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Evidence of Mitogenic Activity in Periodontitis-Associated Bacteria

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This study examines several periodontitis-associated bacterial isolates for the presence of mitogenic activity, as indicated by their capacity to stimulate unsensitized lymphocytes to undergo blastogenesis. Germfree mouse spleen cells responded vigorously to all of the bacterial sonic extracts tested. The kinetics and dose responses to these activators in germfree mouse spleen cell cultures paralleled those seen with the standard murine B-cell mitogen, *Escherichia coli* lipopolysaccharide. In contrast, Streptokinase-Streptodornase (Varidase; Lederle Laboratories) antigen elicited no response. Human cord blood lymphocytes also responded upon stimulation with these same bacterial isolates but failed to respond to Streptokinase-Streptodornase. The frequency, magnitude, and kinetics of these cord blood lymphocyte responses were remarkably similar to those seen with adult peripheral blood lymphocytes. However, in this and previous studies, individuals with unresponsive peripheral blood lymphocytes have been observed. Studies were initiated to determine whether these unresponsive leukocyte preparations truly lacked the capacity to respond to these bacteria or whether unresponsiveness reflected the presence of a regulatory cell population in these cultures. After the removal of the adherent cells from unresponsive peripheral blood lymphocyte cultures, the nonadherent cells were found to be responsive. Therefore, peripheral blood lymphocyte responsiveness appears to be regulated via an adherent cell population. The removal of adherent cells from unresponsive cord blood lymphocyte preparations resulted in a less consistent alteration to responsiveness. However, cord blood lymphocyte preparations unresponsive at a standard cell density were shown to be responsive at altered cell densities.

Models of lymphocyte activation via-antigen-specific or polyclonal nonspecific stimulation by periodontitis-associated bacteria have been proposed to explain the initiation and progression of periodontal disease (18, 29). In support of the polyclonal nonspecific model are numerous recent studies reporting the presence of polyclonal B-cell activators in periodontitis-associated bacteria (3, 6, 9, 22, 29), the presence of mitogenic activity in a number of nonoral isolates (2, 19, 26–28), and the presence of mitogenic activity in purified preparations of peptidoglycans or teichoic acids extracted from bacterial cell walls (8, 12–14, 25). In the past, two major observations have been cited as evidence arguing against polyclonal nonspecific mechanisms as a probable explanation for the reported blastogenic responsiveness seen in lymphocyte preparations stimulated with periodontitis-associated bacteria. First, human umbilical cord blood lymphocytes (CBLs) have been reported to be generally unresponsive after stimulation with dental plaque or bacterial preparations (1, 16, 17, 24). Second, peripheral blood lymphocyte (PBL) preparations taken from some periodontally diseased or healthy subjects have not responded after stimulation with dental plaque or bacterial preparations (1, 10, 11, 16, 17, 24, 31). If the bacteria possess mitogenic activity, both CBLs and PBLs should respond after stimulation regardless of the presence or absence of prior sensitization to the given bacterial stimulant. The objectives of this study were to examine: (i) periodontitis-associated bacteria for their capacity to induce mitogenesis in germfree mouse spleen cells, human CBLs, and adult PBLs and (ii) unresponsive PBL and CBL preparations to determine whether their unresponsive state reflects a complete inability of lymphocytes to respond or whether it reflects the presence of a regulatory cell population in these unresponsive cultures. By a microtiter blastogenesis assay, whole-cell sonic extracts of several periodontitis-associated bacteria were found to be consis-

tently capable of eliciting blast transformation in germfree mouse spleen cells and CBL and PBL cultures. Although a number of CBL and PBL preparations initially failed to respond under standard culturing conditions, subsequent experiments indicated that the unresponsive PBL preparations could be rendered responsive after the removal of the adherent cell population and the CBL preparations which failed to respond under standard culturing conditions were also capable of responding at altered cell densities.

MATERIALS AND METHODS

Bacterial preparation procedures. Bacterial stimulants consisted of whole-cell sonic extracts of *Actinomyces israelii* D12B11, *Actinomyces viscosus* D34B24, *Peptostreptococcus micros* D31C23, *Actinobacillus actinomycetemcomitans* 13127, *Bacteroides gingivalis* D11B26, *Bacteroides intermedius* D16B17, *Bacteroides denticola* D2B18, and *Fusobacterium nucleatum* D30A9. All strains except *A. actinomycetemcomitans* 13127 were isolated and cultured from periodontal microflora by L. V. Holdeman and W. E. C. Moore of the Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg (VPI). *A. actinomycetemcomitans* was obtained from A. C. R. Tanner, Forsyth Dental Center, Boston, Mass., and mass cultivated at the VPI laboratory.

All bacterial cells were cultivated, harvested, washed twice in phosphate-buffered saline and once in a mixture of 1 part glycerol and 2 parts phosphate-buffered saline, frozen, stored, and shipped from VPI to our laboratory. Bacteria were later thawed, washed three times in phosphate-buffered saline, and sonicated (Bronwill Biosonik Sonicator) for maximum disruption. The degree of disruption was assessed by phase-contrast microscopy. Protein concentrations were determind by the method of Lowry et al. (21). The sonic extracts were maintained in a frozen state until used.

Purified Streptokinase-Streptodornase Varidase (SK/SD; Lederle Laboratories) was chosen as a nonmitogenic negative control stimulant in the germfree mouse spleen cell and CBL cultures. Stock SK/SD (5 ml) was dialyzed for 72 h against three 100-ml changes of RPMI 1640 medium to remove preservatives. *Escherichia coli* lipopolysaccharide (LPS; Difco Laboratories), a standard murine B-cell mitogen, was used as a positive control stimulant in the germfree mouse spleen cell cultures. Concanavalin A (ConA; Calbiochem-Behring Corp.), a standard human T-cell mitogen, was selected as a positive control stimulant in both CBL and PBL cultures.

Germfree mouse spleen cell cultures. Germfree C3H mice (4- to 5-week-old females) were obtained from Harland Sprague-Dawley Laboratories. These guaranteed-germfree mice were delivered in a sterile disposable container with bedding and food; they were sacrificed immediately after breaking the seal on the container. Spleens were excised, and the cells were dispersed through a fine wire mesh screen. The dispersed spleen cells were washed three times in RPMI 1640 medium, counted, and divided into two portions. The washed cells for serum-containing spleen cell cultures were suspended to the appropriate cell concentration in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 0.005 M HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid). Standard culturing conditions consisted of 5×10^5 cells per well at 37°C in a 5% CO_2 humidified incubator. Washed cells for serum-free spleen cell cultures were suspended and cultured as above, with only the FCS being omitted from the culturing medium.

Human CBL cultures. The following lymphocyte separation techniques were used based on previous reports and preliminary experiments in our laboratory. Previous investigators have reported that the blastogenic response, particularly when the microtiter blastogenesis assay is used, is susceptible to inhibition by the presence of polymorphonuclear leukocytes in lymphocyte preparations (23). Initial experiments in which various cord blood separation techniques were examined confirmed this finding and indicated that neither dextran sedimentation nor Ficoll-Hypaque separation alone was ideal (data not shown). CBL separation by dextran sedimentation, a standard technique for polymorphonuclear leukocyte isolation, yielded a lymphocyte preparation heavily contaminated with polymorphonuclear leukocytes. A combination of dextran sedimentation followed by Ficoll-Hypaque separation appeared to be more ideal for consistently yielding CBL preparations free of both polymorphonuclear leukocytes and other nonlymphocyte blood elements. Based on these findings, the following procedure was used. Human umbilical cord blood was collected from placenta afterbirths after normal deliveries. Cord blood was collected directly into syringes containing preservative-free heparin (final dilution, 25 U/ml) and 6% dextran (Dextran-200, pyrogen-free) in RPMI 1640 medium. The final syringe volume was composed of 1 part blood, 1 part 6% dextran in RPMI 1640 medium, and 2 additional parts RPMI 1640 medium. Syringes were inverted to mix the contents, and the leukocyte-rich plasma resulting from gravity sedimentation of the erythrocytes was removed, layered over Ficoll-Hypaque lymphocyte separation medium, and separated using standard lymphocyte separation techniques (4). Separated CBLs were washed three times in RPMI 1640 medium and suspended to 2.5×10^6 cells per ml in culture medium consisting of RPMI 1640 medium supplemented with 10% heat-inactivated pooled human A serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. A single lot of human A serum and the same bacterial and mitogen preparations were used for all CBL and PBL cultures compared in this study.

Human PBL cultures. PBLs were obtained from subjects determined to be free of periodontitis and severe gingivitis by clinical examination. Peripheral blood was obtained by venipuncture, and PBLs were separated, stimulated, and cultured using the same methods and reagents as those used for CBL cultures.

Preparation of nonadherent PBLs. Nonadherent PBL preparations were obtained using a standard adherence procedure. Briefly, polystyrene tissue culture plates (100 by 20 mm) were prewetted with 10 ml of RPMI 1640 medium containing 10% human A serum. The PBL suspension was adjusted to 10^7 cells per ml, and 5 ml of the suspension was added to each plate. Incubation proceeded for 90 min at 37°C in a 5%

CO_2 humidified incubator. After incubation, the plates were gently decanted, 5 ml of RPMI 1640 medium containing 10% human A serum was added to each dish, and the medium was gently decanted again. This decanting procedure was repeated five times to yield the nonadherent cell preparations. The residual adherent cells were harvested from the plates, using a Teflon scraper and vigorous washing with RPMI 1640 medium containing 10% human A serum. After harvesting, the unseparated, the adherent, and the nonadherent cell preparations were washed by centrifugation, suspended in culture medium and examined for viability and nonspecific esterase staining (32). All PBL and CBL preparations had greater than 95% viability. Esterase-positive cell percentages for the unseparated, the nonadherent, and the adherent PBL preparations averaged 20.0 ± 1.2, 1.7 ± 0.3, and 60.1 ± 4.8, respectively.

Blastogenesis assay procedure. Blastogenic responses to the various bacterial preparations, antigens, and standard mitogens were assayed using the following protocol unless otherwise stated. Cells were suspended in the standard culture medium and aliquoted into flat-bottom microtiter plates. Each well received 5×10^5 cells of unseparated PBLs in 200 μl of suspension. The cell concentration of nonadherent cell cultures was slightly less than 5×10^5 per well due to removal of the adherent cells. Cultures were immediately stimulated by adding 20 μl of the stimulant suspension to triplicate wells at each dose. All mouse spleen cell, CBL, and adult PBL cultures were stimulated over at least a three-log range of dilutions. Final concentrations in the stimulated spleen cell, CBL, and adult PBL cultures were: 5, 50, and 500 μg of protein per ml of bacterial sonic extracts; 1, 10, and 100 μg of protein per ml of *E. coli* LPS; 1, 10, and 100 μg of protein per ml of ConA; and 1:1,000, 1:100, and 1:10 dilutions from dialyzed stock SK/SD. Cultures were then covered and incubated at 37°C in a 5% CO_2 atmosphere for the designated time period. All CBL and PBL cultures were harvested on day 6, except the ConA-stimulated cultures which were harvested on day 3. At 4 h before harvesting, each well received 1.25 μCi of [*methyl*-^{3}H]thymidine (specific activity, 50 to 60 Ci/mmol) in a 50-μl amount. After the labeling period, cells were harvested on glass-fiber filter paper with a MASH II multiple automated sample harvester. Samples were dried, placed in vials with scintillation fluid, and counted on a Beckman LS-3150T scintillation counter. The stimulation index (SI), that is, the ratio of experimental counts to control counts, was determined for each subject at each dose. The difference (Δcpm) between mean stimulated counts and mean background counts for each set of replicate tubes was also determined. The frequency of responses, the optimal dose for responses, and the magnitude of responses were examined and compared using peak SI and Δcpm values for each stimulant.

RESULTS

Germfree mouse spleen cell responses. Peak blastogenic responses of germfree mouse spleen cells after stimulation with several bacterial sonic extracts are shown in Fig. 1. Strong blastogenic responses were elicited in the presence or absence of FCS, indicating that the blastogenic activity could be directly attributed to bacterial stimulation, as opposed to nonspecific stimulation by FCS. All seven bacterial sonic extracts tested elicited strong blastogenic responses in these unsensitized cells, reflecting the presence of mitogenic activity. Maximum stimulation often approached in magnitude the response to *E. coli* LPS, a standard murine B-cell mitogen and the positive mitogenic control. Both gram-positive and gram-negative isolates possessed this ability to stimulate unsensitized cells, indicating that this activity was not limited to the gram-negative LPS-containing isolates. The kinetics of all seven responses paralleled the *E. coli* LPS response, peaking early on day 2 and then sharply falling. Day 4 and 5 data indicated that these responses continued to decline, with no subsequent peaks being observed (data not shown). In all cases, responses elicited by these bacterial sonic extracts were significantly greater than those seen against SK/SD, a strict antigen and the negative control.

Human CBL and PBL responses. To begin a comparative analysis of CBL and adult PBL responses, each CBL and PBL preparation was designated as either responsive (peak SI ≥ 2.0) or unresponsive (peak SI < 2.0) to each stimulant tested under the standard culturing condition. An SI of ≥2.0 was generally higher than was necessary to demonstrate a statistically significant difference ($P < 0.05$) between stimulated and background counts in these and previous data from our laboratory. As the biological significance might be questioned for some statistically significant responses with low SIs, this strigent criterion of a doubling of background counts upon stimulation was adopted to increase confidence for concluding biological responsiveness. Table 1 indicates the frequencies of responder CBL and PBL preparations for each stimulant. Although the CBL and PBL response frequencies to the gram-positive isolates were essentially identical, CBLs appeared to respond more frequently than did PBLs to the gram-negative isolates and to *E. coli* LPS. However, a 2 × 2 chi-square analysis of the data indicated that only in the case of *E. coli* LPS ($P < 0.01$) was the frequency of response between CBLs and PBLs statistically significant. As expected, the frequency of response to the strict antigen SK/SD was reversed, with PBLs tending to respond more frequently. If additional CBL and PBL SK/SD responses tested in related studies performed in this laboratory are included with these data, the response frequency for CBLs becomes 1/18 and that for PBLs becomes 12/25. Chi-square analysis comparing these ratios indicated a significant difference in responsiveness to SK/SD ($P < 0.001$), reflecting the effect of

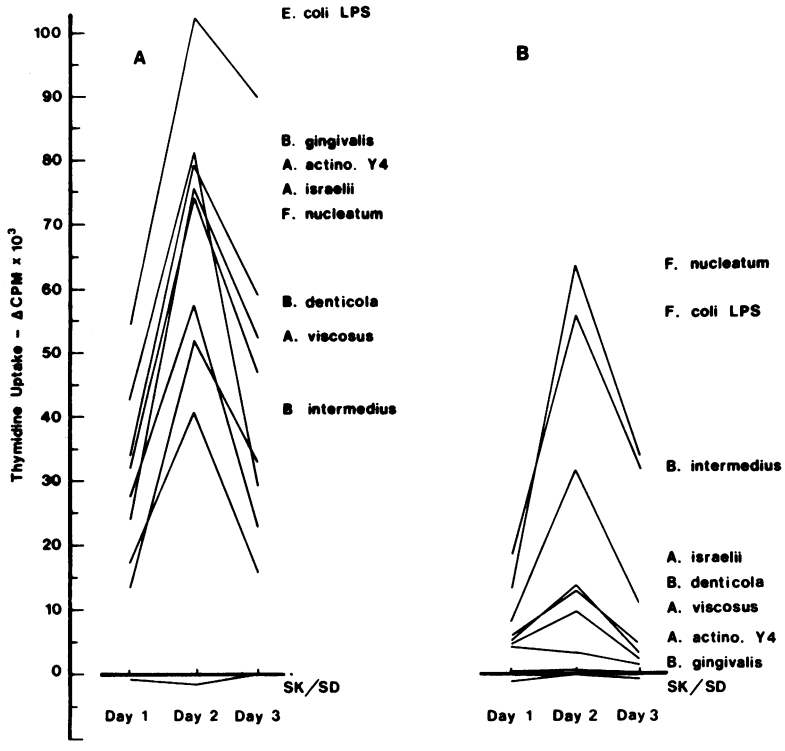

FIG. 1. Comparison of the peak blastogenic responses elicited by bacterial stimulants in germfree mouse spleen cell cultures. Cells were cultured in 10% FCS-supplemented (A) and unsupplemented (B) culturing medium. Stimulants are indicated directly to the right of the peak day 2 response, giving an indication of the magnitude and ranking of each response. Day 1, 2, and 3 background counts in unstimulated cultures were, respectively, 1,992 ± 74, 1.983 ± 51, and 685 ± 45 in the presence of FCS and 403 ± 82, 363 ± 22, and 182 ± 28 in the absence of FCS.

prior exposure and sensitization to SK/SD on the blastogenic responses of adult PBLs.

A comparison of the CBL and PBL sensitivity to stimulation by these sonic extracts was made by examining the dose response distribution of the responder subjects in each group. Table 1 indicates the distribution of the maximum stimulatory doses for each stimulant. With all bacterial isolates tested, there was an apparent tendency for CBLs to respond to lower stimulant doses. For example, although the CBL and PBL response frequencies to *A. israelii* were similar (8/11 and 9/11, respectively), the CBL response distribution showed maximum stimulation at the lower doses of 5 and 50 μg/ml, whereas the maximum stimulatory dose in PBLs always occurred at the maximum dose of 500 μg/ml.

A comparison of the magnitude of the peak Δcpm responses of responding individuals in each group is seen in Fig. 2. The mean peak Δcpm values from responsive CBL preparations were similar in magnitude to those seen from PBL preparations. In no case was the response of PBLs significantly greater than the CBL response. In fact, in one case, the Student *t* test showed that the CBL response to *A. actinomycetemcomitans* was modestly elevated as compared with the PBL response ($P < 0.01$). The ConA-stimulated CBL and PBL cultures also showed no differences in either the magnitude or time kinetics of the elicited responses. All CBL and PBL cultures showed strong stimulation in response to ConA, with responses peaking at day 3 and subsequently falling (data not shown). Examination of the above data also revealed a trend toward individual responsiveness or unresponsiveness. This trend was particularly striking in the CBL responses. Of the 11 CBL preparations, 6 responded to all stimulants except SK/SD. One CBL preparation responded to all bacteria except one, and three CBL preparations failed to respond to any of the bacterial stimulants tested despite responsiveness to ConA.

In this and previous studies (10, 11, 31), we found that under the standard culturing conditions of 5×10^5 cells per well, ca. 70% of periodontally diseased and healthy adult subjects and ca. 80% of more than 80 cords examined were responsive after stimulation with

TABLE 1. Comparison of CBL and adult PBL responses to bacterial stimulants

Bacterial stimulant	Lymphocyte response							
	Response frequency[a]		Distribution of maximum stimulatory doses[b]					
			CBL[c]			PBL[c]		
	CBL	PBL	LD	MD	HD	LD	MD	HD
A. israelii	8/11	9/11	5	3	0	0	0	9
A. viscosus	8/11	10/11	3	4	1	0	0	10
P. micros	6/9[d]	5/11	3	3	0	1	1	3
A. actinomycetemcomitans	7/11	3/11	5	2	0	2	0	1
B. gingivalis	7/11	3/11	6	1	0	1	1	1
B. denticola	7/11	3/11	7	0	0	1	0	2
F. nucleatum	7/11	4/11	2	5	0	0	0	4
E. coli LPS	7/11[e]	1/11	4	3	0	0	0	1
SK/SD	1/11[e]	4/11	0	0	1	2	2	0

[a] Response frequency is the ratio of responders to total subjects tested. Responders were designated as subjects with a peak SI of ≥2.00.

[b] Distribution of maximum stimulatory doses indicates the distribution of maximum stimulatory doses in responder subjects.

[c] The low dose (LD), medium dose (MD), and high dose (HD) of protein, respectively, were: for all bacterial isolates, 5, 50, and 500 μg/ml; for *E. coli*, 1, 10, and 100 μg/ml; and for SK/SD, a 1:1,000, 1:100, and 1:10 dilution from dialyzed stock SK/SD.

[d] Two CBL preparations were not tested against *P. micros* due to insufficient cells.

[e] The CBL response frequency to *E. coli* LPS was significantly elevated as indicated by a chi-square analysis ($P < 0.01$). Additional SK/SD responses from CBL and healthy adult PBL subjects were tested in related studies. When these data are included, the response frequencies are 1/18 for CBLs and 12/25 for PBLs, with $P < 0.001$.

these bacteria. However, central to establishing the presence of mitogenic activity in these bacteria is the demonstration that lymphocytes from essentially all subjects should respond after stimulation. Therefore, studies were initiated examining unresponsive PBL and CBL preparations to determine whether a more defined subset of cells was capable of responding to these bacteria and whether a regulatory cell population existed in these unresponsive cultures. Previous studies had reported a major role for adherent cells in regulating the extent of lymphocyte responsiveness to periodontitis-associated bacteria (22, 30). Several unresponsive sub-

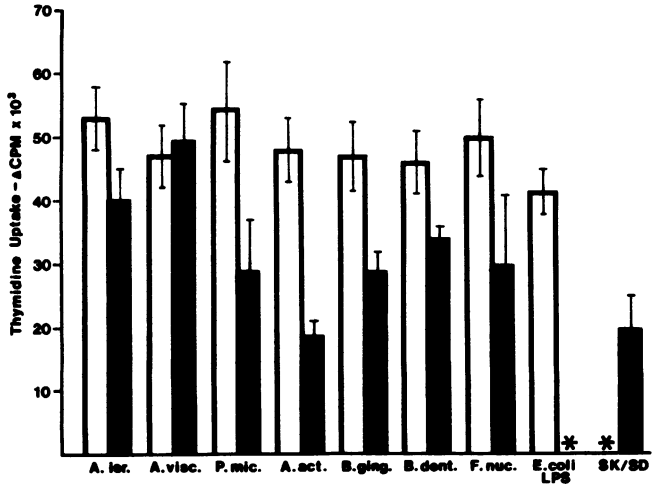

FIG. 2. Comparison of the magnitude of the peak blastogenic responses elicited on day 6 in CBLs (□) and PBLs (■) by bacterial stimulants. Mean ± standard error values represent the mean peak Δcpm response of responder subjects. Analysis by the Student *t* test showed that the CBL response to *A. actinomycetemcomitans* was significantly elevated above the PBL response ($P < 0.01$). *, No means ± standard error were determined for the *E. coli* LPS PBL response and the SK/SD CBL response since only one preparation responded to each. Unstimulated CBL and PBL control counts were 24,855 ± 2331 and 17,898 ± 3279, respectively.

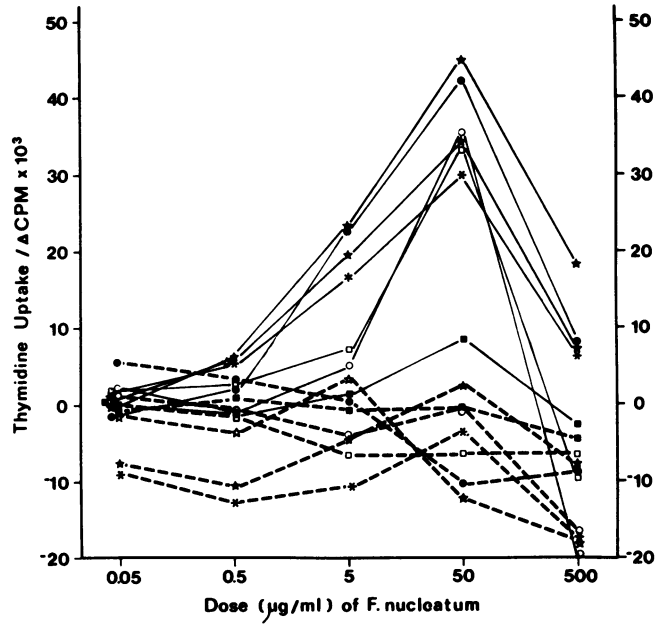

FIG. 3. Comparison of the peak blastogenesis responses elicited by *F. nucleatum* on day 6 in the unseparated and the nonadherent PBL cultures. Peak Δcpm responses at the designated doses of *F. nucleatum* are shown for the unseparated (- - -) and the nonadherent (——) cultures for each of seven individuals. Mean background counts for the unseparated and the nonadherent cultures were 18,633 ± 4011 and 10,867 ± 2597, respectively ($P = 0.14$).

jects from previous studies were identified, PBLs were prepared by standard techniques, and a portion of PBLs was further separated by adherence onto polystyrene tissue culture plates. Typical unseparated and nonadherent PBL responses to these bacteria are shown in Fig. 3. Figure 3 shows Δcpm values for the unseparated and the nonadherent preparations from seven unresponsive subjects at various doses of *F. nucleatum* at 6 days of culture. PBLs from all seven subjects were converted from an unresponsive to a responsive state after the removal of the adherent cell population. The mean SI values for the unseparated and the nonadherent cultures were 0.72 ± 0.13 and 4.40 ± 0.43, respectively. The mean peak Δcpm responses of the unseparated and the nonadherent cultures of the seven subjects were 4,486 ± 2,024 and 32,501 ± 4,471, respectively. These differences were not a result of differences between background counts, since the unseparated and the nonadherent PBL background counts were 18,633 ± 4,011 and 10,867 ± 2,597, respectively ($P = 0.14$).

Additional studies were initiated to examine the effects of the removal of the adherent cell population in unresponsive CBL preparations. Although adherence was effective in reducing the esterase-positive cell count to 5% or less, this reduction rendered many, but not all, unresponsive CBL preparations responsive. However, previous PBL cell density studies performed in our laboratory indicated that, whereas 5×10^5 PBLs per well appeared to be most consistent for detecting positive PBL blastogenic responses to these bacteria, occasionally PBL preparations which were unresponsive at this cell density would respond at other cell densities. Typical experiments demonstrating the effects of cell density on the peak CBL blastogenic responses to *F. nucleatum* are reported in Table 2. This table indicates the peak Δcpm and SI responses elicited in five CBL preparations when tested over a three-log dose range of *F. nucleatum* or *B. denticola* at the designated densities. Note that unresponsive CBL cultures (SI < 2.0) can be converted to responsive CBL cultures (SI ≥ 2.0) simply by altering the cell density.

DISCUSSION

Results of this study indicate that numerous periodontitis-associated bacteria possess the capacity to induce mitogenesis in germfree mouse spleen cells, human umbilical CBLs, and PBLs from periodontally healthy adults. Although the majority of CBL and PBL preparations responded under the standard culturing conditions, additional experiments indicate that even the unre-

TABLE 2. Effect of cell density on the blastogenic responses of CBLs

Cell density	Blastogenic response of CBLs in expt no.[a]:				
	1	2	3	4	5
10.0×10^5	69,692 (1.48)[b]	2,073 (1.02)	11,718 (1.11)	28,424 (1.65)	34,574 (0.44)
5.0×10^5	83,724 (2.85)	64,910 (1.57)	17,962 (1.17)	38,753 (1.68)	50,368 (1.83)
2.5×10^5	83,100 (2.94)	54,974 (1.62)	21,224 (1.27)	69,688 (3.96)	65,994 (2.56)
1.2×10^5	70,439 (7.17)	55,413 (2.17)	45,241 (3.04)	54,083 (4.22)	19,574 (1.76)
0.6×10^5	36,175 (24.67)	35,936 (2.30)	20,234 (3.96)	31,286 (5.78)	12,453 (1.99)
0.3×10^5	10,708 (21.95)	24,237 (2.96)		12,502 (7.38)	60,670 (1.73)

[a] The stimulants were sonic extracts of *F. nucleatum* in experiments 1, 2, and 3 and of *B. denticola* in experiments 4 and 5.
[b] Δcpm (SI).

sponsive CBL and PBL preparations are capable of responding after the removal of the adherent cell population or under altered culturing conditions. These observations, combined with numerous reports indicating nonspecific activators in a variety of bacteria, argue that periodontitis-associated bacteria do possess mitogenic activity.

Initial experiments demonstrated that the blastogenic responses elicited by both gram-positive and gram-negative bacterial sonic extracts in germfree mouse spleen cell cultures were similar in both kinetics and magnitude to the response elicited by the standard murine B-cell mitogen, *E. coli* LPS. In contrast, SK/SD antigen elicited no response in these unsensitized animals. These results confirm previous murine studies by demonstrating mitogenic activity in a variety of bacteria (5, 7, 15, 20) and extend this list of organisms to include numerous periodontitis-associated bacteria. The responsiveness seen in human umbilical CBLs and adult PBLs to these bacteria is consistent with numerous previous studies reporting the presence of polyclonal B-cell activators and mitogenic activators in a variety of bacterial isolates (2, 3, 6, 8, 9, 12–14, 19, 22, 25, 27–29). Clagett and Engel (6) summarized several early reports demonstrating that certain bacteria possess polyclonal B-cell activators for many mammalian species. Several recent reports from this and other laboratories confirm these early reports by demonstrating that numerous periodontitis-associated bacteria possess potent polyclonal B-cell activators, as measured by either a modified Jerne plaque assay or antibody synthesis (3, 6, 9, 22, 29). That some bacteria also possess the capacity to elicit blast transformation is now well established. Sakane and Green (28), as well as Kasahara et al. (19), have demonstrated the B-cell mitogenesis properties of staphylococcal protein A in human CBL. Räsänen et al. (26) have likewise reported CBL mitogenic activity in *Staphylococcus aureus*, *E. coli*, a diptheroid organism, alpha-hemolytic streptococci, group A beta-hemolytic streptococci, and *Mycobacterium bovis*. Our present study extends this list to include several periodontitis-associated bacteria. A number of additional studies have also reported mitogenicity, immunomodulating activity, or adjuvant effects of purified peptidoglycan preparations from a variety of bacteria in a variety of mammalian systems (8, 12–14, 25). Räsänen and Arvilommi (25) recently reported that cell wall preparations and purified peptidoglycans and teichoic acids from *Bacillus subtilis* and *S. aureus* were capable of eliciting blastogenic activation in T and B lymphocytes from adult PBL and CBL preparations. In light of these numerous reports of nonspecific activators, it seems highly improbable that such activities would not also be present in the highly complex periodontitis-associated flora.

Previous studies have cited the observations that not all PBL or CBL cultures respond after stimulation with these bacterial isolates as primary evidence arguing against nonspecific activation as an explanation for blastogenic responsiveness. Data reported in this study indicate that unresponsive PBL preparations can be converted to responsive PBL preparations simply by the removal of the adherent PBL population using polystyrene tissue culture plates. In light of numerous studies indicating a primary role for monocytes in regulating blastogenesis or antibody responses (22, 30), monocytes remain the likely candidate for a possible regulatory cell population. Unresponsive CBL preparations appeared to be less consistently rendered responsive via the removal of the adherent cell population but were capable of responding at altered cell densities.

In summary, several gram-positive and gram-negative periodontitis-associated bacteria appear to possess mitogenic activity, as assessed by their ability to elicit blast transformation in unsensitized cells. To attribute the blastogenic responses seen in this study to antigen-specific mechanisms of activation, one would need to argue that both the germfree mice and the human

umbilical cord blood cells have been exposed and sensitized to the numerous periodontitis-associated bacteria tested or to cross-reactive strains. When the germfree environment in which the mice were raised, the reported immunologically privileged environment of the CBL source, and the number of unique bacterial strains tested are considered, it seems highly improbable that responsiveness reflects antigen-specific responses. These observations support models proposing a role for polyclonal nonspecific activation in periodontal disease. The ability of these isolates to activate lymphocytes in the absence of prior exposure and sensitization may explain in part the blastogenic responsiveness frequently observed in PBLs from periodontally healthy and diseased patients.

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