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Nonspecific Induction of Immunoglobulin M Antibodies to Periodontal Disease-Associated Microorganisms After Polyclonal Human B-Lymphocyte Activation by *Fusobacterium nucleatum*

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The production of antibodies to oral bacteria was determined in lymphocyte cultures stimulated with sonicated *Fusobacterium nucleatum*, a potent inducer of polyclonal B-cell activation. After 9 days the cultures were examined by a micro-enzyme-linked immunosorbent assay for immunoglobulin M (IgM) antibodies to *F. nucleatum*, *Bacteroides gingivalis*, *Actinomyces viscosus*, and *Streptococcus sanguis*. Antibodies to these four bacteria were detected in cultures stimulated with polyclonal B-cell activation-inducing concentrations of *F. nucleatum*. However, significant concentrations of antibodies to *F. nucleatum*, but not to the other three microorganisms, were produced in cultures that received suboptimal polyclonal B-cell activation-inducing doses of *F. nucleatum*. Absorption studies indicated the specificity of the antibodies to each of the bacteria tested. IgM antibody production induced by *F. nucleatum* was enhanced by the addition of T cells. The production of IgM antibodies to the bacteria was reproducible in cultures from a single person tested on 3 consecutive days. The concentration of antibodies in replicate cultures, however, fluctuated greatly. To obtain consistent responses on successive days, multiple replicate cultures were required. These results suggest that *F. nucleatum*, which is frequently present in subgingival plaque, could induce the production of antibodies not only to *F. nucleatum*, but also to other microorganisms associated with periodontal diseases.

Chronic periodontitis is histologically characterized by a substantial accumulation of antibody-secreting plasma cells (16, 25), and it is speculated that microorganisms associated with this disease may play a role in activating these B lymphocytes (B cells) (5). Several recent studies have demonstrated that microorganisms isolated from chronic periodontitis lesions non-specifically stimulate multiple clones of human B cells in vitro (1, 17, 18, 26). Such stimulation is referred to as polyclonal B-cell activation (PBA). In vitro PBA is typically measured by the production of relatively large amounts of immunoglobulin or an increase in the number of hemolytic immunoglobulin-secreting cells (8). However, with the exception of anti-sheep erythrocyte antibodies, the specificities of the antibodies secreted as a result of PBA induced by periodontal disease-associated microorganisms are largely unknown.

The purpose of the present study was to determine whether PBA induced by one oral microorganism would result in the production of antibodies to other oral microorganisms. The production of such antibodies could have beneficial or deleterious effects on the host (11). We recently reported that *Fusobacterium nucleatum*, a gram-negative bacterium frequently isolated from the subgingival plaque of periodontitis patients, is a potent PBA stimulant, inducing the production of large amounts of immunoglobulins (18). However, the specificity of the immunoglobulins (antibodies) produced in these cultures was not determined. The results from this report indicate that the supernatants from human peripheral blood lymphocyte cultures stimulated with PBA-inducing concentrations of *F. nucleatum* contain immunoglobulin M (IgM) antibodies that react with *Bacteroides gingivalis*, *Actinomyces viscosus*, *Streptococcus sanguis*, and *F. nucleatum*. In contrast, suboptimal PBA-inducing concentrations of *F. nucleatum* stimulated the production of significant amounts of antibodies to *F. nucleatum*, but not of antibodies to the other three microorganisms.

MATERIALS AND METHODS

Bacteria. *F. nucleatum* Per-1 and 191, *B. gingivalis* S.2, *A. viscosus* GA, and *S. sanguis* type II were isolated from the subgingival plaque of patients with

chronic periodontitis. Bacteria were cultured anaerobically as previously described (18). The bacteria were washed in sterile phosphate-buffered saline (PBS; 0.15 M NaCl, 0.05 M PO_4), pH 7.4. With the exception of *F. nucleatum* 191, which was suspended in RPMI (10%, vol/vol), the bacteria were suspended in sterile distilled water and sonicated. The sonicate was then centrifuged at 12,000 × *g* for 30 min. The lyophilized supernatant and pellet fractions were used in the experiments on a weight/volume basis. The pellet from *F. nucleatum* 191 was not lyophilized and was suspended to volume in RPMI. PBA was predominantly associated with the pellet fraction of *F. nucleatum* (18); therefore, this fraction was used to stimulate IgM production in the cultures.

Mononuclear cell isolation. Mononuclear leukocytes from healthy persons were isolated and depleted of monocytes as described previously (17, 18).

In vitro immunoglobulin synthesis. Lymphocytes were cultured in 10% fetal bovine serum–RPMI medium as previously described (18). Lymphocyte cultures stimulated with *F. nucleatum* were monocyte depleted and cultured at 2×10^5 cells per well. Cultures stimulated with pokeweed mitogen (PWM) were not depleted of monocytes and were cultured at 3×10^5 cells per well. The cultures were incubated in 5% CO_2 at 37°C with high humidity for 9 days. Initially, we centrifuged the cultures at 250 × *g* for 10 min and assayed the culture supernatants for IgM. However, subsequent comparison tests indicated that the IgM concentration was identical in samples from uncentrifuged cultures and culture supernatants. Therefore, IgM was routinely assayed in uncentrifuged cultures.

Immunoglobulin assay. A micro-enzyme-linked immunosorbent assay (micro-ELISA) was used to measure IgM production and was described previously (17, 18). Polystyrene microtiter plates were coated with 0.1 ml of heavy-chain specific rabbit anti-human IgM (Bio-Rad Laboratories, Richmond, Calif.; 2 μg/ml) per well or bacterial sonicate supernatant fractions (10 μg/ml) for 1 to 2 h at 37°C. Test cultures were diluted in PBS containing 0.05% polyoxyethylene-(20)-sorbitan monolaurate (Tween 20) and 0.02% NaN_3 (PBS-Tween). A portion (0.1 ml) of each dilution was added to washed duplicate or triplicate assay wells. After 3 h at 22°C, the plates were washed again, and 0.1 ml of heavy chain-specific rabbit anti-human IgM conjugated with alkaline phosphatase was added to each well; 16 h later unbound conjugate was washed away and 0.1 ml of alkaline phosphatase substrate (Sigma 104; Sigma Chemical Co., St. Louis, Mo.) in $MgCl_2$–sodium carbonate buffer was added to each well. The plates were incubated for 30 min (total immunoglobulin) or 90 min (anti-bacterial IgM antibodies) at 22°C, after which the absorbance at 405 nm was measured (Multiskan; Flow Laboratories, McLean, Va.). The replicate assays were consistently within 10% of the mean.

The concentration of total IgM in the cultures was determined from a regression line calculated from dilutions of known amounts of human IgM. The concentrations of antibodies reactive with each bacterial isolate was estimated as follows. (i) Twofold dilutions of a pooled human plasma sample were added to wells coated with the bacterial preparations. On the same plate, known amounts of IgM were added to wells sensitized with anti-human IgM. The micro-ELISA was performed as described above, developing the color for 90 min. The concentration of anti-bacterial IgM antibody in the plasma sample was then determined from a regression line calculated from the IgM standards. (ii) Dilutions of the plasma were included as standards on the anti-bacterial IgM antibody assay plates.

Antibody absorption. *F. nucleatum*-stimulated cultures were pooled, aliquotted, and mixed 1:2 with PBS-Tween containing 10-fold serial dilutions (0.1 to 100 μg/ml) of bacterial sonicate supernatants or PBS-Tween (control). The mixtures were incubated at 37°C for 15 min and then assayed by micro-ELISA for remaining IgM antibody reactive with each bacteria.

Statistics. The results were compared by using a two-sided Student *t* test or a correlation matrix.

RESULTS

Production of IgM antibodies after PBA by *F. nucleatum*. Employing culture conditions which provided optimal *F. nucleatum*- or PWM-induced PBA (18), the production of total IgM and IgM antibodies reactive with *F. nucleatum*, *B. gingivalis*, *A. viscosus*, and *S. sanguis* was determined (Fig. 1). The dose-response profile in *F. nucleatum* cultures was similar to the results previously reported for total IgM production (18). Maximal total IgM production was obtained in cultures stimulated with *F. nucleatum* concentrations between 0.5 and 50 μg/ml. In general, the dose response for anti-bacterial IgM antibody production paralleled total IgM production. However, in contrast to the production of IgM antibodies reactive with *B. gingivalis*, *A. viscosus*, and *S. sanguis*, a significant amount (compared with unstimulated control cultures) of IgM reactive with *F. nucleatum* was detected in cultures stimulated with suboptimal PBA-inducing concentrations (0.05 μg/ml) of *F. nucleatum*. PWM, a well-studied polyclonal B-cell activator, also stimulated the production of IgM antibodies reactive with the four bacteria tested.

Specificity of secreted antibodies. The antibodies which react with *B. gingivalis*, *A. viscosus*, and *S. sanguis* may represent antibodies cross-reactive with *F. nucleatum*. Such antibodies would be produced if *B. gingivalis*, *A. viscosus*, and *S. sanguis* shared identical or similar epitopes with those found on *F. nucleatum*. To determine the degree of cross-reactivity, samples of cultures stimulated with *F. nucleatum* were absorbed with the various bacterial antigen preparations. After absorption, the concentration of the remaining reactive antibodies in the culture media was measured by micro-ELISA. As shown in Fig. 2, absorption with each bacterial preparation resulted in a dose-dependent decrease in the amount of detectable homologous antibody (presented as absorbance at 405 nm). A small decrease in heterologous antibodies was found in preparations receiving high

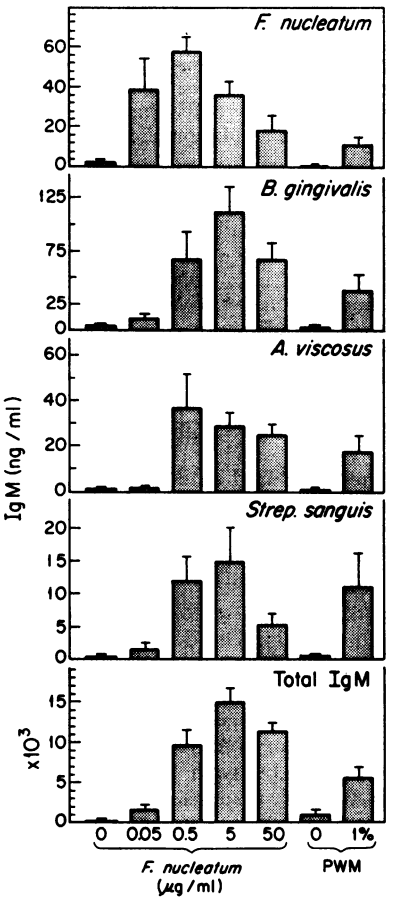

FIG. 1. Production of IgM reactive with oral microorganisms and total IgM in cultures stimulated with either *F. nucleatum* or PWM. Cultures receiving *F. nucleatum* were monocyte depleted, whereas PWM-stimulated cultures were unfractionated. (i.e., contained monocytes). Note that the IgM scale on the *y* axis varies for each panel, and that the total IgM concentration is presented as nanograms $\times 10^3$ per milliliter. The results represent the means (± standard errors of the mean) of 11 to 14 individual experiments with each stimulant concentration tested in 3 to 12 replicates.

concentrations (100 μg/ml) of *B. gingivalis* or *F. nucleatum*. An increase in both heterologous and homologous antibodies was often detected when low concentrations (0.1 to 1 μg/ml) of antigens were added. The reason for this phenomenon was not investigated in this study. Antigen-antibody interactions are mediated by several types of noncovalent molecular bonds (reviewed in reference 20). These bonds may be strengthened in solutions containing the low concentrations of the bacterial preparations.

Kinetics of antibody secretion. The kinetics of production of IgM antibodies reactive with the various bacteria may differ in the *F. nucleatum*-stimulated cultures. Differences in kinetics might occur if subpopulations of B cells were activated at different time points during stimulation of the cultures. Therefore, the release of antibodies in cultures stimulated with an optimal PBA-inducing concentration of *F. nucleatum* was followed over a 14-day period (Fig. 3). The kinetics of release of IgM antibodies reactive with the microorganisms paralleled that of the total IgM in the cultures (18). IgM production was first detected on day 6, increased rapidly between day 6 and day 8, and plateaued thereafter. Thus, anti-bacterial antibody production appears to occur in the cultures at approximately the same time during PBA induced by *F. nucleatum*.

Reproducibility of antibody production in a single donor. The concentration of antibodies produced in vitro after PBA may reflect the number of circulating antigen-specific B cells. The number of antigen-specific B cells in the peripheral circulation increases after reimmunization with the antigen and then returns to

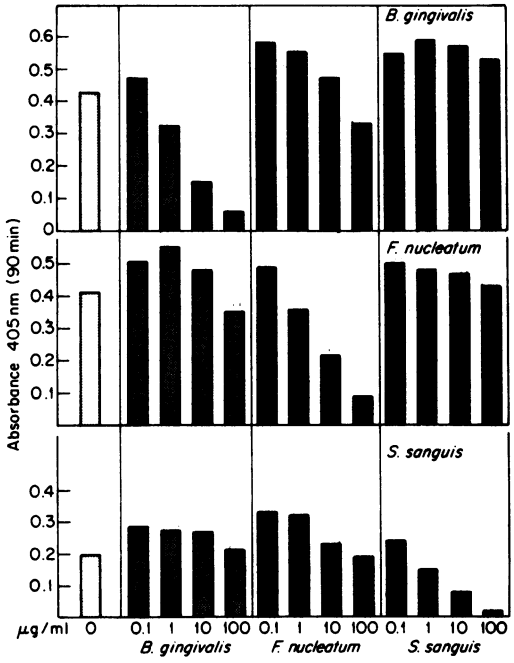

FIG. 2. Absorption of IgM anti-bacterial antibodies by bacterial antigens. Cultures stimulated with *F nucleatum* (5 μg/ml) were pooled, aliquotted, and mixed 1:2 with PBS-Tween containing various concentrations of *B. gingivalis*, *F. nucleatum*, *S. sanguis* sonicate extracts or with PBS-Tween alone (control). The remaining IgM antibody reactive with each microorganism was then measured by micro ELISA. The results are from one experiment and are representative of four similar absorption experiments.

preimmunization levels approximately 1 to 2 months later (14, 29). Thus, an increased amount of antibody produced in vitro may prove to be diagnostic of recent exposure to an antigen provided a stable preexposure baseline concentration value could be established for an individual. Thus, we next examined the reproducibility of in vitro antibody production. Blood was drawn from a person on 3 consecutive days. The cultures set up each day were stimulated with *F. nucleatum*, and 9 days later the total IgM and IgM anti-bacterial antibodies were measured (Fig. 4). IgM antibody production was found to be statistically equivalent ($P > 0.05$) on each of the 3 days. Although the total IgM produced on 1 day tended to be statistically different from total IgM produced on the other 2 days, the concentration of total IgM released in the *F. nucleatum*-stimulated cultures typically varied by less than 10% of the mean when tested on consecutive days. Thus, the IgM released as a result of *F. nucleatum*-induced PBA was reproducible within reasonable (10%) biological limits, and a baseline (healthy control) value could most likely be established for an individual.

To determine whether two different isolates of *F. nucleatum* would stimulate similar, reproducible IgM antibody production, cultures were stimulated with optimal PBA-inducing concentrations of *F. nucleatum* isolates Per-1 and 191. The total IgM and IgM reactive with *B. gingivalis* and *A. viscosus* were statistically equivalent on each of 3 consecutive days (Fig. 5). Similar equivalent anti-*F. nucleatum* and anti-*S. sanguis* IgM responses were obtained in cultures stimulated with these isolates of *F. nucleatum* (data not presented).

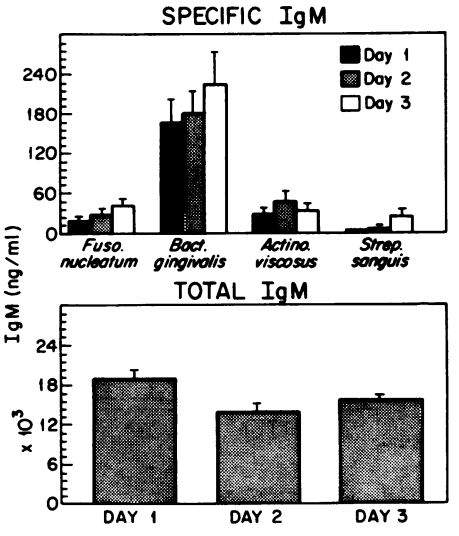

FIG. 4. IgM production in cultures from the same person on 3 consecutive days. Cultures set up each day were stimulated with *F. nucleatum* (5 μg/ml) for 9 days. The results represent the means (± standard errors of the mean) of 12 replicate cultures.

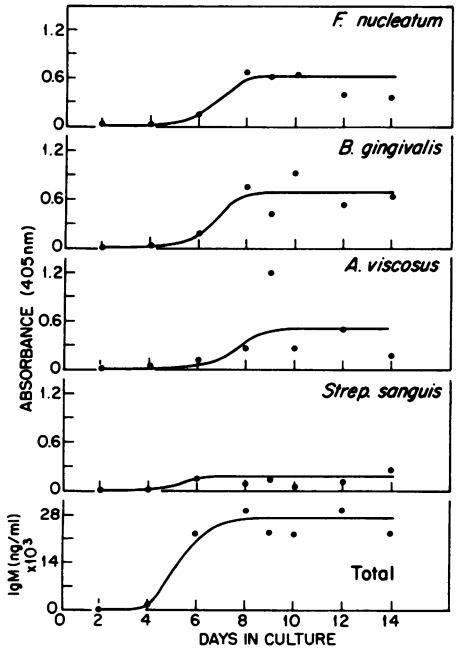

FIG. 3. Kinetics of IgM production over a 14-day period. Cultures were stimulated with *F. nucleatum* (1 μg/ml) or RPMI (control) on day 0. Results are expressed as the pooled average of triplicate cultures (minus the control value) and are representative of two similar experiments.

Variation of antibody production in replicate cultures. The results presented in Fig. 1 were obtained from 11 to 14 persons and indicated an average response to stimulation by various concentrations of *F. nucleatum*. The average responses obtained from 12 replicate cultures from one person also showed this typical dose-response profile (Fig. 6). However, as also shown in Fig. 6, the production of IgM reactive with *B. gingivalis* fluctuated greatly in each of the replicate wells. Variations in the amount of IgM reactive with *F. nucleatum*, *A. viscosus*, and *S. sanguis* were also found in replicate cultures (data not shown). Total IgM production was more consistent, typically ranging within 10% of the mean. A high positive correlation ($P < 0.01$) was found between the concentration of a particular IgM antibody and the total IgM. Thus, the more IgM produced in the culture, the more likely it was that a particular IgM antibody was produced in concentrations high enough to be detected.

Effect of T cells on specific IgM production. We

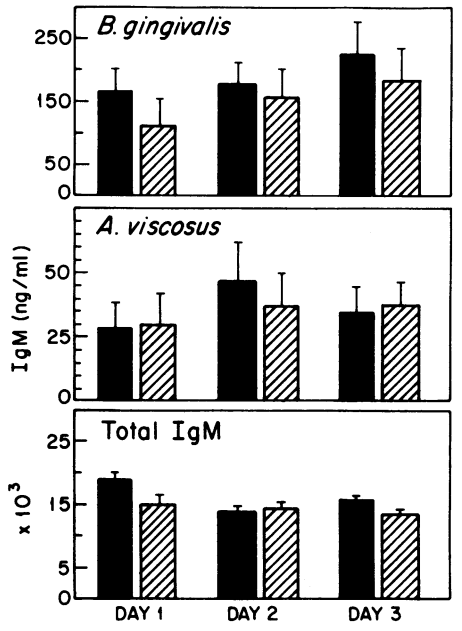

FIG. 5. IgM production in cultures from a person stimulated with *F. nucleatum* Per-1 (■) and 191 (▨) on 3 consecutive days. Cultures set up each day were stimulated with an optimal total IgM-inducing concentration of the sonicate pellet fraction from each isolate for 9 days. The results represent the means (± standard errors of the mean) of responses from 12 replicate cultures.

previously found that *F. nucleatum*-induced total IgM production (i.e., PBA) was enhanced by T cells. The maximal IgM response occurred at a B cell/T cell ratio of 1:1 (18). The production of IgM antibody to *B. gingivalis* was also enhanced by adding T cells to a B cell-enriched preparation (Fig. 7). Similarly, IgM antibody to *F. nucleatum*, *A. viscosus*, and *S. sanguis* was enhanced by T cells (data not presented). Maximal antibody production occurred in cultures having a B cell/T cell ratio of either 3:1 or 1:1 depending on the donor.

DISCUSSION

Antibodies (IgM) reactive with *F. nucleatum*, *B. gingivalis*, *A. viscosus*, and *S. sanguis* were found in the cultures stimulated with *F. nucleatum*. The concentrations of antibody to *B. gingivalis*, *A. viscosus*, and *S. sanguis* were higher in cultures stimulated with *F. nucleatum* at concentrations of 0.5 to 50 μg/ml, which were also the optimal total IgM-inducing doses of *F. nucleatum* (18). At 0.05 μg/ml, *F. nucleatum* induced a small amount of total IgM and almost no antibodies to the other microorganisms, whereas there was a significant concentration of antibody produced to *F. nucleatum*. Thus, at low concentrations, *F. nucleatum* induces poor PBA, whereas a good *F. nucleatum*-specific IgM response is generated. At high concentrations, *F. nucleatum* induces both good PBA and good *F. nucleatum*-specific antibody responses. The dichotomy between antigen-specific and polyclonal antibody production induced by low and high concentrations of antigen, respectively, has been reported by others using tetanus toxoid (10, 14, 31), keyhole limpet hemocyanin (14, 32), and purified protein derivative (2). However, these previous reports have indicated that PBA-inducing concentrations of tetanus toxoid or keyhole limpet hemocyanin fail to stimulate specific antibodies to these antigens (14, 31, 32). The suppressed antigen-specific response in the polyclonally activated cultures was not due to absorption of antibodies with the stimulating antigen. Cultures in which the high concentrations of stimulant were removed before antibody secretion still produced a polyclonal response, yet failed to release specific antibody. In contrast, we detected antibodies to *F. nucleatum* even in the cultures receiving PBA-inducing concentrations of *F. nucleatum*. Although high concentrations of *F. nucleatum* (>0.5 μg/ml) appeared to depress anti-*F. nucleatum* IgM production, this decreased IgM production may be largely artificial since anti-*F. nucleatum* antibodies are absorbed by the *F. nucleatum* stimulant (Fig. 2). Therefore, the actual amount of IgM reactive with *F. nucleatum* is most likely

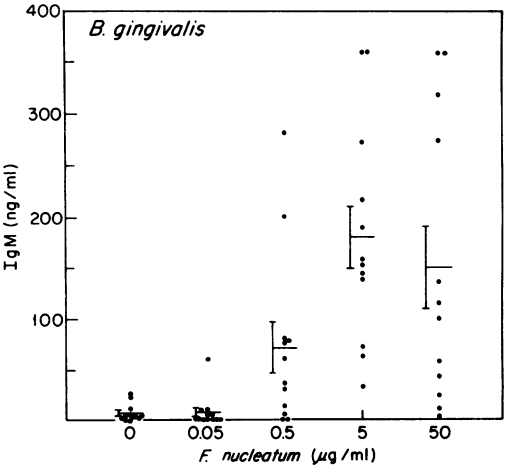

FIG. 6. Production of anti-*B. gingivalis* IgM in replicate culture wells. Cultures were stimulated with various concentrations of *F. nucleatum*. The means and standard errors of the mean are indicated for the 12 replicate cultures. The data are from one person and are representative of 10 similar experiments.

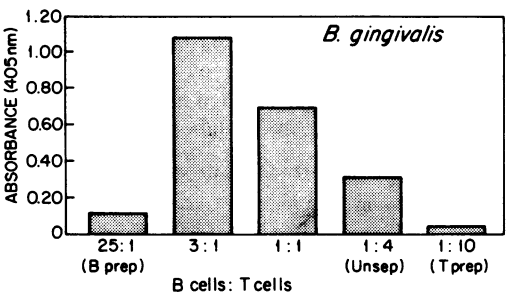

FIG. 7. Effect of the B cell/T cell ratio on anti-*B. gingivalis* IgM production. B cell-enriched preparations (B prep), T cell-enriched preparations (T prep), mixtures of B- and T-cell preparations, or unseparated, monocyte-depleted lymphocytes (Unsep) were cultured at 100,000 cells per well. B cell/T cell ratios are indicated for each culture preparation. Cultures received either *F. nucleatum* (10 μg/ml) or RPMI (unstimulated controls). The results are presented as the stimulated response minus the unstimulated, control value, (which never exceeded an absorbance of 0.24). The results are the averages of triplicate cultures and are representative of two similar experiments.

greater than that shown in Fig. 1, particularly at high stimulating concentrations of *F. nucleatum*.

The total IgM released in the cultures receiving the higher doses of *F. nucleatum* appears to be a collection of immunoglobulin molecules having specificities for a variety of antigens. An estimated 1 to 5% of these antibodies react specifically with *B. gingivalis*, *A. viscosus*, and *S. sanguis* (Fig. 2). There was no evidence that these antibodies represent substantial amounts of cross-reacting antibodies with the stimulating *F. nucleatum*. Absorption experiments showed a small decrease in heterologous antibodies at high absorbing concentrations (100 μg/ml) of *B. gingivalis* and *F. nucleatum*. In contrast, however, over 80% of the homologous antibody activity was removed by this concentration of homologous bacteria.

The production of anti-bacterial IgM antibodies in PBA cultures from a person was statistically reproducible over a 3-day period. Assuming that exposure of this healthy person to the oral microorganisms tested did not change during the 3-day period, the reproducibility of the response seems reasonable. However, it must be emphasized that the concentration of antibody produced in each replicate culture varied quite extensively (Fig. 6). Therefore, to obtain consistent results, it was necessary to test in multiple replicates. The variation of IgM in replicate cultures may reflect the number of antigen-specific B cells within each culture. The average response of the replicate cultures would be proportional to the number of antigen-specific B cells in the peripheral blood at the time the blood was obtained. The absolute number of specific B cells circulating in the peripheral blood at any point in time is reported to be influenced by when the person was last exposed to the antigen (14, 29). B cells are released into the circulation during a short interval (approximately 2 weeks) after antigen exposure and then decrease to preexposure levels within 4 weeks. Thus, the B cells in peripheral blood may primarily reflect the recent immune history of an individual and not the total immune potential. The spleen and lymph nodes may contain a pool of (memory) B cells that are more representative of the antigens to which the person has been exposed (27–29). In this regard, although we found that all normal, orally healthy adults typically have IgM antibodies to the four bacterial isolates used in this study (6; unpublished results), we were unable to correlate the serum IgM antibody titers of these persons with the magnitude of the IgM antibody produced in vitro after stimulation with either *F. nucleatum* or PWM.

In general, we found that *F. nucleatum*-induced PBA cultures produced more anti-*B. gingivalis* antibody than antibody to the other bacteria tested in this study. There may be several explanations for this. First, the IgM produced may reflect the number of circulating B cells specific for *B. gingivalis*, i.e., there are more B cells specific for *B. gingivalis* than for the other bacteria. Second, the subset of B cells specific for *B. gingivalis* may be more sensitive to activation by *F. nucleatum*. The work of Gronowicz and Coutinho (12) indicates that polyclonal B-cell stimulants can activate different subpopulations of B cells. Third, the micro-ELISA may selectively measure antibodies to certain antigens. Differences in the binding of the bacterial antigens to the polystyrene plates could artificially elevate or depress the antibody concentrations. Although we determined the maximum micro-ELISA sensitizing concentrations, bacterial preparations that bind in higher concentrations or more avidly to the plastic would yield higher IgM concentrations (7). Alternatively, the affinity or avidity of the antibodies produced in vitro may be different for each bacterium. Equal concentrations of antibodies having different affinities result in unequal quantitative micro-ELISA determinations of total antibody levels since antibody of low affinity is not detected (3, 15). From the data presented in the current study, it is clear that antibodies to the microorganisms tested are produced after PBA by *F. nucleatum* and that the antibody concentrations are influenced by the stimulating concentration of *F. nucleatum*. However, comparisons between the absolute magnitudes of the IgM antibody concentrations should be avoided.

Preliminary studies indicated that IgG-reactive antibodies were also produced in the *F. nucleatum*-stimulated cultures (data not presented). However, the concentration of IgG antibodies was typically very low, and frequently we were unable to measure anti-bacterial IgG antibodies in the cultures. This suggests that IgM is the predominant antibody isotype produced in *F. nucleatum*-stimulated cultures. This may be due to the production of higher concentrations of total IgM than IgG in these cultures (18). Statistical analysis of our data indicated that the more total immunoglobulin produced in the culture, the more likely it was that anti-bacterial antibodies would be detected. Therefore, we measured only IgM antibody concentrations in this study in an attempt to maximize detecting antibodies reactive with the oral microorganisms. Optimal stimulation of IgG by *F. nucleatum* may require unique culture conditions not employed in these experiments. It is also possible that in vivo booster immunization with the bacterial antigens may be required for optimal generation of an IgG antibody response in vitro (29).

Many microorganisms associated with periodontal diseases are capable of stimulating PBA and therefore should be able to stimulate heterologous antibody production as demonstrated in the present study. In preliminary studies with *A. viscosus* as a polyclonal B-cell activator (17), we were able to detect antibodies to *B. gingivalis* and *F. nucleatum* (unpublished data). This indicates that *F. nucleatum*, *A. viscosus*, and possibly other bacteria can non-specifically stimulate the release of antibody to antigenically nonrelated microorganisms. Hence, there is a tremendous potential for B cells entering the gingival tissues to become activated, even in the absence of specific antigen in the subgingival plaque at that time. This nonspecific B-cell activation may explain in part the high number of plasma cells in chronically diseased periodontal tissues.

The role of PBA in vivo is only speculative at this time. Concentrations of *F. nucleatum* that stimulate PBA (approximately 0.5 to 50 μg/ml) may easily accumulate in subgingival plaque and non-specifically stimulate B cells. This stimulation would result in the release of antibodies, some of which would react with other microorganisms to which the person has been exposed. These polyclonal responses might keep the immunological system primed and provide the person with enhanced protection against infection (19, 21, 22). On the other hand, an uncontrolled polyclonal B-cell response may have deleterious effects on the host. Such effects might include (i) release of bone-resorbing lymphokines (4), (ii) production of immunosuppressive (blocking) antibodies (13, 21, 30), (iii) activation of forbidden clones of B cells capable of secreting autoimmune antibodies (9, 23, 24), or (iv) suppression of memory B cells (19). Studies on the stimulation and regulation of microbe-induced PBA are continuing in our laboratory.

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