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Suppression of Fibroblast Proliferation by Oral Spirochetes

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Soluble sonic extracts of several strains of *Treponema denticola* and *Treponema vincentii* were examined for their abilities to alter proliferation of both murine and human fibroblasts. We found that sonic extracts of all tested strains of *T. denticola* caused a dose-dependent inhibition of murine and human fibroblast proliferation when assessed by both DNA synthesis ([^{3}H]thymidine incorporation) and direct cell counts. *T. vincentii* had only a minimal inhibitory effect at comparable doses. No inhibition was observed when sonic extracts were added simultaneously with [^{3}H]thymidine, indicating that suppression was not due to the presence of excessive amounts of cold thymidine in the extract, nonspecific effects on thymidine utilization by the cells (transport and incorporation), or degradation of label. RNA ([^{3}H]uridine incorporation) and protein ([^{3}H]leucine incorporation) synthesis were similarly altered after exposure to the *T. denticola* sonic extracts. There was no effect on cell viability as measured by trypan blue exclusion. Inhibition could be reversed by extensive washing of the cells within the first few hours of exposure to sonic extracts. Preliminary characterization and purification indicated that the inhibitory factor(s) is not endotoxin since it is heat labile, and elutes in a single, well-defined peak on a Sephadex G-150 chromatography column corresponding to a molecular weight of approximately 50,000. Since oral spirochetes have been implicated in the pathogenesis of periodontal disorders, it is possible that they contribute to the disease process by inhibition of fibroblast growth and therefore may, at least in part, account for the loss of collagen seen in diseased tissue.

Periodontal diseases represent a group of chronic inflammatory disorders that lead to the destruction of tooth-supporting tissue. The mechanisms responsible for this tissue injury are not known, but subgingival bacterial infection is a major etiological factor contributing to the disease process. Elevated numbers of oral spirochetes have been demonstrated in the subgingival plaque of patients with both gingivitis and various forms of periodontitis (9, 11, 12, 14). In acute necrotizing ulcerative gingivitis, for example, spirochetes appear to invade the underlying connective tissue (10), and penetration of host tissue by spirochetes may also occur in advanced phases of adult periodontitis (15). Successful treatment of periodontal disease is associated with a significant reduction in the number of plaque spirochetes (13). For these reasons, spirochetes or their products are believed to contribute to the development of periodontal disease, but possible mechanisms of virulence are not completely understood. Since the loss of collagen is a major feature of the disease process, we initiated studies to determine whether oral spirochetes can alter fibroblast growth and function. In this paper, we report that sonic extracts of several strains of *Treponema denticola* cause a dose-dependent inhibition of both human and murine fibroblast proliferation with no effect on cell viability. If these organisms act in vivo as they do in vitro, spirochetes could alter fibroblast proliferation and interfere with normal tissue homeostasis. This conceivably could contribute to the loss of the tooth-supporting collagenous components in periodontal lesions.

MATERIALS AND METHODS

Preparation of bacterial sonic extracts. In this study, we employed several of our own laboratory strains of *T. denticola*, designated LL2513, LL2516, LL2519, LL2533, and LL2535. These strains were isolated from pathological gingival sulci of patients with adult periodontitis. Additional oral strains, CD-1 (*T. denticola*) and LA-1 (*T. vincentii*), were kindly provided by B. Laughon (University of Michigan, Ann Arbor). Spirochetes were maintained and grown according to the procedures outlined in the Virginia Polytechnic Institute Anaerobic Laboratory Manual (7). Briefly, organisms were grown anaerobically in a medium containing Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.), yeast extract, veal heart infusion, glucose, volatile fatty acids, and heat inactivated rabbit serum for 3 to 4 days at 35°C in an anaerobic chamber (80% N_2, 10% CO_2, 10% H_2), harvested by centrifugation at 15,000 × *g* for 10 min (4°C), and washed twice with sterile saline. Spirochete suspensions were then sonicated for a total of 2 min (4°C) at 30-s intervals with a Branson sonicator (model 350, Branson Sonic Power Co., Danbury, Conn.) in the presence of glass beads. After settling of the glass beads, the sonic extract was collected and centrifuged at 7,000 × *g* for 30 min (4°C). The supernatant (sonic extract) was extensively dialyzed against phosphate-buffered saline and stored at −20°C. The dose of sonic extract added to fibroblast cultures was based on micrograms of protein per milliliter of cell culture as determined with the Bio-Rad protein assay (Bio-Rad Laboratories, Rockville Centre, N.Y.) with bovine serum albumin as a standard (3). Sonic extracts were dialyzed against Eagle minimal essential medium (GIBCO Laboratories, Grand Island, N.Y.) for 24 h and filter sterilized through a 0.45-μm Millipore filter (Millipore Corp., Bedford, Mass.) before being added to the cell cultures.

Growth of fibroblasts. A murine cell line (L-929) was maintained as previously described (16); briefly, cells were cultured in minimal essential medium containing 5% heat-inactivated fetal bovine serum (GIBCO), 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 2 mM glutamine in 75-cm^2 flasks (Costar, Cambridge, Mass.). The cells were subcultured every 3 to 4 days, harvested by trypsinization, and washed. Approximately 20% of the recovered fibroblasts were placed into new flasks with fresh medium. The rest of the cells were used for experiments as described below.

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Human skin fibroblasts were provided by J. Rosenbloom (School of Dental Medicine, University of Pennsylvania, Philadelphia) (2). These cells were grown in minimal essential medium containing the supplements described above, together with 10% fetal bovine serum. Every 3 to 4 days, 10 ml of the culture supernatant was removed and replaced by fresh medium. Fibroblasts were used between passages 16 and 20. Both murine and human fibroblasts were incubated at 37°C in humidified air containing 5% CO_2.

Fibroblast proliferation assays. DNA, RNA, and protein synthesis were assayed by monitoring the incorporation of [^{3}H]thymidine ([^{3}H]TdR), [^{3}H]uridine, and [^{3}H]leucine, respectively. Stock cultures of fibroblasts were harvested by trypsinization, washed, and suspended in medium (see above) to a concentration of 5×10^4 cells per ml (murine) and 2×10^5 cells per ml (human). Cells (0.1 ml) were placed into each well of a 96-well flat-bottom microculture plate (Costar), and the cells were incubated for 24 h at 37°C in humidified air containing 5% CO_2. Spirochete sonic extracts or medium (control cultures) in a volume of 0.1 ml was then added to the wells, and the cells were incubated for an additional 72 h (or as indicated). [^{3}H]TdR (0.25 μCi; specific activity, 54 Ci/mmol; Amersham Corp., Arlington Heights, Ill.), [^{3}H]uridine (0.25 μCi; specific activity, 50.4 Ci/mmol; New England Nuclear Corp., Boston, Mass.), or [^{3}H]leucine (0.50 μCi; specific activity, 50.4 Ci/mmol; New England Nuclear) was added for the last 2 h of incubation of murine cells and for the last 18 h of incubation of human cells, as previously described (16). The medium was then carefully removed and replaced with 0.1 ml of a solution containing 0.5 mg of trypsin and 0.2 mg of EDTA per ml. After trypsinization for 10 min at room temperature, the cells were harvested onto fiber glass filters with an automatic cell harvester (model 24V; Brandel, Rockville, Md.). Incorporation of the radiolabels was determined by counting in a Packard Tri-Carb Prias liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). The data are expressed as a percentage of radiolabel incorporation in experimental cultures as compared with control cells.

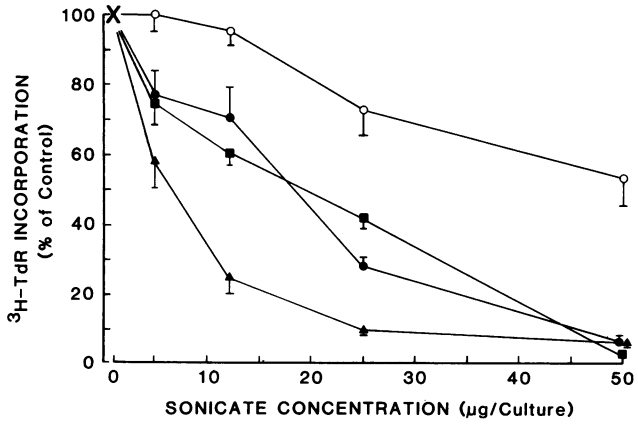

FIG. 1. Effects of different spirochete strains on murine fibroblast proliferation. Various amounts of the sonic extracts (micrograms of protein per milliliter of culture) from three different *T. denticola* strains and one *T. vincentii* strain were examined for their effects on the proliferation of the murine cell line L-929. Symbols indicate *T. denticola* CD-1 (●), LL2516 (■), LL2533 (▲), and *T. vincentii* LA-1 (○). DNA synthesis was measured by [^{3}H]TdR incorporation. Results are plotted as a percentage of [^{3}H]TdR incorporation in control cultures; incorporation in control cultures averaged 76,070 cpm. Each point represents the mean ± standard error (SE) of triplicate cultures in two experiments.

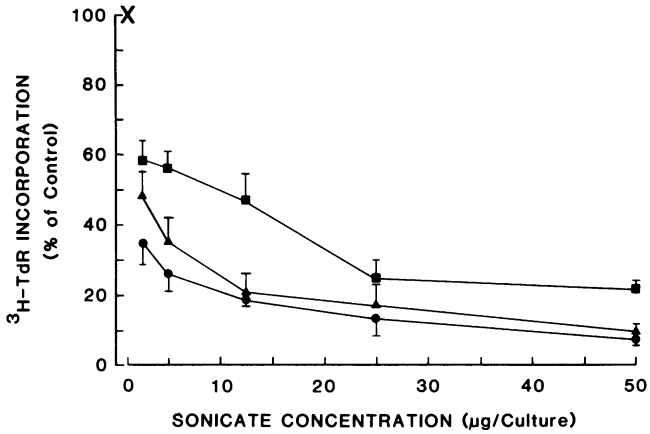

FIG. 2. Effects of different *T. denticola* strains on human fibroblast proliferation. Various amounts of sonic extracts (micrograms of protein per milliliter of culture) from three different *T. denticola* strains were examined for their effects on the proliferation of human skin fibroblasts. Symbols indicate *T. denticola* CD-1 (●), LL2516 (■), and LL2533 (▲). DNA synthesis was measured by [^{3}H]TdR incorporation. Results are plotted as a percentage of [^{3}H]TdR incorporation in control cultures; incorporation in control cultures averaged 1,530 cpm. Each point represents the mean ± SE of triplicate cultures in two experiments.

In other experiments, cell proliferation was determined by direct cell counts. Cultures were established and incubated with sonic extracts as described above. After the 72-h incubation period, the supernatant was carefully removed and replaced with 0.1 ml of trypsin-EDTA solution. After an additional 10 min of incubation, the cells were transferred into 10 ml of Hematall solution (Fisher Scientific Co., Philadelphia, Pa.) containing 0.1% fetal bovine serum, and the total number of cells per well was determined with a Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla.).

Gel filtration chromatography. Spirochete sonic extracts were concentrated with a Diaflo PM10 membrane (Amicon Corp., Danvers, Mass.) and applied to a Sephadex G-150 column (2.5 by 85 cm; Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) equilibrated with 50 mM Tris buffer (pH 7.4) containing 100 mM NaCl. Then 3-ml fractions (flow rate, 15 ml/h) were collected and assessed for fibroblast inhibitory activity, lymphocyte inhibitory activity, and absorption at 280 nm.

Assay for lymphocyte inhibitory activity. Column fractions were tested for the presence of the *T. denticola* immunosuppressive factor as described previously (16a). Briefly, human peripheral blood lymphocytes from 100 to 200 ml of heparinized venous blood from healthy donors were isolated by buoyant-density centrifugation on Ficoll-Hypaque. The lymphocytes were washed and suspended at a cell concentration of 2×10^6 cells per ml in medium consisting of RPMI 1640 (GIBCO), 2% heat-inactivated pooled AB serum, 2 mM L-glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Lymphocytes (0.1 ml) were placed into each well of flat-bottomed microculture plates (Costar), and various dilutions (diluted in medium) of column fractions were added. An optimal mitogenic dose of concanavalin A (2 μg per culture; Calbiochem-Behring, La Jolla, Calif.) was added after 30 min of incubation. The cells were then incubated for

TABLE 1. Effect of *T. denticola* CD-1 on murine fibroblast numbers

Sonic extract concn (μg/ml)	No. of fibroblasts[a]	% of control[b]
0	45,590 ± 450	(100.0)
5	49,260 ± 1,640	(108.1)
12	45,510 ± 1,930	(99.8)
25	38,370 ± 1,170	(84.2)
50	28,800 ± 2,210	(63.2)
100	21,880 ± 2,800	(48.0)

[a] After a 3-day incubation in the presence of strain CD-1 sonic extract, the number of fibroblasts in each culture was determined with a Coulter Counter as described in the text. Each value represents the mean ± SE of two experiments, each performed in triplicate.

[b] Percentage of cells in experimental cultures when compared with control cultures, which were not exposed to spirochete extracts.

72 h at 37°C in humidified air containing 5% CO_2. [^{3}H]TdR (0.25 μCi) was added for the last 4 h of culture. The cells were harvested, and the amount of radiolabeled thymidine incorporation was determined as described above.

RESULTS

Soluble sonic extracts of spirochetes caused a significant, dose-dependent suppression of [^{3}H]TdR incorporation in murine fibroblasts (Fig. 1). All *T. denticola* sonic extracts reduced the cell proliferation more than 90% when 50 μg of protein per ml of sonic extract was employed, whereas *T. vincentii* LA-1 caused only 40% inhibition at a comparable concentration. Human fibroblast proliferation was similarly affected by the sonic extracts; Fig. 2 shows the dose-dependent inhibitory effects of sonic extracts of strains LL2516, LL2533, and CD-1 on human foreskin fibroblasts. Different strains of *T. denticola* varied in their ability to inhibit fibroblast growth. In the experiments described below, sonic extracts from *T. denticola* CD-1 were employed for illustrative purposes.

Since [^{3}H]TdR incorporation measures only one aspect of cell proliferation, we also assessed whether CD-1 sonic extracts altered fibroblast numbers or synthesis of RNA ([^{3}H]uridine incorporation) and protein ([^{3}H]leucine incorporation). Reduction of [^{3}H]TdR incorporation in fibroblasts exposed to CD-1 was corroborated by a similar effect on the total cell number (Table 1). Exposure to sonic extracts caused an inhibition of cell proliferation during the 72 h of incubation in a dose-dependent fashion. Control cultures, which contained medium only, reached a total number of 45,590 cells per well during this period. However, the addition of sonic extracts (25, 50, and 100 μg of protein per ml) to similar cultures after the cells had been established for 24 h inhibited the further proliferation of the fibroblasts to various degrees (Table 1). Furthermore, strain CD-1 caused a profound and dose-dependent inhibition of RNA synthesis (Fig. 3), with inhibition ranging from 25% in the presence of 5 μg of sonic extract to 80% in the presence of 100 μg of sonic extract. Similar dose-dependent inhibition was observed for protein synthesis. Even the highest concentrations of sonic extract which inhibited DNA synthesis by more than 90% did not totally inhibit RNA and protein synthesis, indicating that the cells were active and viable. Furthermore, direct assessment of cell viability by trypan blue exclusion and microscopic examination also failed to demonstrate any effects on cell viability or cytopathic alterations after treatment with bacterial sonic extracts (data not shown).

Kinetic experiments were performed to rule out the possibility that the suppression of DNA synthesis was due to the presence of excessive amounts of cold thymidine or simply involved interference with the ability of the cells to utilize and incorporate [^{3}H]TdR. Simultaneous addition of the sonic extracts with [^{3}H]TdR for the last 2 h of culture did not produce a significant effect on label incorporation (Table 2); the sonic extract had to be available for at least 24 h to cause suppression. We also examined whether this suppression could be reversed by washing the cells after they had been exposed for various periods to the extracts. Suppression was totally reversed when the cells were washed within the first 10 h (Fig. 4) and partially reversed after 30 h of exposure to the sonic extracts. The effect was no longer reversible by washing after 48 h of incubation. These data suggest that the effect of the sonic extract was not immediate and most likely occurred between 12 and 24 h after exposure.

Preliminary characterization of the inhibitory activity demonstrated that the activity was nondialyzable. Heat lability was determined by exposing samples of sonic extracts to several temperatures for 30 min, followed by re-equilibration at 4°C and testing for inhibitory activity. All activity was lost when sonic extracts were heated to 100°C. Exposure to 80°C partially destroyed the inhibitory effect of spirochete sonic extracts, but heating to 37 and 56°C did not have an effect on the suppressive activity when compared with a control sample kept at 4°C. These results strongly suggest that the inhibitory effects are not due to endotoxin. Since spirochete extracts have already been shown to contain a factor capable of suppressing lymphocyte proliferation (16a), we wanted to ascertain whether the fibroblast inhibitory factor(s) and the lymphocyte suppressive factor were the same. Crude extracts were fractioned by gel filtration chromatography (Sephadex G-150) (Fig. 5), and the fractions were tested for their ability to alter both fibroblast and lymphocyte proliferation. Fibroblast inhibitory activity appeared in a single, well-defined peak corresponding to a

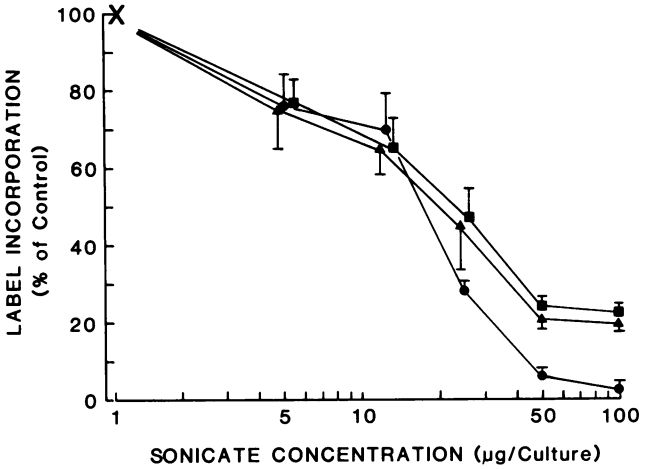

FIG. 3. Effects of *T. denticola* on fibroblast proliferation. Murine fibroblasts were incubated with various amounts of CD-1 sonic extracts (micrograms of protein per milliliter of culture) for 72 h. DNA (●), RNA (■), and protein (▲) syntheses were then assessed by incorporation of [^{3}H]TdR, [^{3}H]uridine, and [^{3}H]leucine, respectively. Results are plotted as a percentage of radiolabeled precursor incorporation in control cultures receiving medium only. Each point represents the mean ± SE of triplicate assays in two experiments. Incorporation in control cultures averaged 84,970 cpm ([^{3}H]TdR), 13,330 cpm ([^{3}H]uridine), and 3,760 cpm ([^{3}H]leucine).

TABLE 2. Kinetics of murine fibroblast inhibition by *T. denticola* CD-1

Time (h) of addition[a]	[^{3}H]TdR incorporation (% of control)[b]
0	1.2 ± 0.4
6	1.9 ± 0.9
10	9.3 ± 3.9
24	9.5 ± 6.2
30	13.7 ± 5.0
48	77.2 ± 10.3
54	90.8 ± 4.1
60	102.3 ± 10.1
72	97.1 ± 10.7

[a] Fibroblast cultures were established as described in the text; 10 μg of sonic extract was then added to the cultures at various times. The final addition (72 h) was made simultaneously with [^{3}H]TdR.

[b] Incorporation of [^{3}H]TdR in cultures exposed to sonic extracts is expressed as a percentage of incorporation in control cultures. Each value represents the mean ± SE of three experiments, each performed in triplicate.

molecular weight of approximately 50,000, whereas the lymphocyte activity appeared in a peak corresponding to a molecular weight of 100,000 (Fig. 5). These results demonstrate that the inhibitory activities are most likely separate entities. Further purification and characterization of these factors are currently being carried out.

DISCUSSION

In the present paper, we report on the ability of *T. denticola* to suppress murine and human fibroblast growth in vitro. Proliferation of both the murine fibroblasts (L-929) and the human skin fibroblasts (CC-102) were inhibited by sonic extracts of *T. denticola* in a dose-dependent fashion. Minimal inhibitory activity was also detected in *T. vincentii* LA-1. The mechanism of action of this factor(s) is not known yet. Although it suppresses DNA, RNA, and protein syntheses, it does not affect cell viability. Based on reversal (washing) and kinetic studies, the effect of the factor is not immediate and most likely occurs within the first 12 to 24 h of exposure. Furthermore, we can rule out nonspecific effects on [^{3}H]TdR incorporation (such as degradation of the label, dilution by cold thymidine in the sonic extract, or transport of label through the cell membranes) since simultaneous addition of sonic extracts with [^{3}H]TdR did not affect label incorporation. The data from direct cell counts also confirmed that the observed inhibition was not due to nonspecific effects on the ability of the cells to utilize [^{3}H]TdR. The inhibitory activity is not derived from the spirochete culture medium since the broth itself did not affect the fibroblast proliferation (data not shown). The inhibitory activity is not endotoxin since it is heat labile and elutes in a well-defined peak in gel filtration chromatography corresponding to a molecular weight of ca. 50,000. It appears that the fibroblast inhibitory factor is an entity distinct from the previously described immunosuppressive factor, which has a molecular weight of approximately 100,000 (16a).

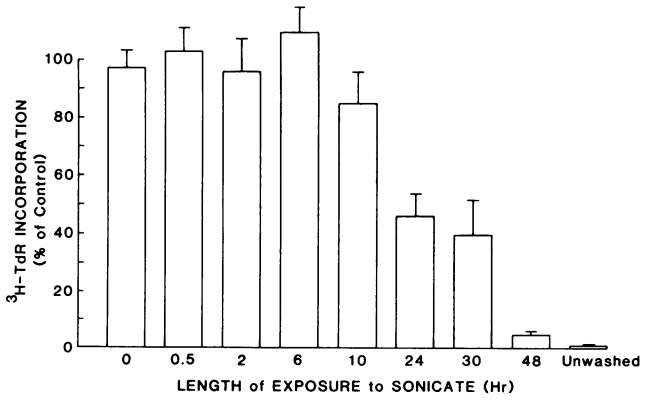

FIG. 4. Reversibility of inhibitory effects of spirochete extracts. The reversibility of *T. denticola* inhibition was assessed by adding strain CD-1 sonic extracts (50 μg per culture) to murine fibroblasts for various periods. The cultures were then washed with warm medium, and the incubation was continued for a total of 72 h in 0.2 ml of medium. DNA synthesis was measured by [^{3}H]TdR incorporation as described in the text and is expressed as a percentage of the incorporation observed in control cultures; incorporation in control cultures averaged 78,420 cpm. Each point represents the mean ± SE of triplicate cultures in three experiments.

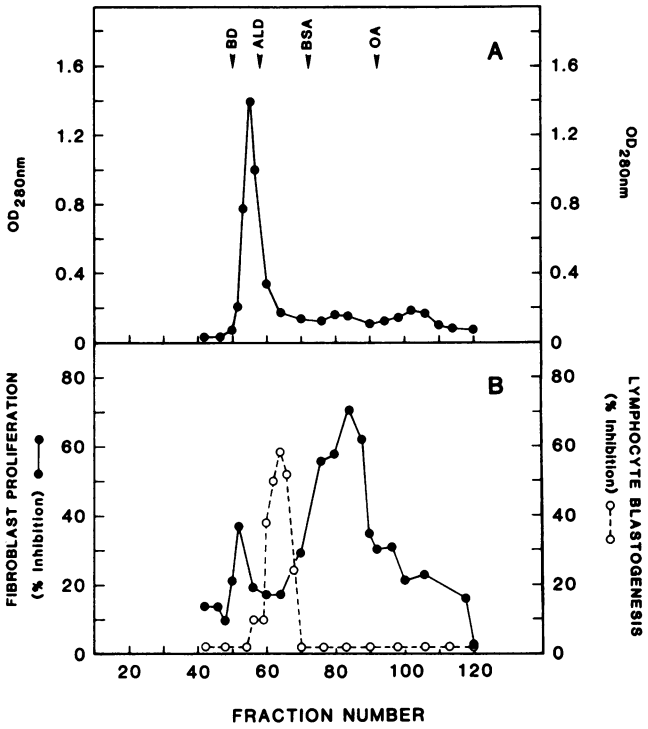

FIG. 5. Sephadex G-150 gel filtration chromatography of *T. denticola* CD-1. Sonic extract was prepared and applied to the column as described in the text. The profile of optical density ($OD_{280\ nm}$), together with molecular weight markers, is shown in (A): BD, blue dextran (2,000,000); ALD, aldolase (160,000); BSA, bovine serum albumin (66,000); OA, ovalbumin (45,000). (B) Alternate fractions were assayed for their ability to inhibit murine fibroblast proliferation and lymphocyte blastogenesis.

Current understanding of the etiology of periodontal disease strongly implies that oral bacteria or their products or both play a major role in the pathology of the disease. Several different gram-positive and gram-negative oral bacteria have been shown to suppress in vitro proliferation of fibroblasts. Shenker et al. (16) and Stevens et al. (19) have reported a fibroblast inhibitory factor in *Actinobacillus actinomycetemcomitans*. Other workers have found in vitro modulation of fibroblast growth and structure by oral bacteria such as *Actinomyces viscosus* (4, 6), *Streptococcus mutans* and *Streptococcus intermedius* (6), and *Capnocytophaga sputigena* (20), but not for *Actinomyces naeslundii*, *Streptococcus sanguis*, and several strains of *S. mutans* (6).

Extracts of *A. viscosus* have even been shown to be cytotoxic to rat embryo fibroblasts (5). A recent report of Larjava et al. (8) describes fibroblast inhibitory factors that seem to be endotoxin extracted from whole dental plaque. Other bacterial substances, such as mucopeptides and certain low-molecular-weight metabolic end products, have been shown to be present in plaque or produced by plaque bacteria (18). Lipopolysaccharide from *Escherichia coli* was reported to be inhibitory to mouse fibroblast growth at a level of 100 μg/ml (1), but others report that 50 μg/ml of lipopolysaccharide actually had a stimulatory effect on mouse embryo fibroblast growth (17). Collectively, these observations are particularly relevant to the pathogenesis of periodontal disease since there is severe loss of collagen in diseased tissue. The ability of spirochete sonic extracts and other organisms to inhibit growth of fibroblasts could be at least one alteration to explain the loss of collagen at these sites. The presence of spirochetes or their products in diseased tissue and their ability to suppress both fibroblast proliferation and the immune response (16a) in vitro provide indirect evidence to suggest an active role for these organisms in the pathogenicity of periodontal disease.

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