INFECTION AND IMMUNITY, Aug. 1978, p. 567-574
0019-9567/78/0021-0567$02.00/0
Copyright © 1978 American Society for Microbiology
Vol. 21, No. 2
Printed in U.S.A.

Inhibitory Effects of Extracellular Products from Oral Bacteria on Human Fibroblasts and Stimulated Lymphocytes†

THOMAS B. HIGERD,* DAVID H. VESOLE, AND JEAN-MICHEL GOUST

Department of Basic and Clinical Immunology and Microbiology, Medical University of South Carolina, Charleston, South Carolina 29401

Received for publication 11 May 1978

Extracellular products of 12 strains of *Streptococcus mutans* and 5 additional species of oral bacteria were analyzed for their ability to inhibit proliferation of fibroblastoid cells (HeLa and AV3) and blast transformation of human peripheral blood lymphocytes obtained from normal individuals. Products from *S. mutans* strains AHT and BHT, *Streptococcus intermedius*, and *Actinomyces viscosus* inhibited [^{3}H]thymidine uptake by fibroblastoid cells and phytohemagglutinin-stimulated lymphocytes. Products from *S. mutans* E49, *Streptococcus salivarius*, and *Actinomyces naeslundii* inhibited blast transformation of human lymphocytes but did not significantly inhibit the growth of fibroblastoid cells. Preparations from *S. intermedius* gave the greatest inhibitory activity against both target cell types; initial characterization of this preparation suggested a single factor active in both assays, in that the heat lability and Sephadex G-200 elution profile were similar for the inhibitory activity seen with the two cell types. The molecular weight of the inhibitor, estimated by gel filtration on Sephadex G-200 and Ultragel AcA34, was approximately 160,000. The results strongly suggest that oral bacteria produce heat-labile substances that interfere with fibroblast proliferation and alter the lymphocytic immunological response.

The pathogenesis of dental disease of the soft tissue is poorly understood. Gingivitis, the clinical manifestation following the accumulation of bacterial plaque in the gingival crevice area (21, 31), usually progresses to periodontal disease, and thus it is reasonable to conclude that dental plaque contains the primary factor(s) responsible for the initiation of periodontal disease.

Many studies (8, 28, 29) have been concerned with the detection and quantitation of a wide variety of microorganisms contained in the debris from the human gingival crevice, but only recently have investigations focused on the host's immune response to plaque material. Considerable evidence is accumulating that plaque microorganisms and/or their products initiate periodontal inflammation by altering the immune response of the host (3). Although the precise role of humoral (antibody-mediated) immunity in the pathogenesis of periodontal disease is obscure, serum antibodies against oral bacteria have been detected in patients with periodontal disease (4, 6, 18, 22, 30). More recently, the importance of cell-mediated immunity in periodontal disease has been suggested (10, 23). Ivanyi and Lehner (12, 13), Baker et al. (1), Horton et al. (9), and Gaumer et al. (7) have reported the presence of bacterial factors capable of transforming lymphocytes and, presumably, enhancing antibody production. These substances have been demonstrated in sonically treated material (11, 12, 14, 15, 25, 27), extracts (1, 7, 17), and heated culture fluids (24) from oral bacteria as well as sonically treated material or extracts of dental plaque (9, 13, 17, 26) and saliva (9). Correlation of the lymphocyte response to the crude extracts and the presence, absence, or severity of periodontal disease remains questionable.

It is reasonable to assume a priori that extracellular products of oral bacteria may be involved in the pathogenesis of periodontal disease, since bacteria are rarely, if ever, found within gingival tissues. These products might also be assumed to interfere with the function of fibroblasts, since early loss of collagen in periodontal disease may be directly related to the ability of fibroblasts to produce or catabolize collagen. We have found that extracellular products from cultured oral bacteria are capable of inhibiting both proliferation of human fibroblastoid cell lines and blast transformation of stimulated human peripheral lymphocytes from normal individuals, suggesting that such products may be implicated in the pathology of periodon-

† Publication no. 201 from the Department of Basic and Clinical Immunology and Microbiology, Medical University of South Carolina.

tal disease and/or may interfere with the immune defense of the host against the invading microorganisms.

MATERIALS AND METHODS

Organisms and media. *Actinomyces* sp. were furnished by John K. Dyer, Department of Oral Biology, University of Nebraska, Lincoln, and *Streptococcus sanguis* 10556 was obtained from the American Type Culture Collection. All other strains were the gift of Robert H. Staat, Department of Oral Biology, University of Louisville, Louisville, Ky. The microorganisms were grown anaerobically in tryptone-glucose agar consisting of 20 g of tryptone, 5 g of glucose, 4 g of K_2HPO_4, 1 g of KH_2PO_4, 2 g of NaCl, 250 mg of $MgSO_4 \cdot 7H_2O$, 17 mg of $MnSO_4$, and 15 g of agar dissolved in 1 liter of distilled water. In each case, the organisms were grown at 37°C for 24 h.

Bacterial products. For the analysis of extracellular products, disks of sterile dialysis membranes (12,000- to 14,000-molecular-weight cutoff) of the same diameter as the petri dish (100 mm) were placed on the agar surface of fresh plates. A sample (0.1 ml) of a bacterial cell suspension (10^8 cells per ml) was spread over the dialysis membrane with a cotton swab. The plates were incubated at 37°C under anaerobic conditions. After 24 h of incubation, the dialysis membranes were removed and washed with a minimal volume of 0.05 M potassium phosphate buffer, pH 7.5. The wash was clarified by centrifugation at 29,000 × *g* for 15 min and concentrated about 10-fold by positive-pressure ultrafiltration, using an Amicon PM-10 filter. The final solution was designated "extracellular products." Since concentrated washes of sterile disks harvested from tryptone-glucose agar medium did not result in detectable protein bands after electrophoresis, or biological activity, contamination from the medium was considered negligible. No attempt was made to solubilize proteins that were bound to cells or cellular debris.

Characterization of inhibitory factor. Preparations of *Streptococcus intermedius* and *Actinomyces naeslundii* were dialyzed against phosphate-buffered saline overnight and applied to a chromatographic column of (i) Sephadex G-200 (Pharmacia Fine Chemicals, Piscataway, N.J.) equilibrated with the same buffer (0.15 by 60 cm; 2-ml fractions collected) or (ii) LKB Ultragel AcA34 (LKB Produkter AB, Bromma, Sweden) equilibrated with the same buffer (0.25 by 91 cm; 8.4-ml fractions collected).

Thin-layer sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out by the procedure described by Karam and Bowles (16), and the gel was stained with a 0.02% Coomassie brilliant blue solution.

Assay of fibroblast inhibitory factor. HeLa (established from human carcinoma of the cervix) and AV3 (established from human amnion) cell lines were grown in monolayer culture. At confluency, 0.25% trypsin-ethylenediaminetetraacetate (Grand Island Biological Co., Grand Island, N.Y.) in sterile phosphate-buffered saline was added to the culture flask. After detachment, the cells were centrifuged, washed twice in Eagle minimal essential medium (MEM), and resuspended in MEM with 10% fetal calf serum at a final concentration of 10^4 cells per ml.

Each well of a Falcon microtiter plate was seeded with 100 μl of the suspension (10^4 cells), followed by incubation for 2 to 4 h at 37°C to allow the cells to attach. Then 100 μl of the bacterial product to be tested, in twofold serial dilutions with MEM containing 20% fetal calf serum, was added to each well. the plates were incubated for 5 days at 37°C in an air-CO_2 (95%:5%) incubator and pulsed with 0.2 μCi of [^{3}H]-thymidine (specific activity, 1.9 Ci/mmol; New England Nuclear Corp., Boston, Mass.) for the last 18 h. The supernatants were removed, and 0.2 ml of 0.25% trypsin-ethylenediaminetetraacetate in sterile phosphate-buffered saline was added to each well. After complete detachment from the bottom of the wells, the cells were harvested onto glass fiber strips, using a multiple automated sample harvester (Mash II; Microbiological Associates, Bethesda, Md.), dried, and transferred to vials containing 2 ml of Omnifluor before counting in a beta scintillation counter (Packard Tri-Carb, no. 3380). The formula used to calculate percent inhibition (%I) was: %I = 100 × (counts per minute, experiment/counts per minute, control) − 100.

Assay for inhibition of blast transformation. Heparinized peripheral blood from healthy volunteer blood donors was obtained through the courtesy of H. L. Taylor (American Red Cross Transfusion Center of the Lowcountry, Charleston, S.C.). Mononuclear cells were separated on Ficoll-Isopaque gradients and washed three times in MEM. The cells were resuspended at a final concentration of 2×10^6 cells per ml in MEM with 10% fetal calf serum, and 100 μl of the cell suspension was added to each well of a Falcon microtiter plate. Controls with and without phytohemagglutinin (PHA) were placed in cultures simultaneously with test cells, which were stimulated with PHA and to which the bacterial products to be tested were added in twofold serial dilutions (in MEM with 20% fetal calf serum) to a final volume of 0.2 ml. The cells were incubated at 37°C for 2 days before pulsing overnight with [^{3}H]thymidine as described above. The lymphocytes were collected with the automatic harvester onto glass fiber strips and dried, and the radioactivity was determined in a scintillation counter.

The formula used to calculate %I of lymphocyte blast transformation was: %I = 100 × (counts per minute, experiment − counts per minute, unstimulated)/(counts per minute stimulated with PHA alone − counts per minute, unstimulated controls) − 100. A semilogarithmic relationship was found between dilution and %I. By applying the formula $y = e^x$, with y = log (dilution) and x = %I, each experiment was converted to a linear regression curve of the form $y = -ax + b$. The regression coefficient was calculated and considered as fitting the experimental results for values $1.10 < r < 0.90$. From the experimental curve, the dilution producing 25% inhibition was interpolated, and the maximum inhibitory effect was extrapolated.

Direct cytotoxicity of each preparation was assayed by simple trypan blue exclusion tests. Lymphocyte viability was consistently found to be greater than 90% even after 5 days of incubation with the inhibitory products.

RESULTS

The technique of growing microorganisms on

dialysis membranes is commonly used as a method of collecting extracellular products, and the ability to separate the products from intracellular constituents depends on the degree of cell lysis. To confirm that microbial lysis was minimal by this technique, we determined UV absorption profiles of *S. intermedius* preparations after exhaustive dialysis against distilled water. A single peak of absorbance occurred at 275 nm, with minimal absorbance at 256 nm, suggesting insignificant nucleic acid contamination and therefore negligible cell lysis (Fig. 1).

Initially, the extracellular products from 12 strains of *Streptococcus mutans* and 5 additional species of oral bacteria were tested for their ability to inhibit [^{3}H]thymidine uptake by established human fibroblastoid cell lines. The products were diluted at least 1:40 in MEM to prevent pH or salt effects from interfering with the assay. In addition, all samples were diluted so that 2 μg of protein was added to each well. When the %I was not greater than 15, the results were regarded as insignificant. Only four strains of *S. mutans*, AHT, BHT, 10449, and IB, appeared to produce factors that significantly inhibited fibroblast proliferation as measured by [^{3}H]thymidine uptake (Table 1). In no instance did products from all strains belonging to a given serotype (2) cause inhibition. The bacterium isolated from human dental plaque and classified by the Communicable Disease Center (Atlanta, Ga.) as *S. intermedius* consistently gave the highest %I. *Actinomyces viscosus* but not *A. naeslundii* produced substances that inhibited proliferation of fibroblasts. The results for AV3 and HeLa cell lines were similar (only those for HeLa cells are shown in Table 1).

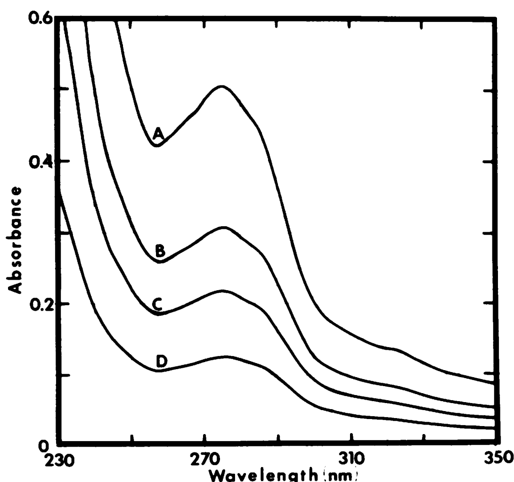

FIG. 1. *Wavelength profile of the dialyzed preparation from S. intermedius at (A) 0.42, (B) 0.26, (C) 0.18, and (D) 0.10 mg of protein per ml.*

TABLE 1. *Inhibition of [^{3}H]thymidine uptake by fibroblastoid cell lines and PHA-stimulated lymphocytes in the presence of extracellular products from various oral bacteria*[a]

Bacterial strain	Serotype[b]	Inhibition (%)	
		FCL (AV3)	PHA-stimulated lymphocytes
S. mutans AHT	*a*	24	22
S. mutans OMZ 61	*a*	—[c]	—
S. mutans E49	*a*	—	42
S. mutans BHT	*b*	72	45
S. mutans Fa-1	*b*	—	16
S. mutans 10449	*c*	17	—
S. mutans GS5	*c*	—	—
S. mutans IB	*c*	22	—
S. mutans SL1	*d*	—	—
S. mutans OMZ 176	*d*	—	—
S. mutans LM7	*e*	—	—
S. mutans B2	*e*	—	—
S. intermedius		84	85
S. salivarius		—	38
S. sanguis 10556		—	—
A. naeslundii 12164		—	69
A. viscosus M100		19	32

[a] Assayed in the presence of 2 μg of protein per well as described in Materials and Methods. FCL, Fibroblastoid cell line.

[b] See reference 2.

[c] —, Not significant (inhibition < 15%).

Sonic extracts of oral bacteria and plaque have been shown previously to stimulate [^{3}H]thymidine uptake by Ficoll-Isopaque-purified human mononuclear cells under certain conditions. Using PHA-stimulated lymphocytes as targets, identical samples of the same preparations used in the previous experiment with fibroblasts were added to microtiter wells containing mononuclear cells, and the %I of [^{3}H]thymidine incorporation after blast transformation was calculated (Table 1). Preparations from *S. mutans* strains AHT, E49, BHT, and Fa-1 caused significant inhibition. Again, *S. intermedius* products were the most effective, with a correlation coefficient for linear regression of 0.988. From the experimental curve (Fig. 2), 210 ng of the *S. intermedius* product gave 25% inhibition, and approximately 2 μg was calculated to give complete inhibition of [^{3}H]thymidine uptake by PHA-stimulated lymphocytes. Both *A. naeslundii* and *A. viscosus* were able to inhibit thymidine uptake, as was *S. salivarius*. Of particular interest was the observation that *S. mutans* E49, *S. salivarius*, and *A. naeslundii* appeared to elaborate substances that inhibited blast transformation of peripheral lymphocytes but lacked the ability to inhibit fibroblasts.

Since all preparations were extensively dialyzed against phosphate-buffered saline, it was

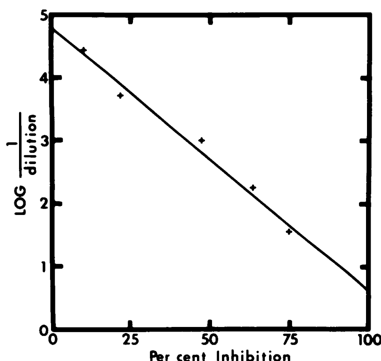

FIG. 2. *Inhibitory activity of various dilutions of a crude preparation (100 μg of protein per ml) of extracellular products from S. intermedius, assayed in cultures of PHA-stimulated human peripheral blood lymphocytes.*

not likely that the inhibition of the label was due to competition with cold thymidine. In addition, the effect of the preparation from *S. intermedius* on lymphocytes and fibroblasts has been subjected to morphological and cytofluorographic analysis. The results (manuscript in preparation) indicate the absence of blast transformation and decreased DNA and RNA synthesis in both fresh human peripheral blood lymphocytes and cultured lymphocytes and fibroblasts.

Partial purification of the inhibitory factor(s) was achieved by passage of a sample of the crude preparation from *S. intermedius* through a Sephadex G-200 column. The chromatographic profile (Fig. 3) revealed a single peak of biological activity when the fractions were assayed for the capacity to inhibit [^{3}H]thymidine incorporation by fibroblasts or PHA-stimulated lymphocytes. The molecular weight of this fraction was estimated to be approximately 160,000 by comparison with the elution profiles of proteins of known molecular weight. In addition, gel filtration of the crude preparation on Ultragel AcA34 (Fig. 3) provided a similar estimate for the molecular weight of the biological activity.

To assess purity, both the crude *S. intermedius* preparation and concentrated fractions from Sephadex G-200 possessing biological activity were subjected to sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis. The resulting protein pattern (Fig. 4) revealed the presence of about 40 proteins in the crude preparation and approximately 25 proteins in the G-200 fraction that exhibited biological activity.

Samples of the crude preparation from *S. intermedius* were placed at various temperatures for 10 min. All samples were cooled in an ice bath, diluted appropriately, and assayed for residual biological activity. As is evident from Fig. 5, the inhibitory activities for fibroblasts and for PHA-stimulated lymphocytes had similar heat lability profiles, in that the activity was retained after heating at 50°C for 10 min but was lost at temperatures above 70°C.

DISCUSSION

Because of the apparent absence of 260-nm-absorbing material in preparations from *S. intermedius* obtained by the dialysis membrane technique, it appears unlikely that the inhibition seen in these studies was caused by intracellular metabolites resulting from bacteriolysis. In addition, the well-defined elution pattern from gel filtration chromatography suggests that the inhibitor from this organism probably is not capsular material or endotoxin. It should be noted, however, that cell surface material may have been present in our preparations. Until the inhibitory factors have been purified and characterized, we have tentatively called our preparations "microbial products" in that microbial lysis was not evident, control preparations harvested under identical conditions but lacking microorganisms did not exhibit biological activity, and a well-defined population of proteins existed.

Current understanding of the etiology of periodontal disease strongly implies that oral bacteria and/or their "products" play a decisive role in the pathology of the disease. Despite the popularity of this statement, very little attention has been devoted to preparations of bacterial products as a source of material for use in experiments designed to answer fundamental questions concerning periodontal disease. As is evident from this communication, products from oral bacteria are easily obtained without denaturing treatments and possess interesting biological properties.

The pathology of gingivitis suggests that dental plaque contains substances which exert a destructive effect directly on the gingival epithelium (5). Extracts of human dental plaque prevent the growth of several types of mammalian cells in culture (19). More recently, Levine et al. (20) partially characterized the substances into two fractions: one was heat labile at 100°C and had a monomeric molecular weight of 30,000, and the second was not inactivated by boiling. We report the existence of extracellular products from several oral bacteria that can alter the growth of HeLa and AV3 cells. The extracellular product from one of the bacteria, *S. intermedius*, was heat labile and had a molecular weight of approximately 160,000. Whether our bacterial products are similar to the cytotoxic substances

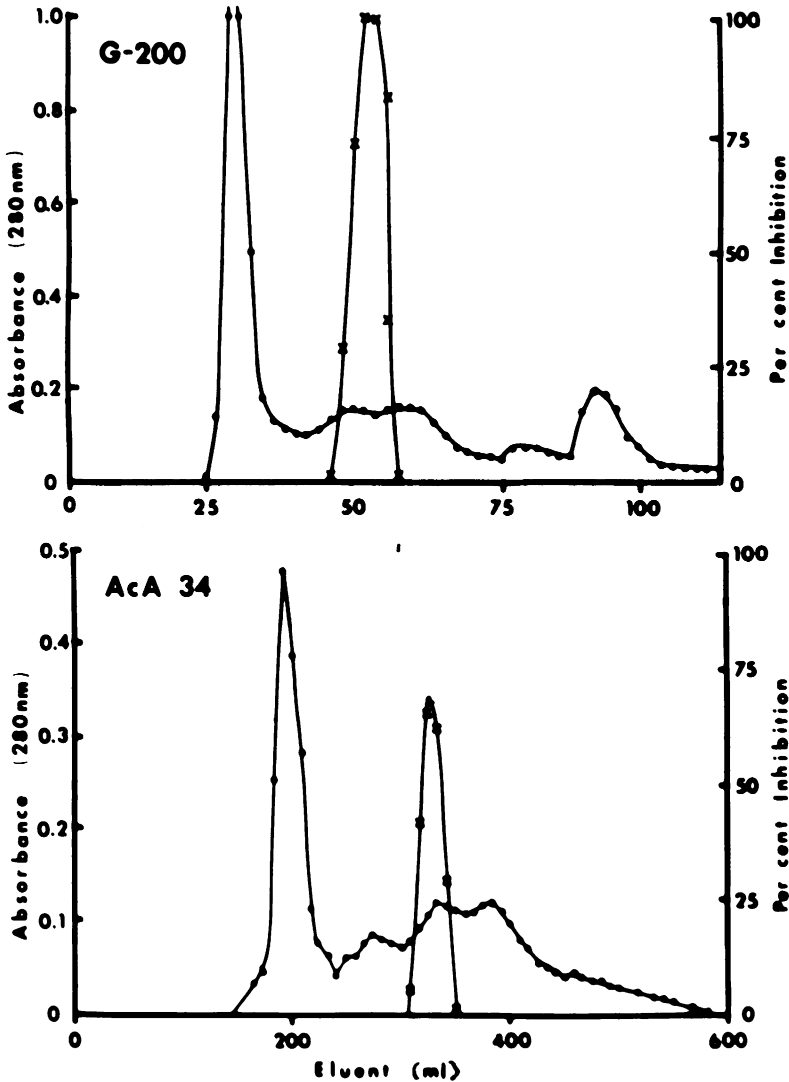

FIG. 3. *Fractionation of inhibitory activity from a crude preparation of S. intermedius on Sephadex G-200 and Ultragel AcA34. Absorbancy at 280 nm (●) was analyzed for each fraction, and a sample of each fraction was diluted 1:10 in MEM, sterilized by membrane filtration (Millipore Corp.), and assayed for inhibitory capacity against PHA-stimulated lymphocytes* (×) *as described in the text. When AV3 fibroblasts were used as target cells, the peak of inhibitory activity obtained from both chromatographic columns was indistinguishable from the profile for the activity assayed against stimulated lymphocytes.*

in human dental plaque reported by Levine et al. (20) must await further investigation. The association of dental plaque with gingivitis leads to the speculation that the cytotoxic factors from plaque may be involved in the initial tissue destruction that permits entry of high-molecular-weight substances into the adjacent gingival connective tissue. These factors may also be involved in the destruction of collagen by inhibiting the ability of fibroblasts to synthesize the amount of collagen needed to balance the rate of collagen turnover. A better understanding of these factors will be necessary to evaluate the biological events occurring in the disease state.

Of equal interest is the observation that oral bacteria produce extracellular substances that inhibit blast transformation of peripheral lymphocytes from normal individuals. Several investigators have used a variety of crude microbial or plaque extracts for lymphocyte proliferation assays and have reported a stimulatory

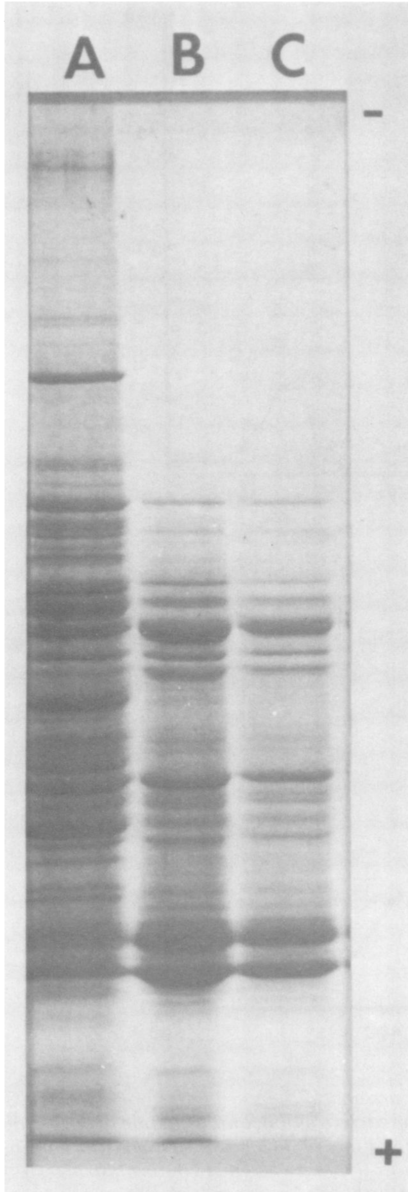

FIG. 4. *Coomassie brilliant blue staining for protein after sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the crude preparation (A) and the biologically active fraction from Sephadex G-200 (B, C) from S. intermedius.*

effect with peripheral lymphocytes from patients with gingivitis or mild periodontitis, whereas no significant proliferative response was observed with lymphocytes derived from patients with severe periodontal disease or normal controls (1, 7, 9, 10, 12, 13, 25, 26). Kiger et al. (17), however, reported the transformation of lymphocytes from normal individuals. All of these investigations have used extremely crude preparations consisting of extracts of whole bacteria or dental plaque, and thus it is difficult to determine the nature of the stimulus. It is of interest that in our experiments preparations from *S. intermedius* added to unstimulated lymphocytes did not cause increased cell proliferation. In addition, viability (determined with trypan blue, 0.1%) of stimulated or unstimulated lymphocytes incubated with the crude preparation from *S. intermedius* was consistently above 90%.

The ability of extracellular products from oral bacteria to inhibit blast transformation of PHA-stimulated lymphocytes suggests that microorganisms within the oral cavity are capable of altering the host's immune response. In this study, the alteration resulted in the ability of normal human lymphocytes to incorporate [^{3}H]thymidine into cellular constituents as a result of PHA stimulation. In the case of *S. intermedius*, the extracellular products inhibited fibroblast growth as well as blast transformation. On the other hand, *A. naeslundii* produced substances that inhibited the lymphocyte response to PHA but had a negligible effect on fibroblast proliferation. The greatest inhibitory activity against either fibroblastoid cell lines or peripheral lymphocytes was obtained from preparations of *S. intermedius*. Purification of the inhibitory substances from this microorganism is

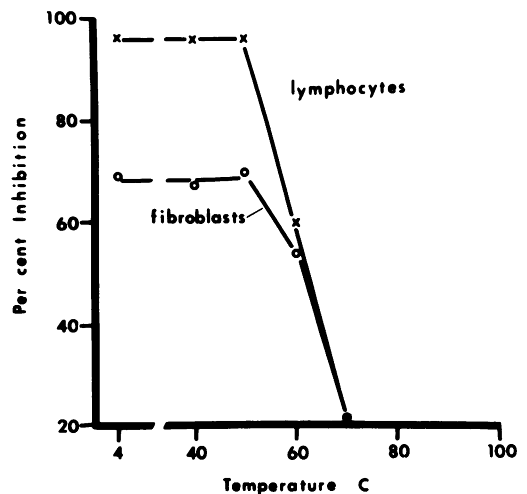

FIG. 5. *Effect of heat on biological activity of samples from the crude S. intermedius preparation. Samples were heated at each temperature for 10 min, cooled to 4°C, and assayed for residual inhibitory capacity against AV3 fibroblasts* (O) *and PHA-stimulated lymphocytes* (×).

being pursued, and it is reasonable to assume that both target cells are affected by the same inhibitory substance, in that the biological activities could not be distinguished by gel filtration or differential heat lability.

The evidence presented herein indicates that a variety of oral bacteria are capable of elaborating substances that inhibit the proliferation of human fibroblastoid cell lines and stimulated human lymphocytes. Initial characterization of one of the inhibitors suggests that the factors are heat labile and are relatively high-molecular-weight material. These substances may be exotoxins that have not been described previously; however, their biochemical characterization must await further investigation. It is interesting to speculate on their role in the healthy human oral cavity, but this role, if any, and any possible role in the pathogenesis of gingivitis or periodontal disease remain conjectural.

ACKNOWLEDGMENTS

We thank Marilyn Orvin and Gina Ciabattari for excellent technical assistance, H. Hugh Fudenberg and James W. Fett for valuable discussions, and Charles L. Smith for critical review of the manuscript.

This research was supported by a grant from the South Carolina State Appropriation for Biomedical Research, Medical University of South Carolina.

LITERATURE CITED

1. **Baker, J. J., S. P. Chan, S. S. Socransky, J. J. Oppenheim, and S. E. Mergenhagen.** 1976. Importance of *Actinomyces* and certain gram-negative anaerobic organisms in the transformation of lymphocytes from patients with periodontal disease. Infect. Immun. **13**:1363–1368.
2. **Bratthall, D.** 1970. Demonstration of five serological groups of streptococcal strains resembling *Streptococcus mutans*. Odontol. Rev. **21**:143–152.
3. **Burnett, G. W., and H. W. Scherp.** 1968. Oral microbiology and infectious diseases, 3rd ed., p. 409–466. The Williams & Wilkins Co., Baltimore.
4. **Courant, P. R., and R. J. Gibbons.** 1967. Biochemical and immunological heterogeneity of *Bacteroides melaninogenicus*. Arch. Oral Biol. **12**:1605–1613.
5. **Cowley, G. C., and M. Levine.** 1972. The effect of plaque on gingival epithelium. Oral Sci. Rev. **1**:103–127.
6. **Evans, R. T., S. Spaeth, and S. E. Mergenhagen.** 1966. Bactericidal antibody in mammalian serum to obligatorily anaerobic Gram-negative bacteria. J. Immunol. **97**:112–119.
7. **Gaumer, H. R., P. Holm-Pedersen, and L. E. A. Folke.** 1976. Indirect blastogenesis of peripheral blood leukocytes in experimental gingivitis. Infect. Immun. **13**:1347–1353.
8. **Gibbons, R. J., S. S. Socransky, S. Sawyer, B. Kapsimalis, and J. B. MacDonald.** 1963. The microbiota of the gingival crevice area of man. II. The predominant cultivable organisms. Arch. Oral. Biol. **8**:281–289.
9. **Horton, J. E., S. Leiken, and J. J. Oppenheim.** 1972. Human lymphoproliferative reaction to saliva and dental plaque deposits: an *in vitro* correlation with periodontal disease. J. Periodont. **43**:522–527.
10. **Horton, J. E., J. J. Oppenheim, and S. E. Mergenhagen.** 1974. A role of cell-mediated immunity in the pathogenesis of periodontal disease. J. Periodontol. **45**:351–360.
11. **Ivanyi, L., S. J. Challacombe, and T. Lehner.** 1973. The specificity of serum factors in lymphocyte transformation in periodontal disease. Clin. Exp. Immunol. **14**:497–500.
12. **Ivanyi, L., and T. Lehner.** 1970. Stimulation of lymphocyte transformation by bacterial antigens in patients with periodontal disease. Arch. Oral. Biol. **15**:1089–1096.
13. **Ivanyi, L., and T. Lehner.** 1971. Lymphocyte transformation by sonicates of dental plaque in human periodontal disease. Arch. Oral. Biol. **16**:1117–1121.
14. **Ivanyi, L., and T. Lehner.** 1971. The significance of serum factors in stimulation of lymphocytes from patients with periodontal disease by *Veillonella alcalescens*. Int. Arch. Allergy **41**:620–627.
15. **Ivanyi, L., J. M. A. Wilton, and T. Lehner.** 1972. Cell-mediated immunity in periodontal disease; cytotoxicity, migration inhibition and lymphocyte transformation studies. Immunology **22**:141–145.
16. **Karam, J. D., and M. G. Bowles.** 1974. Mutation to overproduction of bacteriophage T4 gene products. J. Virol. **13**:428–438.
17. **Kiger, R. D., W. H. Wright, and H. R. Creamer.** 1974. The significance of lymphocyte transformation responses to various microbial stimulants. J. Periodontol. **45**:780–785.
18. **Lehner, T., and E. D. Clarry.** 1966. Acute ulcerative gingivitis. An immunofluorescent investigation. Br. Dent. J. **121**:366–370.
19. **Levine, M., R. L. P. Adams, and G. C. Cowley.** 1973. Effect of dental plaque extracts on mammalian cells *in vitro*. J. Periodontal Res. **8**:296–303.
20. **Levine, M., R. L. P. Adams, C. G. Cowley, and D. K. Mason.** 1974. Some characteristics of the cytotoxic material in human dental plaque extracts. Arch. Oral. Biol. **19**:1145–1152.
21. **Loe, H., E. Theilade, and S. B. Jensen.** 1965. Experimental gingivitis in man. J. Periodontol. **36**:177–187.
22. **Mergenhagen, S. E.** 1967. Nature and significance of somatic antigens of oral bacteria. J. Dent. Res. **46**:46–52.
23. **Nisengard, R.** 1974. Immediate hypersensitivity and periodontal disease. J. Periodontol. **45**:344–347.
24. **Nisengard, R., E. H. Beutner, and S. P. Hazen.** 1968. Immunologic studies of periodontal diseases. IV. Bacterial hypersensitivity and periodontal disease. J. Periodontol. **39**:329–332.
25. **Patters, M. R., R. J. Genco, M. J. Reed, and P. A. Mashimo.** 1976. Blastogenic response of human lymphocytes to oral bacterial antigens: comparison of individuals with periodontal disease to normal and edentulous subjects. Infect. Immun. **14**:1213–1220.
26. **Reed, M. J., M. E. Neiders, and R. J. Genco.** 1976. Synergistic effects on DNA synthesis in peripheral blood lymphocytes of mitogens and dental plaque sonicates in man and macaque monkeys. Arch. Oral Biol. **21**:441–445.
27. **Reed, M. J., M. R. Patters, P. A. Mashimo, R. J. Genco, and M. J. Levine.** 1976. Blastogenic response of human lymphocytes to oral bacterial antigens: characterization of bacterial sonicates. Infect. Immun. **14**:1202–1212.
28. **Rosebury, T., J. B. MacDonald, and A. R. Clark.** 1950. A bacteriological survey of gingival scrapings from periodontal infections by direct examination, guinea pig innoculation and anaerobic cultivation. J. Dent. Res. **29**:718–731.
29. **Socransky, S. S., R. J. Gibbons, A. C. Dale, L. Bortnik, E. Rosenthal, and J. B. MacDonald.** 1963. The microbiota of the gingival crevice area of man. I. Total microscopic and viable counts, and counts of specific

organisms. Arch. Oral. Biol. **8**:275–280.
30. **Steinberg, A. I., and S. N. Gershoff.** 1968. Quantitative differences in spirochetal antibody observed in periodontal disease. J. Periodontol. **39**:286–289.
31. **Svomi, J. D., J. C. Green, J. R. Vermilion, J. Doyle, J. J. Chang, and E. C. Leatherwood.** 1971. The effect of controlled oral hygiene procedures on the progression of periodontal disease in adults: results after the third and final year. J. Periodontol. **42**:152–160.
32. **Warburg, O., and W. Christian.** 1942. Isolierung und Kristallisation des Garungsfragments Enolase. Biochem. Z. **310**:384–421.