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Blastogenic Response of Human Lymphocytes to Oral Bacterial Antigens: Characterization of Bacterial Sonicates

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Soluble sonicate supernatant preparations were made from *Actinomyces viscosus* (ATCC 19246), *A. naeslundii* (ATCC 12104), two strains of *Veillonella alcalescens* (strain HV-1 and a human oral isolate), *Streptococcus sanguis* (ATCC 10556), *S. mutans* (strain 6715-T2), *Bacteroides melaninogenicus* (strain K110), and *Leptotrichia buccalis* (isolated from human dental plaque). These supernatants were characterized with reference to their chemical and antigenic components and their biological activity determined by using in vitro lymphocyte blastogenesis as a measure of the host's cellular immune response. The sonicate supernatant of each bacterium contained protein, neutral sugars, methylpentose, and nucleic acids. Protein was the major component in all except *L. buccalis,* in which neutral sugars predominated. The antigenic components in each supernatant were detected by using rabbit antisera prepared against the whole bacteria and the sonicate supernatant. The supernatants showed a complex antigenic distribution on immunoelectrophoretic analysis. The supernatants were shown to be antigenic and not mitogenic in nature, since neither cord blood lymphocytes nor all adult lymphocytes were stimulated. The supernatant antigen preparations showed a reproducible, dose-dependent, and kinetic response in vitro, which was similar to that seen with the antigen preparation streptokinase-streptodornase.

Studies by Ivanyi and Lehner (6, 7) Horton and co-workers (5), and more recently Baker and co-workers (1) have indicated a role for the host's cellular immunity in the immunopathology of periodontal disease. These investigators used the technique of in vitro lymphocyte blastogenesis to measure in vivo cellular immunity and found that peripheral blood lymphocytes from patients with periodontal disease underwent transformation when stimulated with various preparations from oral bacteria. Lymphocytes from selected normal individuals did not transform under similar culture and stimulus conditions.

Among antigen preparations used for in vitro stimulation of periodontal patients' lymphocytes have been sonicates of dental plaque or sonicates of specific oral microorganisms (5-7). However, little information about the antigenic composition or chemical properties of these sonicates is available from these studies. To understand immune reactions and their role in disease, whether they are antigen-antibody reactions or the reactions of antigens with sensitized lymphocytes as in cellular immunity, a knowledge of the antigens involved is essential.

We have selected as a major objective the determination of the specificity of the reactions between oral bacterial antigens and the host's sensitized lymphocytes. It is necessary to have well-characterized antigens to accomplish this. The purpose of this study was to analyze and describe the sonicates of specific oral bacteria with respect to the effects of time of sonication upon basic chemical composition, antigenic composition, and biological activity when assayed by in vitro lymphocyte blastogenesis.

MATERIALS AND METHODS

Preparation of antigens. (i) Bacteria and culture conditions. *Actinomyces viscosus* (AV; ATCC 19246), *A. naeslundii* (AN; ATCC 12104), and *Veillonella alcalescens* (VAI; strain HV-1) were grown in Trypticase soy broth for 72 h in batch cultures of up to 20 liters. *V. alcalescens* (VAII) was grown in FM-3 medium for 72 h (11). The bacteria were harvested and washed three to five times with 0.005 M phosphate buffer, pH 7.2, or phosphate-buffered saline, pH 7.2, by centrifugation at 16,300 × *g* for 30 min at 4°C. The final pellet was stored at −20°C until used.

Streptococcus sanguis (SS; ATCC 10556) and *Streptococcus mutans* (SM; strain 6715-T2) were grown in Trypticase soy broth for 48 h. Harvest was

as described above. *Bacteroides melaninogenicus* (BM; strain K110) was grown in EX-1 medium supplemented with hemin (11). *Leptotrichia buccalis* (LB), isolated from human dental plaque, was grown in EX-1 medium. Again after harvesting and washing, the bacteria were stored at −20°C. All bacteria were grown at 37°C in an atmosphere of air except for *L. buccalis,* which was grown in an atmosphere of CO_2 (11).

(ii) Sonication procedure. Bacteria were disrupted by using a Heat Systems Sonifier, model W185 (Branson Ultrasonics, Plainview, Long Island, N.Y.), working at approximately 95-W output with appropriate probe in suspensions held in a rosette sonifying vessel. The vessel was kept in ice water during the process. The temperature of the solution in the vessel ranged from 0 to 10°C during sonication. The bacteria were suspended in either 0.005 M phosphate buffer, pH 7.2, or RPMI 1640 culture medium (Associated Biomedic Systems, Inc., Buffalo, N.Y.) containing penicillin (100 U/ml), streptomycin (50 μg/ml), and 2 mM glutamine. These suspensions contained a concentration of either 10 or 100 mg (wet weight) of bacteria/ml in a volume of 150 ml. The suspensions were sonicated until more than 95% breakage of microorganisms had occurred as determined by phase-contrast microscopy.

To test the effect of time of sonication upon the antigen preparations, cells of AV, AN, and VAI were sonicated for 0.5, 5, 10, 20, 40, 80, and 120 min in a 15-ml rosette cell.

The sonicates were centrifuged at 12,000 × *g* for 30 min at 4°C, and the clear supernatants were removed. The concentration of protein (9) (using bovine serum albumin as standard), neutral sugar (4) (using glucose as standard), and methylpentose (3) (using fucose as standard) and the optical density at 230, 260, and 280 nm were determined on each sonicate supernatant. The supernatants were divided into small aliquots and stored at −20°C until used.

Preparation of antisera to whole bacteria and to sonicate supernatants. Antisera were prepared in rabbits against both the whole live bacteria and the sonicate supernatant. For immunization with the whole organisms, cultures were grown in 15 ml of appropriate medium and washed three times with pyrogen-free saline, and the pellet was made up to a volume of 3 ml with saline. The immunization schedule was that described by McCarty and Lancefield (10). A 0.5-ml amount of these suspensions was injected via the marginal ear vein on 3 successive days. After a 4-day rest, three more injections of 1 ml each were given. Two additional series of three 1-ml injections were given, each at a 4-day interval from the preceding one. A trial bleeding was taken 4 days later. Serum was tested for antibodies, using immunoelectrophoresis (IEP) with sonicate preparations as antigen. The sonicates were used at a concentration of approximately 7 mg of protein per ml. IEP was carried out at 6 V/cm for 50 to 60 min in veronal buffer, pH 8.4. The animals were then immunized for a subsequent week, rested for 1 week to 10 days, and then exsanguinated by cardiac puncture.

When immunizing with sonicate supernatant as antigen, the solution was mixed 1:1 with complete Freund adjuvant (Difco Laboratories, Detroit, Mich.) and then injected subcutaneously into the back of the rabbit in two well-separated sites (1.5 ml/site). At each immunization, each rabbit was given 21 mg (AV), 19.5 mg (AN), and 36 mg (VA) of total protein. The rabbits were trial bled after 1 month, and the sera were tested in IEP. The animals were reimmunized as above if required. Antisera were stored in small aliquots in sealed tubes at −20°C.

Biological activity of the sonicate antigen preparations. A microassay technique of in vitro lymphocyte blastogenesis (13) was used to measure biological activity. Experiments were designed to measure activity of the several bacterial sonicates and to test the reproducibility of the microassay system, using multiple samples taken successively from the same individuals.

Microassay technique. Venous blood was drawn from human volunteers and heparinized (20 U/ml). The mononuclear cells were separated by Ficoll-Hypaque density gradient centrifugation (2). They were washed three times with RPMI 1640 and resuspended at a concentration 1×10^6/ml in RPMI 1640 containing 15 mM HEPES buffer (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (Calbiochem, La Jolla, Calif.) and 20% heat-inactivated autologous plasma. Cultures were set up in triplicate groups containing 2×10^5 cells in 200 μl of medium per round-bottom well in microplates (no. 1-211-24-1, Cooke Laboratory Products, Alexandria, Va.).

Control (RPMI 1640), mitogen (phytohemagglutinin [PHA]; Burroughs-Wellcome, Research Triangle Park, N.C.), or antigens at appropriate dilutions were added to triplicate wells in 20-μl volumes. The cultures were maintained at 37°C in a humid atmosphere of air containing 5% CO_2. The cultures were incubated for 4 days, the final 24 h of which was in the presence of 0.2 μCi of [^{3}H]thymidine (specific activity, 2 Ci/mmol; Amersham/Searle, Arlington Heights, Ill.) added in 20 μl of medium.

The viability of randomly selected cultures was tested at the end of the culture period, using the trypan blue exclusion procedure. Viability was greater than 95% in all cultures tested.

The cells were harvested, using distilled water onto glass fiber filter paper disks (Skatron, A-S, 3401 Lierbyen, Norway), with the Skatron cell harvester. These were then dried for 1 h at 60°C and transferred to a scintillation vial containing 5 ml of a mixture of Scintiprep II and toluene (Fisher Scientific Co., Rochester, N.Y.; 60 ml of Scintiprep II and 1 liter of toluene). Counting was done in a Packard Tri-Carb liquid scintillation spectrometer.

A stimulation index (SI) was computed as follows:

$$SI = \frac{\text{mean cpm in stimulated cultures}}{\text{mean cpm in control cultures}}$$

Using normal distribution theory and a pooled estimate of variance, SIs exceeding 2.5 were significant at the 95% level.

Reproducibility of the microassay procedure was

determined by using samples from four male subjects, all of whom were reactive to antigens prepared from AV, AN, and VAI and to streptokinase-streptodornase (SK-SD; Lederle, Pearl River, N. Y.; prepared at 2,500 SK units/ml of RPMI 1640). All subjects had gingival indexes of 1 to 1.5, using the Löe and Silness (8) index. One subject was tested 14 times over a 7-week period, two others on six occasions over a 3-week period, and one subject nine times over a 4-week period. In all instances, blood was taken at the same time of day from each individual. The mitogen (PHA) and antigen preparations were prepared in aliquots before the experiment, each aliquot being of sufficient volume for one experimental sample. All experiments were carried out by one person using the same preparative procedures.

Antigen preparations from SS, SM, BM, and LB were used to test individuals with gingivitis and periodontitis (12). In addition, the stimulatory effects of antigen preparations from AV, AN, and VAI and the standard SK-SD preparation were assayed with human cord blood mononuclear cells.

RESULTS

Effects of sonication time. The results of sonication for various periods of time are shown in Fig. 1 for VAI, AN, and AV. With the AN and AV, maximum breakage occurred after approximately 2 h of sonication under the conditions described. Maximum breakage of VAI occurred after only 40 min of sonication. The increases in the amount of protein and in the optical density at wavelengths indicated were correlated directly with increase in breakage of the microorganism. Little carbohydrate was detected in the sonicate supernatants at that time, and there was only slight

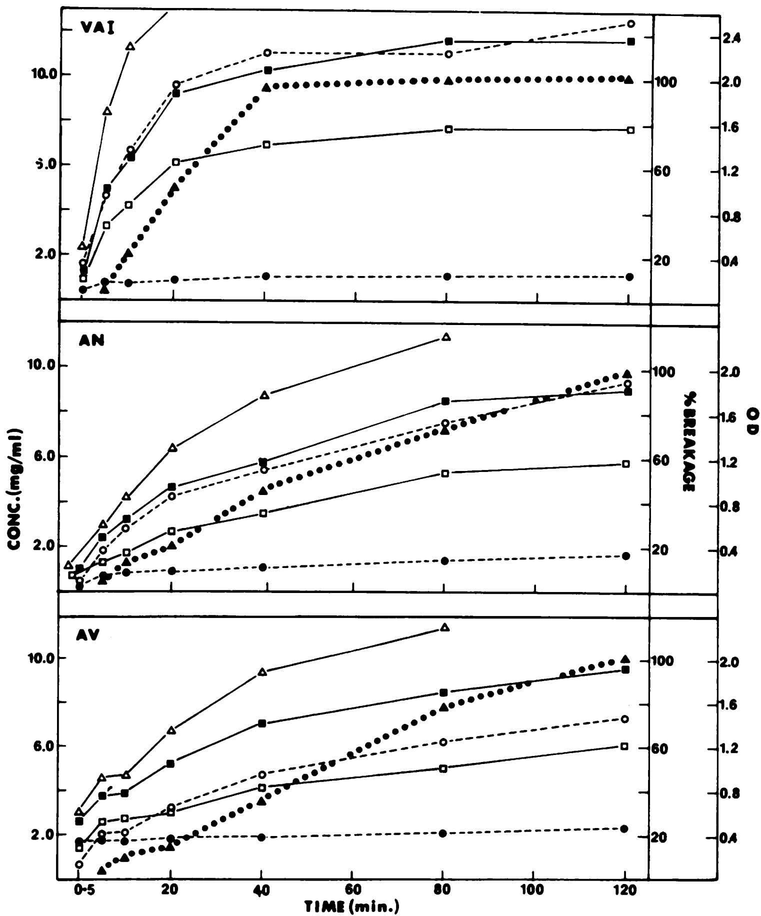

FIG. 1. *Effect of time upon breakage and components in the sonicate supernatants of VAI, AN, and AV. OD, Optical density. Symbols:* (□) *280 nm;* (○) *protein;* (■) *260 nm;* (●) *neutral sugar;* (△) *230 nm;* (▲) *percent breakage.*

rise observed with continuing sonication. Overall, sonication resulted in breakage of the organisms, associated with increase in soluble substances in the supernatant.

On the basis of experiments such as the preceding ones, sonication procedures were established for the production of soluble antigens. Sonication was carried out until more than 95% of the microorgansims were broken. For 150 ml of suspension containing 100 mg (wet weight) per ml of bacteria, the time taken was 2.5 to 3 h for the *Actinomyces* species 1 to 1.5 h for VA, SS, and SM, and less than 1 h for BM and LB. The protein and sugar composition of each sonicate supernatant is shown in Table 1. It can be seen that most were rich in protein with the exception of LB, which was predominantly neutral sugar, and SM, which was about equal in neutral sugar and protein. There was relatively little methylpentose detected in the sonicates except for AV and SM, where the methylpentose content was about 1/10 that of the protein.

Antigenic components of sonicate supernatants. Antisera to the whole bacteria and to the sonicate supernatants were used to study antigenic components present in the sonicate supernatants. Figure 2 illustrates typical IEP patterns obtained. The antigen preparation in each case was the sonicate supernatant obtained after maximum breakage of the respective microorganisms. In general, the complexity of the precipitin bands obtained upon IEP using anti-sonicate antisera was greater than that seen with the anti-whole bacteria antisera. This was particularly evident in examining sonicates obtained from AN and VAI (Fig. 2b, and c). It should also be noted from Fig. 2b and 2c that some antigens were detected with one type of antiserum that were not visualized with the other, even though the total number of precipitin arcs was always less in the reactions with antiserum to intact bacteria. Figure 2a shows the patterns obtained with AV. A major electronegative antigen, marked with an asterisk in Fig. 2a (AV) and 2b (AN), and a major antigen of γ-mobility, marked with an asterisk in Fig. 2c (VAI), were detectable in the respective sonicate preparations with the homologous anti-whole bacterial antiserum.

It has been pointed out that as the time of sonication increased, there was a quantitative increase in the supernatant components related to cell breakage. Even though the quantity of soluble antigens increased during the period of sonication, there was no detectable qualitative change in the IEP pattern. This is illustrated in Fig. 3, in which samples collected early in sonication are compared with those taken after cell disruption had been completed. Ten and 20 min of sonication was sufficient for detection of all components using VAI and *Actinomyces* sonicates, respectively.

The antigenic components found in sonicate supernatants from SM, SS, VAII, BM, and LB at maximum breakage are shown in Fig. 4. This illustrates their complexity and the heterogeneity of the rabbit antibodies. In Fig. 5, reactions of antisera to two strains of *Veillonella* are compared. The precipitin patterns observed were very different, with only one cross-reacting antigen detected.

Cellular immunity. The tests that were done of reproducibility of our assay indicated dose dependency of the reactions and also verified that the bacterial substances acted as antigens and not as mitogens. The data in Table 2 and in Fig. 6 illustrate these points. Reproducibility is indicated by the small standard errors observed in these studies. If the sub-

TABLE 1. *Protein, neutral sugar, and methylpentose measurements of bacterial sonicates and amounts tested in in vitro lymphocyte blastogenesis*

Bacteria	Wet wt of bacteria sonicated (mg/ml)	Protein[a]	Neutral sugar[a]	Methyl pentose[a]	Protein/ neutral sugar ratio	Protein/ methylpentose ratio	Concn of protein/ml of culture (μg) in undiluted samples
AV	100	70	22	6.2	3.2	11.3	700
AN	100	95	19	4.3	5.0	22.1	950
VAI	100	120	15	2.8	8.0	42.9	1,200
VAII	100	88	10	0.6	8.8	146.7	880
LB	10	44	70	0.1	0.6	440.0	440
BM	100	143	23	4.4	6.2	32.5	1,430
SM	100	56	52	5.2	1.1	10.8	560
SS	100	51	20	0.8	2.5	63.8	510

[a] Micrograms per milligram of bacteria sonicated.

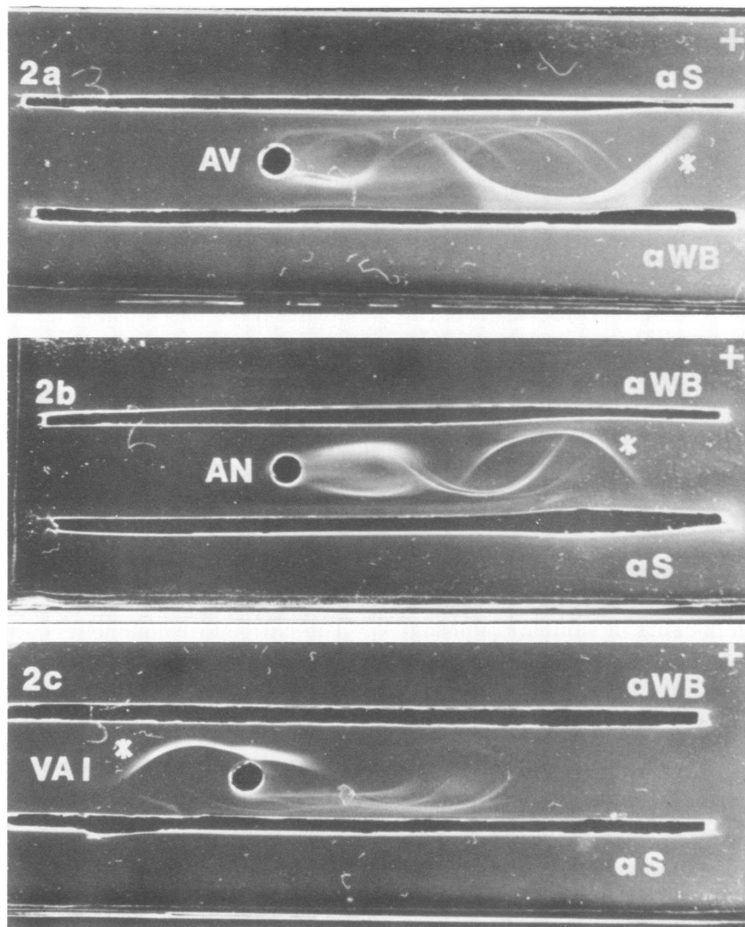

FIG. 2. *(a) IEP of sonicate supernatant of AV (well; 7 mg/ml of protein) obtained after maximum breakage. Abbreviations: aS, Anti-sonicate antiserum in upper trough; aWB, anti-whole bacterial antiserum in lower trough. (b) Same as (a) but with AN (well; 9.5 mg/ml of protein) and aS in lower trough and aWB in upper trough. (c) Same as (a) but with VAI (well; 12 mg/ml of protein) and aS in lower trough and aWB in upper trough.*

stances were stimulatory because they contained mitogens at the concentrations tested, one would expect all individuals to react strongly.

Further verification that the preparations were antigenic and not mitogenic in nature for the peripheral blood lymphocytes is found in Table 3. When lymphocytes from human cord blood were tested with the preparations from AV, AN, and VAI, there was little or no stimulation observed. Again, this was like SK-SD rather than like the mitogen PHA, which did stimulate all subjects.

Table 4 shows the responses to sonicate supernatants from AV obtained after various times of sonication. They were used at an equivalent protein concentration in cultures with adult peripheral blood lymphocytes. The SIs depended only upon antigen dose and not upon preparation time.

As reported in a companion paper (12), results using sonicates of BM,LB, andVAII in the blastogenesis assay were as follows: BM and LB stimulated cells from individuals with various severities of periodontal disease; samples from five of eight individuals with periodontitis and one of seven patients with gingivitis were stimulated by BM; LB significantly stimulated cells from six of eight patients with periodontitis and three of seven with gingivitis; and VAII stimulated cells from four of six patients with gingivitis and from all with periodontitis.

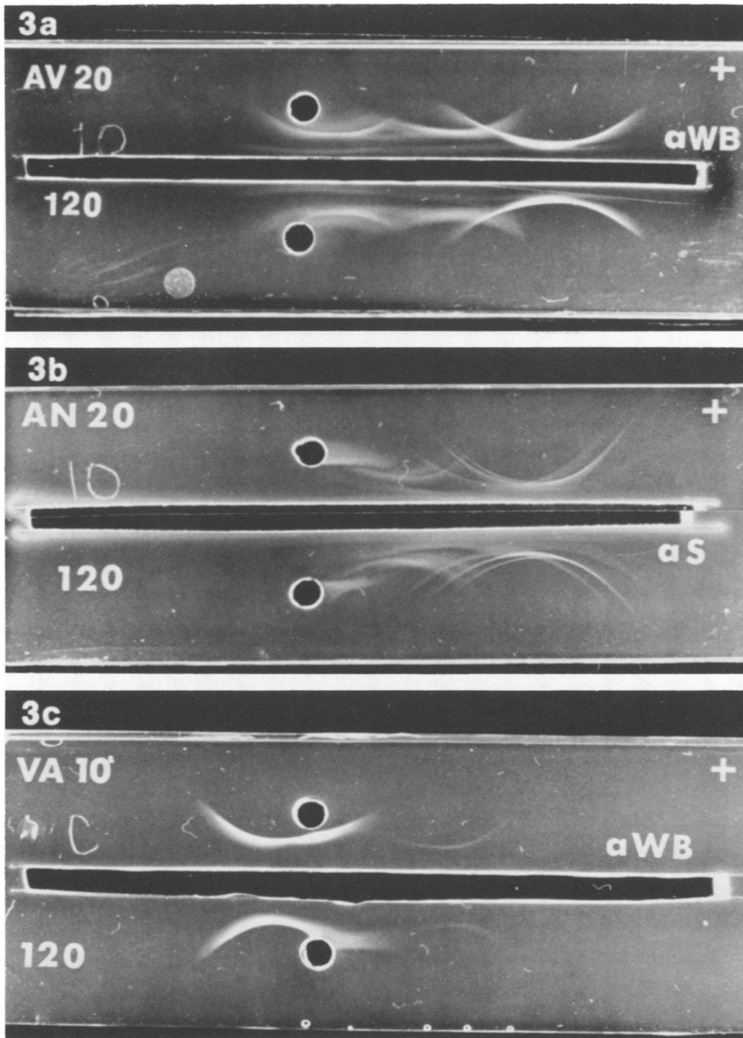

FIG. 3. *(a) Effect of time of sonication upon the production of sonicate supernatant antigens of AV. Anti-whole bacterial antiserum (aWB) shows that antigens present at 120 min of sonication (maximum breakage, lower well) are also present after 20 min of sonication (upper well). Protein concentration in each well was approximately 6 mg/ml. (b) All antigens detectable after 120 min of sonication (lower well) of AN are present after 20 min of sonication, using antiserum to the sonication supernatant obtained after maximum breakage (aS). Protein concentration in each well was approximately 6 mg/ml. (c) Using an anti-whole bacterial antiserum (aWB), all components present after maximum sonication of VAI are also seen after only 10 min of sonication. Protein concentration in each well was approximately 8 mg/ml.*

DISCUSSION

The reagents used to distinguish and identify the components of the sonicate preparations used in this study were antisera raised in rabbits against either washed viable whole organisms or the sonicates prepared from some of these organisms. IEP analysis of the sonicates with those antisera showed the sonicates to contain many antigens. The anti-sonicate antisera generally showed a greater number of distinguishable antigenic components than did antisera to the whole cell. The antisera also served as reagents for checking the consistency of components released within each successive sonicate preparation. Several separate batches of sonicate have now been prepared from the major organisms used in

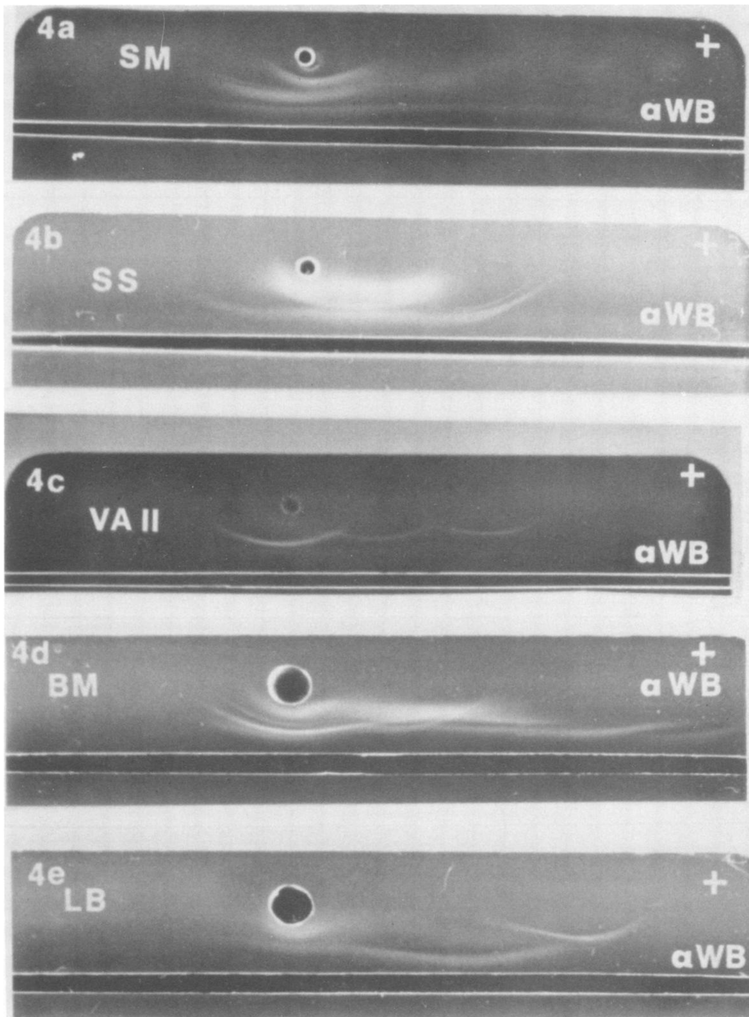

FIG. 4. *Antigenic components found in the sonicate supernatants after maximum breakage, detected by using anti-whole bacterial antiserums of SM (a), SS (b), VAII (c), BM (d), and LB (e). Protein concentration in wells was: SM, 5.6 mg/ml; SS, 5.1 mg/ml; VAII, 8.8 mg/ml; BM, 14.3 mg/ml; and LB, 4.4 mg/ml.*

this study, and they have been shown to be comparable in IEP. This method of cell disruption and antigen release results in a constant product as far as antigenic content detected in this fashion is concerned.

It is interesting that in the case of the AV, AN, and VAI sonicate antigen preparations, a major antigen could be detected with antiserum to the whole organism but not with antisonicate serum. These are marked with asterisks in Fig. 2. Apparently these components are immunogenic when present as part of a whole cell but are nonimmunogenic in the form in which they are recovered as a soluble substance in the sonic supernatants. Alternatively, one may suppose either that some adjuvant effect induced by other components of the bacteria or that the route of immunization is significant in stimulating a rabbit to produce antibodies to these major antigens.

The experiments comparing the effect of sonication time showed that this complexity was an indigenous property of the sonicate preparations and not due to artifactual production of antigens during extended sonication as the result of breakage of large polymeric antigens into smaller components. The constancy of the biological activity of the

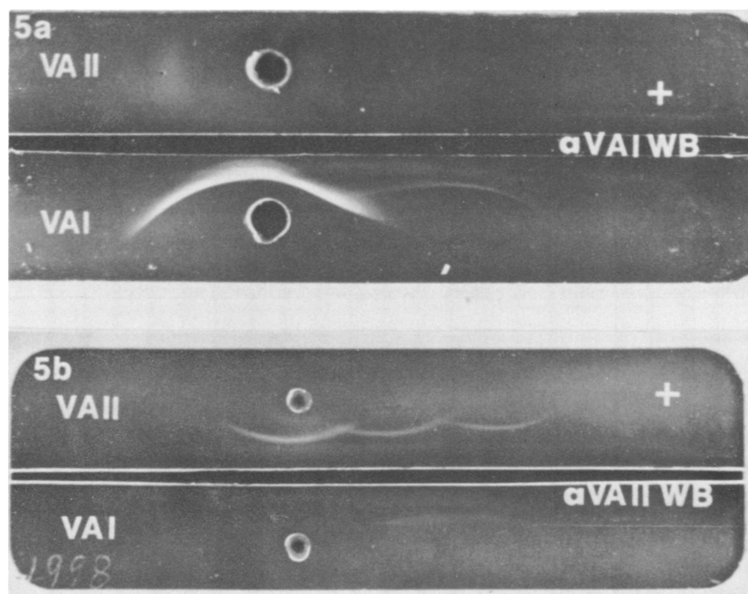

FIG. 5. *Cross-reaction between the two strains of VA. Anti-VAI whole bacterial antiserum (aVAIWB) shows no cross-reaction with VAII supernatant (a; 8.8 mg of protein per ml), whereas a faint cross-reaction is seen with aVAIIWB antiserum with VAI supernatant (b; 12 mg of protein per ml).*

equivalently concentrated timed sonicates of AV also indicates the presence of the major stimulating antigen(s) early in the course of sonication (Table 4).

The chemical analyses showed that the major components released from most bacteria were protein. Usually little neutral sugar was detected. Only in LB was neutral sugar present in greater amounts than protein. The presence of nucleic acids in the sonicate supernatant is suggested by the optical density at 260 nm in Fig. 1. Methylpentose was detected in all sonicate supernatants but was present only in small amounts, and in all cases less than the amount of neutral sugar (Table 1).

In determining the biological activity of the sonicates, a microtechnique for in vitro lymphocyte blastogenesis was used (13). Our experiments demonstrated the consistency and reproducibility of the method, and also that the sonic supernatants contain specific antigens. Nonspecific mitogenic responses are probably not measured under the conditions of our cultures of peripheral blood leucocytes.

The responses detected among individuals were, as expected, variable; however, the response within the samples tested from a single individual were reproducible and appeared to be characteristic of that individual over the time periods tested (Table 2 and Fig. 6).

The response to the sonicates was dose dependent, with the maximum dose not necessarily producing maximum stimulation. The dose-dependent nature of the response with the sonicate preparations was similar to that observed with SK-SD. The maximum stimulation produced by sonicate preparations and with SK-SD was at 4 to 5 days. This was significantly different from the time course of stimulation obtained with the mitogen PHA, which in our experiments was maximum at 2 to 3 days (12). The final indication of the antigenic nature of the cellular response to the antigens comes from the experiments using cord blood lymphocytes. Neither the oral bacterial antigens tested nor SK-SD stimulated these cells significantly, whereas the mitogen PHA did.

Responsiveness of adult lymphocytes to the sonicates from AV, AN, and VA was consistently more ubiquitous than responsiveness to BM and LB sonicates (12). The finding that sonicates of SS and SM were not stimulatory in most adults tested we consider as indicating rarity of sensitization. Although other sorts of antigen preparations might be considered, we believe that preparations as described here contain many of the principal antigens.

The analysis of the antigens in the preparations used in this study represents the initial step in purifying and characterizing them for use in experiments designed to determine the

TABLE 2. *Results of longitudinal experiments designed to determine reproducibility of microtechnique and activity of antigen preparations*

Test reagent/ml of culture	Subject 1, n = 14[a]		Subject 2, n = 9		Subject 3, n = 9		Subject 4, n = 6	
	cpm ± SE[b]	SI ± SE	cpm ± SE	SI ± SE	cpm ± SE	SI ± SE	cpm ± SE	SI ± SE
Control	154 ± 15		155 ± 39		234 ± 77		106 ± 10	
PHA, 10 μg	15,084 ± 832	110.6 ± 13.0	17,649 ± 805	158.4 ± 32.6	10,269 ± 1,004	65.1 ± 14.5	14,304 ± 1,142	140.1 ± 16.1
AV								
Undiluted[c]	2,291 ± 283	15.3 ± 1.3	1,283 ± 243	9.9 ± 1.6	1,250 ± 154	7.7 ± 1.6	806 ± 84	17.6 ± 0.6
1/10	1,144 ± 138	7.2 ± 0.6	803 ± 183	6.3 ± 1.3	1,127 ± 102	6.8 ± 1.6	471 ± 63	4.4 ± 0.2
1/50	683 ± 80	4.5 ± 0.3			707 ± 60	4.4 ± 1.1	446 ± 50	4.1 ± 0.2
1/100	379 ± 69	2.4 ± 0.2			463 ± 15	2.9 ± 0.7	303 ± 68	2.8 ± 0.6
AN								
Undiluted	1,606 ± 161	10.6 ± 0.7	603 ± 161	4.2 ± 0.5	823 ± 112	4.9 ± 1.0	626 ± 66	5.9 ± 0.4
1/10	1,984 ± 261	13.1 ± 2.1	550 ± 101	3.8 ± 0.3	966 ± 178	5.9 ± 1.2	1,140 ± 91	10.9 ± 0.7
1/50	1,149 ± 146	7.9 ± 1.3			732 ± 109	4.5 ± 1.2	652 ± 54	6.2 ± 0.4
1/100	947 ± 124	6.5 ± 1.0			644 ± 87	3.8 ± 0.9	573 ± 43	5.5 ± 0.3
VAI								
1/5	926 ± 95	6.4 ± 0.7	616 ± 112	4.5 ± 0.6	728 ± 90	4.1 ± 0.7	422 ± 54	4.0 ± 0.4
1/10	1,190 ± 197	7.7 ± 0.9	661 ± 151	5.7 ± 0.4	722 ± 91	4.5 ± 1.0	538 ± 54	5.3 ± 0.7
1/100	677 ± 88	4.6 ± 0.5			612 ± 107	3.7 ± 0.8	417 ± 76	3.9 ± 0.5
SK-SO (U)								
100	1,132 ± 173	7.6 ± 1.0	1,946 ± 321	14.2 ± 1.4	983 ± 56	6.0 ± 1.3	688 ± 118	6.6 ± 1.2
50	882 ± 103	5.5 ± 0.5	1,652 ± 237	13.0 ± 1.9	817 ± 64	4.9 ± 1.2	423 ± 56	4.2 ± 0.7
10	425 ± 87	2.7 ± 0.3			520 ± 41	3.5 ± 0.6	219 ± 44	2.1 ± 0.5
1	153 ± 24	1.0 ± 0.1			294 ± 28	1.8 ± 0.4	127 ± 26	1.2 ± 0.2

[a] n, Number of individual test samples.
[b] SE, Standard error.
[c] Concentration used in culture shown in Table 1.

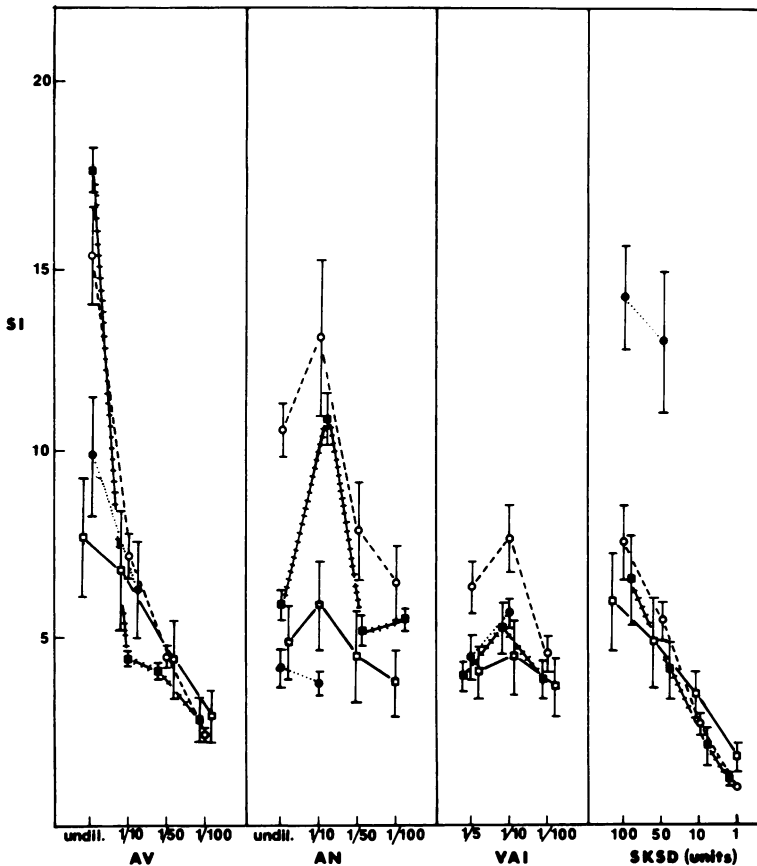

FIG. 6. *Reproducibility and dose response nature of in vitro lymphocyte blastogenesis to the antigen preparations of AV, AN, VAI, and SK-SD. Each point of the graph represents the mean ± 1 standard error of the stimulation index (SI) of the samples tested. Symbols: (○) Subject 1, n = 14; (●) subject 2, n = 9; (□) subject 3, n = 6; (■) subject 4, n = 6.*

TABLE 3. *In vitro response of human cord blood lymphocytes to mitogen and antigens*[a]

Material	Determination (mean ± standard error)	
	cpm	SI
Control	1,304 ± 316	
PHA	13,420 ± 6,254	10.3 ± 4.8
AV	2,738 ± 680	2.1 ± 0.5
AN[b]	11,906 ± 2,269	1.9 ± 0.2
VAI	1,695 ± 130	1.3 ± 0.1
SK-SD	1,564 ± 130	1.2 ± 0.1

[a] Four separate cord bloods were tested.
[b] Determined by using 1×10^6 mononuclear cells cultured in 1 ml of RPMI 1640. The control cpm under these conditions was 6,065 ± 1,030.

specificity of the cell-mediated immunity in periodontal diseases. A preliminary report of this work has been reported (M. J. Reed, P. Black, and D. L. Fisher, J. Dent. Res. 55:B211, Abstr. 598, 1976).

In summary, it has been shown the sonicate supernatant preparations made from several strains of oral bacteria stimulate human peripheral blood lymphocytes to blastogenesis. The preparations are complex mixtures of antigens, mainly protein and generally containing small amounts of neutral sugars or methylpentoses. These antigen preparations evoke a dose-dependent lymphoproliferative response optimal at 4 to 5 days of culture similar to that obtained by using SK-SD.

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TABLE 4. *Biological activity of timed sonicates of A. viscosus*

Time (min)	Dilution	cpm (mean ± SE)[a]	SI (mean ± SE)
0.5	0[b]	1,221 ± 213	6.6 ± 2.00
	1:10	580 ± 185	3.2 ± 1.10
	1:100	250 ± 61	1.2 ± 0.14
5	0	1,428 ± 545	7.6 ± 2.80
	1:10	739 ± 278	3.8 ± 1.30
	1:100	324 ± 125	1.7 ± 0.51
10	0	1,339 ± 507	7.6 ± 3.30
	1:10	681 ± 223	3.8 ± 1.50
	1:100	312 ± 109	1.6 ± 0.43
20	0	1,041 ± 326	6.0 ± 2.40
	1:10	665 ± 146	3.7 ± 1.20
	1:100	322 ± 103	1.6 ± 0.38
40	0	1,247 ± 321	6.6 ± 1.70
	1:10	601 ± 140	3.2 ± 0.89
	1:100	294 ± 106	1.5 ± 0.43
80	0	1,548 ± 250	8.1 ± 1.40
	1:10	704 ± 203	3.3 ± 0.37
	1:100	330 ± 104	1.6 ± 0.38
120	0	1,127 ± 105	6.3 ± 1.50
	1:10	835 ± 221	4.8 ± 1.80
	1:100	326 ± 102	1.8 ± 0.59

[a] SE, Standard error.

[b] Undiluted cultures contained approximately 500 μg per ml of culture.

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