. Most of the preparations were richer in protein than carbohydrate, with the exception of *Eikenella* strains 373 and 374 and *S. sputigena* isolate 289. There was relatively little methylpentose detected in the sonic extracts, except for *A. viscosus* and *A. naeslundii*, in which the methylpentose content was less than 10% of the protein. The values obtained by these chemical assays were useful in determining whether subsequent antigen preparations were equivalent to the original preparations.

Table 1 also shows the time required to break 95% of the organisms with a 95- to 100-W sonic force. The gram-positive bacteria (*A. viscosus*, *A. naeslundii*, and *S. sanguis*) required a minimum of 1.5 h of sonication. The gram-negative organisms, on the other hand, were disrupted in comparatively less time, usually less than 30 min.

Lymphocyte blastogenesis. Figure 1 shows the responses of the patients to the nonplaque

TABLE 1. *Protein, neutral sugar, and methylpentose measurements of bacterial sonic extracts*

Bacterium	Time to complete sonication (min)	Wet wt of bacteria sonicated (mg/ml)	Concn (μg/mg [wet wt] of bacteria sonicated) of:			Protein/ neutral sugar ratio	Protein/ methylpentose ratio	Concn of protein in undiluted sample (μg/ml of cell culture)
			Protein	Neutral sugar	Methylpentose			
A. viscosus	150	100	70	22	6.2	3.2	11.3	700
A. naeslundii	150	100	95	19	4.3	5.0	22.1	950
L. buccalis 19616	20	100	29	25	0.1	1.2	290.0	290
S. sanguis	90	100	51	20	0.8	2.5	63.8	510
F. nucleatum	15	100	21	2	0.1	9.1	210.0	210
S. sputigena GB1	15	100	76	11.6	0.6	6.5	126.3	758
S. sputigena 289	8	50	19	23	0.1	0.8	190.0	95
B. asaccharolyticus 381	10	100	93	28	0.6	3.3	155.0	930
B. assacharolyticus 382	10	100	76	28	0.6	2.7	126.6	760
E. corrodens 373	10	50	7	8	0.2	0.9	35.0	35
E. corrodens 374	10	50	7	8	0.2	0.9	35.0	35
Campylobacter Δ29	8	20	13	6	0.1	2.2	130.0	26
Spirochetes K-1	10	100	20	2	< 0.1	10.0	>200.0	200
Capnocytophaga S-10	12	100	38	8	0.1	4.8	380.0	380

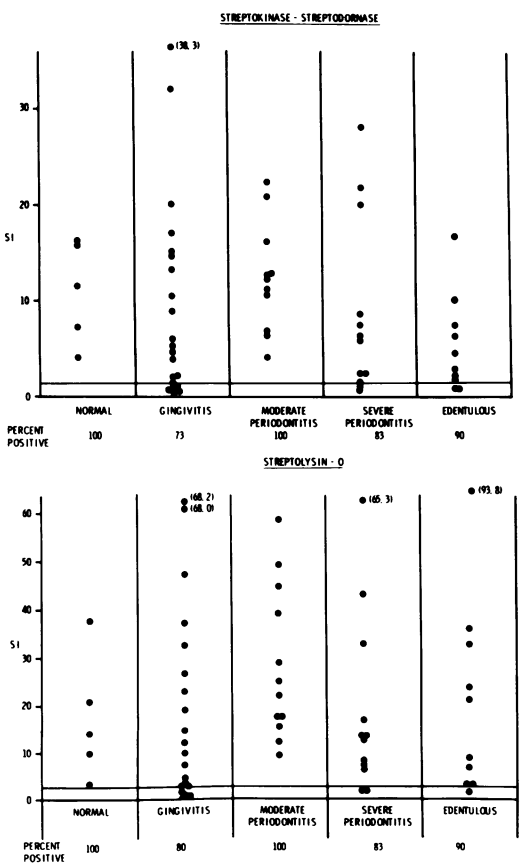

FIG. 1. *Lymphocyte stimulation of patient groups with streptokinase-streptodornase (50 U of streptokinase per ml) and streptolysin O (50 μl/ml). There were no statistically significant differences among the groups. SI, Stimulation index.*

antigens streptolysin O and streptokinase-streptodornase. These antigens stimulated most subjects, and no group differences were found.

Figure 2 shows the blastogenic responses of the groups to sonic extracts of *A. viscosus* and *A. naeslundii*. These antigen preparations elicited strong responses from all groups of subjects tested, although the response of the normal group was significantly less than the response of the diseased groups. No differences were noted in the magnitudes or frequencies of the positive responses among the remaining subject groups.

The responses of the groups to antigen preparations of *L. buccalis* and *F. nucleatum* are shown in Fig. 3. The gingivitis group was significantly more reative to the antigens of *L. buccalis* than either the edentulous group or the normal group. The patients in the combined periodontitis group were significantly more reactive than the patients in the nondiseased group. Similar findings occurred with antigens of *F. nucleatum*; the gingivitis group was significantly more reactive than both the edentulous group and the normal group. The patients in the combined diseased group were significantly more reactive than those in the combined nondiseased group.

Figure 4 shows the blastogenic responses to antigens of *S. sputigena*. Each of the individual diseased groups was significantly more reactive to strain GB1 than the edentulous group. The combined diseased and combined periodontitis groups were also significantly more reactive than the edentulous group.

The overall reactivities of the groups to *S. sputigena* isolate 289 were much less than their reactivities to *S. sputigena* isolate GB1. No sig-

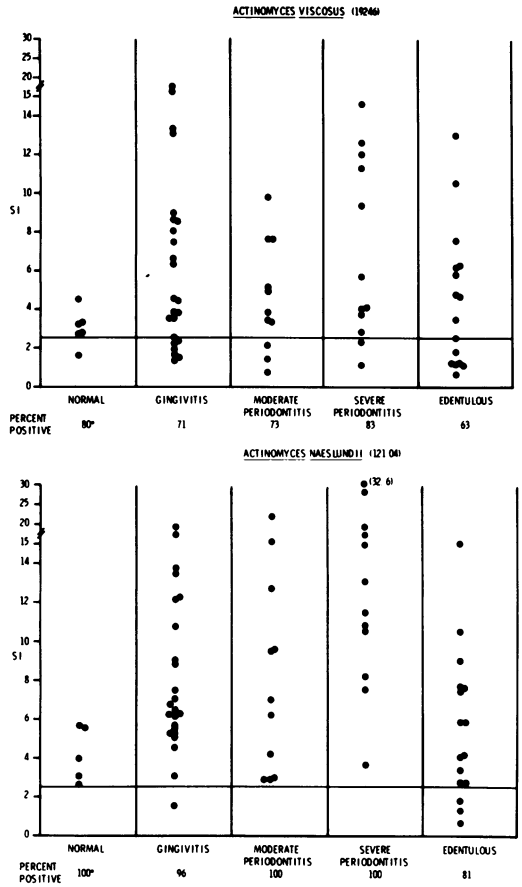

FIG. 2. *Lymphocyte stimulation of patient groups with sonic extract antigens of A. viscosus and A. naeslundii. The responses of the normal group were significantly less than those of the other groups ($P < 0.05$). SI, Stimulation index.*

nificant differences were noted for any of the groups tested.

Figure 5 shows the responses to two isolates of *B. asaccharolyticus*. The mild-to-moderate periodontitis, combined periodontitis, and combined diseased groups were significantly more reactive than the edentulous group. Also, the mild-to-moderate periodontitis group showed greater reactivity than the gingivitis group.

The reactivities of the patient groups to *B. asaccharolyticus* isolate 382 were similar to their reactivities to *B. asaccharolyticus* isolate 381. However, even clearer differences between the groups were noted. Each of the diseased groups was significantly more reactive than the edentulous group. The combined periodontitis and combined diseased groups were likewise more reactive than the edentulous group. In addition, the mild-to-moderate periodontitis group and the combined periodontitis group were significantly more reactive than the gingivitis group.

Figure 6 shows the responses of the groups to antigens of an oral spirochete, *T. denticola*. There was significantly greater stimulation in the severe periodontitis group and the combined periodontitis group compared with the low response in the edentulous group. In addition, the severe periodontitis and combined periodontitis groups were significantly more stimulated than the normal group. The combined diseased group was significantly more reactive than the combined nondiseased group. In response to this antigen preparation, the severe periodontitis group and the combined periodontitis group were significantly more reactive than the gingivitis group.

The responses of the subjects to *E. corrodens* isolates 373 and 374 are shown in Fig. 7. No statistical differences were noted among the tested groups, probably because the level of

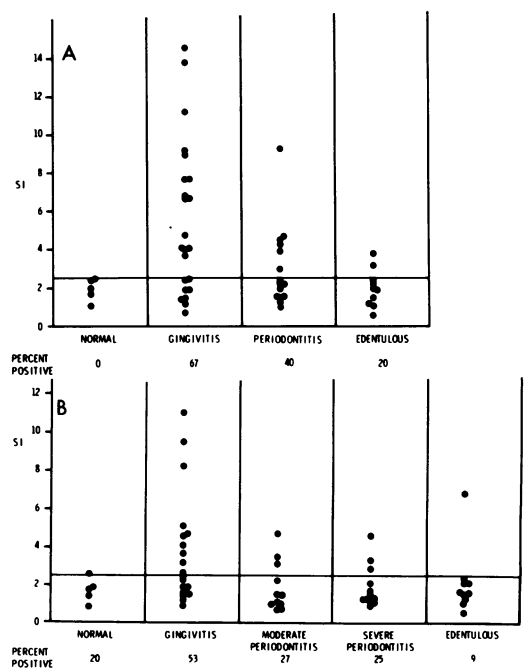

FIG. 3. *Lymphocyte stimulation of patient groups with sonic extract antigens of L . buccalis 19616 (A) and F. nucleatum 25586 (B). Statistically significant differences in the frequencies of positive responses to both L. buccalis and F. nucleatum included the following: gingivitis more reactive than edentulous (L. buccalis, $P = 0.015$; F. nucleatum, $P = 0.019$) or normal (L. buccalis, $P = 0.01$; F. nucleatum, $P < 0.05$); diseased more reactive than nondiseased (L. buccalis, $P = 0.005$; F. nucleatum, $P = 0.03$). SI, Stimulation index.*

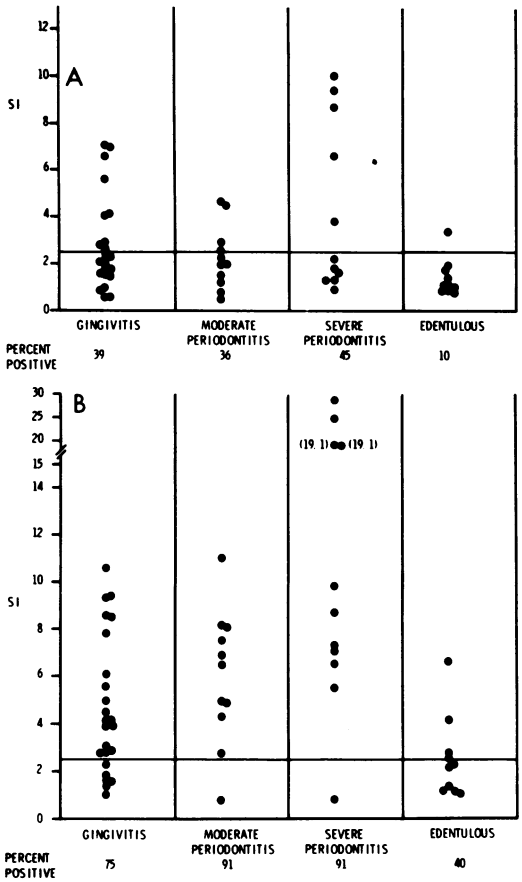

FIG. 4. *Lymphocyte stimulation of patient groups with sonic extract antigens of S. sputigena 289 (A) and GB-1 (B). With strain GB1, each of the individual diseased groups was significantly more reactive than the edentulous group (gingivitis, P = 0.05; moderate periodontitis, P = 0.02; severe periodontitis, P = 0.02). The combined diseased and combined periodontitis groups were also significantly more reactive than the edentulous group (P = 0.007 and 0.005, respectively). SI, Stimulation index.*

response to these organisms was low.

Other oral bacteria which proved to be poor stimulators of lymphocyte blastogenesis in all subject groups included the isolates of *S. sanguis* and *Campylobacter* tested. As Fig. 8 shows, less than 20% of the groups tested were significantly reactive to these antigen preparations.

Finally, we studied the lymphoproliferative responses stimulated by an organism of recent interest, *Capnocytophaga*. Although considerable difficulty was encountered in obtaining this organism in pure culture and in deriving an antigen preparation which lacked inherent toxicity to lymphocytes, one strain (strain S-10) was suitable for study. Figure 9 shows the responses obtained with this organism. *Capnocytophaga* was a potent stimulator of blastogenesis in diseased patients, but no differences in blastogenic responses were detectable among the diseased groups. However, the patients with periodontitis were significantly more frequently reactive to this organism than the edentulous patients.

DISCUSSION

Three general patterns of blastogenic responses to oral bacterial antigens are evident.

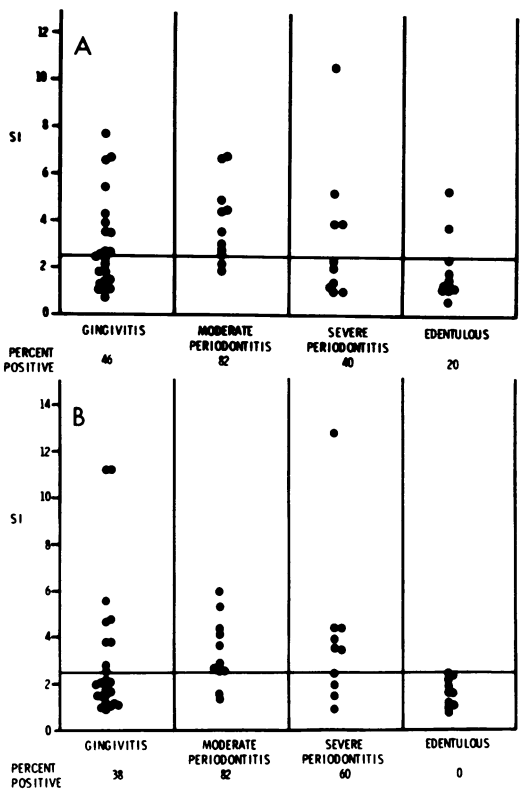

FIG. 5. *Lymphocyte stimulation of patient groups with sonic extract antigens of B. asaccharolyticus 381 (A) and 382 (B). With isolate 381, the moderate periodontitis, combined periodontitis, and combined diseased groups were significantly more reactive than the edentulous group (P = 0.007, 0.03, and 0.04, respectively). The mild-to-moderate periodontitis group showed greater reactivity than the gingivitis group (P = 0.04). With isolate 382, each of the diseased groups was significantly more reactive than the edentulous group (gingivitis, P = 0.025; moderate periodontitis, P = 0.0002; severe periodontitis, P = 0.005). The combined periodontitis and combined diseased groups were more reactive than the edentulous group (P = 0.0002 and 0.001, respectively). The mild-to-moderate periodontitis group and the combined periodontitis group were significantly more reactive than the gingivitis group (P = 0.015 and 0.019, respectively). SI, Stimulation index.*

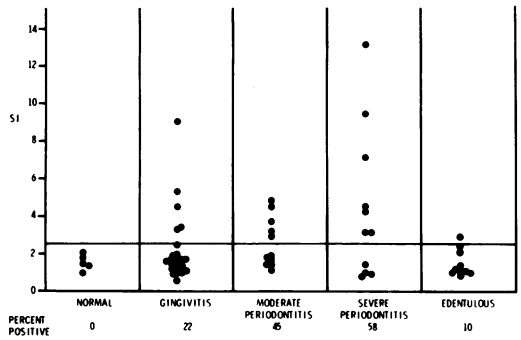

FIG. 6. *Lymphocyte stimulation of patient groups with sonic extract antigens of T. denticola (spirochete K-1). There was significantly greater stimulation in the severe periodontitis group (P = 0.025) and combined periodontitis group (P = 0.02) compared with the edentulous and normal groups (P = 0.04 and 0.04, respectively). The combined diseased group was significantly more reactive than the combined nondiseased group (P = 0.012), and the severe periodontitis and combined periodontitis groups were significantly more reactive than the gingivitis group (P = 0.032 and 0.036, respectively). SI, Stimulation index.*

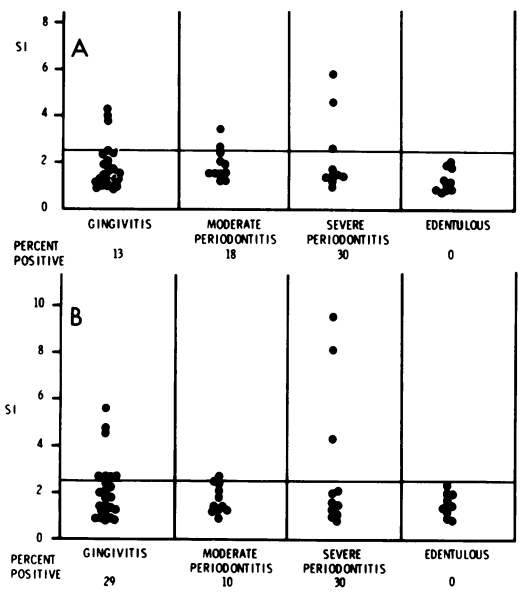

FIG. 7. *Lymphocyte stimulation of patient groups with sonic extract antigens of E. corrodens 373 (A) and 374 (B). There were no statistical differences among the groups tested. SI, Stimulation index.*

The first pattern is represented by the response to *Actinomyces* (Fig. 2). This organism proved to be a potent stimulator of lymphocytes in most patients tested. Although this response pattern might appear to be similar to the response to a nonspecific mitogen and recent evidence has shown that *Actinomyces* contains substances which are B-cell mitogens in rodents (3, 6), we found previously that this preparation stimulates very few human fetal cord bloods and therefore does not appear to be a nonspecific mitogen to human peripheral lymphocytes (19, 23). Alternatively, this reaction might reflect host sensitization caused by the ubiquitous presence of this organism in the oral cavities of humans (31).

Microbiological evidence suggests that *Acti-*

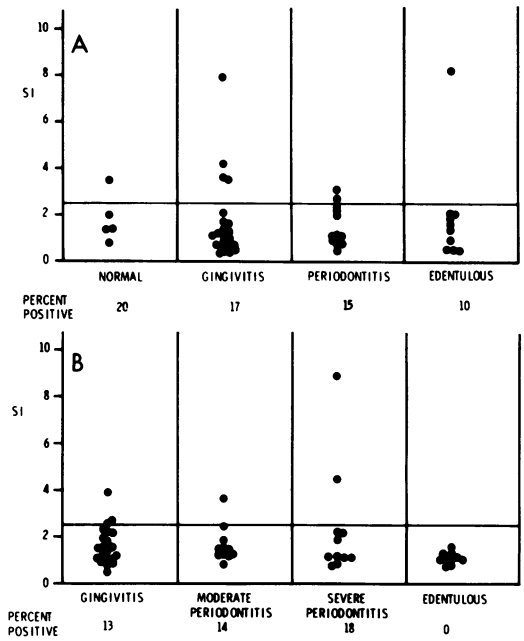

FIG. 8. *Lymphocyte stimulation of patient groups with sonic extract antigens of S. sanguis 10556 (A) and Campylobacter Δ29 (B). There were no statistically significant differences. SI, Stimulation index.*

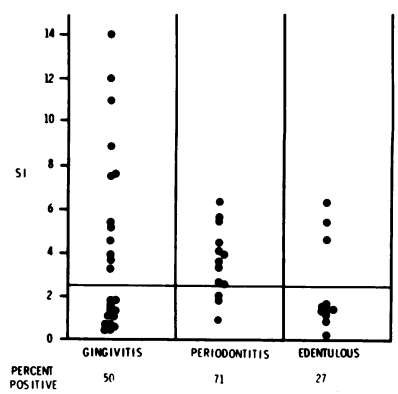

FIG. 9. *Lymphocyte stimulation of patient groups with sonic extract antigens of Capnocytophaga. The combined periodontitis group was significantly more reactive than the edentulous group (P = 0.018). SI, Stimulation index.*

nomyces are present in all dentulous subjects and that their numbers correlate with the presence of periodontal disease (25, 27, 29, 31, 32, 34). This agrees with the lower blastogenic responses obtained from normal subjects, who harbor fewer organisms than diseased subjects. The edentulous subjects who responded strongly may have been continually sensitized due to the presence of *Actinomyces* on artificial dentures (14, 15). In addition, longitudinal studies of cellular immunity to *Actinomyces* during the induction or resolution of gingivitis confirm the relationship between magnitude of blastogenic response and suspected numbers of *Actinomyces* cells in plaque (11, 20, 21, 30). Therefore, the measured response to *Actinomyces* appears to represent a naturally occurring cell-mediated immune response to a commensal organism which sensitizes the host primarily through the oral tissues.

In contrast to the *Actinomyces* response, all groups responded weakly to *S. sanquis* (Fig. 8). Although a commensal organism, this bacterium appears to be unable to stimulate a strong cellular immune response, or else the antigenic preparations used in this study were unable to elicit this response in vitro. Similar findings were obtained with *E. corrodens* and *Campylobacter*, bacteria suspected of being periodontal pathogens.

Finally, a different pattern of reactivity was shown by the blastogenic responses to spirochetes (Fig. 6) and *B. asaccharolyticus* (Fig. 5). Although these organisms appear to be capable of eliciting strong cellular immune responses, these responses seem to be limited to those individuals with destructive periodontitis. Recently, microbiological findings have related the presence of these organisms in subgingival plaque to destructive periodontitis in both humans (26) and monkeys (28).

Therefore, it seems likely that the blastogenic response patterns elicited by plaque organisms are dependent both on the presence of these organisms in plaque and on their ability to stimulate a cell-mediated immune response in the host. Also important is the ability of the antigen preparations derived from such organisms to stimulate sensitized cells in in vitro assays.

This cross-sectional study of blastogenic responses of humans with varying severities of periodontal disease to a large battery of plaque organisms has given the following information. (i) A general increase in the blastogenic responses to antigens of plaque organisms was noted when subjects with periodontal disease were compared with disease-free individuals. This finding is supported by the work of Horton et al. (8), Ivanyi and Lehner (9), Lang and Smith (10), and Baker et al. (1). (ii) The frequency of positive responses or the magnitudes of these responses failed to distinguish varying severities of periodontal disease. Exceptions to this were the responses to *B. asaccharolyticus* and *T. denticola*, which more consistently stimulated lymphocytes of subjects with periodontitis than subjects with gingivitis. These results are supported by the work of Lang and Smith (10), who found that *Bacteroides melaninogenicus* stimulated lymphocytes of subjects with periodontitis. (iii) The in vitro lymphoproliferative response to plaque antigens appears to reflect current antigenic stimulation and not memory of past antigenic encounters. Therefore, lymphocytes from edentulous subjects with a history of periodontitis were generally unreactive to organisms associated with destructive periodontitis, whereas lymphocytes from subjects with the disease were significantly reactive to these same antigenic preparations. And (iv) in general, the pattern of lymphocyte reactivity to plaque organisms correlates with the presence of the organisms in the flora adjacent to periodontal tissues during various periodontal disease states. The studies of the microbiota associated with gingivitis and periodontitis by Slots et al. (25, 26, 29), Socransky (31), and Newman and Socransky (17) provide convincing evidence that as the severity of periodontal disease increases, the flora shifts from primarily gram-positive bacteria to primarily gram-negative bacteria. Our studies suggest that associated with this change in bacterial flora is a change in the specificity of the lymphoproliferative response. Most gram-negative organisms tested stimulated lymphocytes of subjects with periodontitis much more frequently than lymphocytes of subjects without periodontal disease.

These results provide convincing evidence that a cellular immune response to the periodontal flora is present in periodontal disease states and may contribute to the inflammation observed with these diseases. Furthermore, these results suggest that detailed longitudinal studies of cell-mediated immunity to relevant antigens of organisms such as *B. asaccharolyticus* may reveal a temporal relationship between immunity and disease onset or resolution. Such a correlation may be useful in assessing disease activity, monitoring successful treatment, and predicting recurrent episodes of active disease.

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