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Inhibition of Fibroblast Proliferation by *Actinobacillus actinomycetemcomitans*

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We have examined soluble sonic extracts of *Actinobacillus actinomycetemcomitans* for their ability to alter human and murine fibroblast proliferation. We found that extracts of all *A. actinomycetemcomitans* strains examined (both leukotoxic and nonleukotoxic) caused a dose-dependent inhibition of both murine and human fibroblast proliferation as assessed by DNA synthesis ([^{3}H]thymidine incorporation). Addition of sonic extract simultaneously with [^{3}H]thymidine had no effect on incorporation, indicating that suppression was not due to the presence of excessive amounts of cold thymidine. Inhibition of DNA synthesis was also paralleled by decreased RNA synthesis ([^{3}H]uridine incorporation) and by a decrease in cell growth as assessed by direct cell counts; there was no effect on cell viability. The suppressive factor(s) is heat labile; preliminary purification and characterization studies indicate that it is a distinct and separate moiety from other *A. actinomycetemcomitans* mediators previously reported, including leukotoxin, immune suppressive factor, and endotoxin. Although it is not clear how *A. actinomycetemcomitans* acts to cause disease, we propose that one aspect of the pathogenicity of this organism rests in its ability to inhibit fibroblast growth, which in turn could contribute to the collagen loss associated with certain forms of periodontal disease, in particular juvenile periodontitis.

Periodontal disease in general and juvenile periodontitis (JP) in particular represent a group of chronic inflammatory diseases that lead to the destruction of the tooth supporting tissue (9, 14). Although the mechanism(s) responsible for tissue injury is not known, it has been suggested that infection by specific organisms, at least during some stage(s), may be a major factor contributing to the pathogenesis of JP (9, 13, 14). In this regard, *Actinobacillus actinomycetemcomitans* has been isolated in relatively high concentration from the inflamed gingival pockets of JP patients and is now believed to be associated with the pathogenesis of this disease (12, 18). Although it is not clear how *A. actinomycetemcomitans* contributes to the development of JP, it is known that this organism produces several biologically active mediators including a heat-labile leukotoxin, capable of killing human polymorphonuclear leukocytes and monocytes (2, 19), and an endotoxin that promotes bone resorption (8). More recently, we have demonstrated the presence of an immunosuppressive factor present in all strains of *A. actinomycetemcomitans* (16). In this study we examined *A. actinomycetemcomitans* for its ability to alter proliferation of both human and murine fibroblasts. We found that soluble sonic extracts of all tested strains inhibited fibroblast proliferation with no effect on cell viability. It seems reasonable to postulate that inhibition of fibroblast proliferation by *A. actinomycetemcomitans* represents one mechanism by which this organism may act to cause disease and furthermore may account for the loss of collagen observed in oral lesions associated with JP (13).

MATERIALS AND METHODS

Preparation of bacterial sonic extracts. The *A. actinomycetemcomitans* strains used in this study were originally isolated from the gingival sulci of patients with JP by S. S. Socransky and colleagues (Forsyth Dental Center, Boston, Mass.). Bacteria were grown as previously described (2); briefly, the strains were cultured anaerobically (95% N_2 and 5% CO_2) from stock lyophilized cultures in thioglycolate broth (Difco Laboratories, Detroit, Mich.). After 48 h, bacterial cell suspensions were harvested by centrifugation at 16,000 × *g* for 15 min at 4°C and washed three times in phosphate-buffered saline. The equivalent of 10 liters of washed cells was pooled, suspended in distilled water, and sonicated for a total of 10 min at 1-min intervals with a Branson sonicator (model 350, Branson Sonic Power Co., Danbury, Conn.) in the presence of an equal volume of glass beads. After settling of the beads, the supernatants were collected and centrifuged at 4,000 × *g* for 30 min at 4°C. The supernatant (sonic extract) was dialyzed against dis-

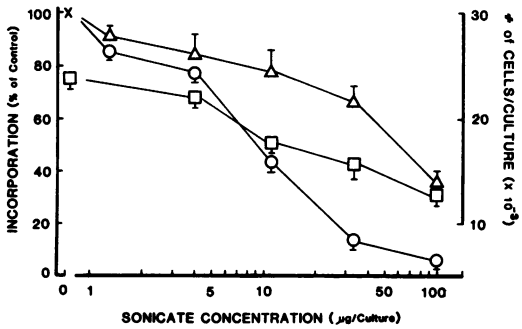

FIG. 1. Effects of *A. actinomycetemcomitans* Y4 on fibroblast proliferation. Murine fibroblasts (L929) were incubated with strain Y4 sonic extract for 48 h; DNA (○) and RNA (△) syntheses were then assessed by [^{3}H]TdR and [^{3}H]uridine incorporation, respectively. Results are plotted as a percentage of radiolabeled precursor incorporation in control cultures receiving medium only; net incorporation in control cultures averaged 82,628 cpm ([^{3}H]TdR) and 6,276 cpm ([^{3}H]uridine). Cell growth was also assessed by direct count as outlined in the text (□). Each point represents the mean value ± standard error of triplicate assays of each of three experiments.

tilled water for 24 h, lyophilized, and stored at −20°C until employed in these studies. The dose of sonic extract that was added to cell cultures was based on milligrams of dry weight per milliliter (different batches of extract contained between 0.1 and 0.2 mg of protein per mg of dry weight).

Growth of fibroblasts. The murine fibroblast cell line (L929) was maintained in Eagle minimal essential medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 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 or 4 days; cells were harvested by trypsinization and washed, and approximately 20% of the recovered fibroblasts were placed into new flasks with fresh medium. The remaining cells were used for experiments as described below.

Normal human skin fibroblasts were provided by J. Rosenbloom (University of Pennsylvania, Center for Oral Health Research); the cells were isolated from skin biopsies of healthy donors as previously described (3). The cells were grown in Eagle minimal essential medium containing 5% fetal bovine serum (heat inactivated), 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Fibroblasts were used between passages 5 and 20. Both murine and human fibroblasts were incubated at 37°C in humidified air containing 5% CO_2.

Proliferation assays. DNA and RNA syntheses were assayed by the incorporation of [^{3}H]thymidine ([^{3}H]TdR) and [^{3}H]uridine, respectively. Stock cultures of fibroblasts (described above) were harvested by trypsinization, washed, suspended in minimal essential medium containing serum and antibiotics (see above), and counted. A 0.2-ml sample of cell suspension (2.5 × 10^4 fibroblasts per ml) was placed in each well of a 96-well flat-bottom microculture plate (Costar). The cells were incubated for 24 h, after which 50 μl of *A. actinomycetemcomitans* sonic extract or medium was added and the cells were incubated for an additional 48 h or as indicated. Preliminary studies indicated that a 48-h incubation was more than sufficient time to demonstrate altered cell proliferation. [^{3}H]TdR (0.25 μCi; specific activity, 14.9 Ci/mmol; Amersham, Arlington Heights, Ill.) or [^{3}H]uridine (0.5 μCi; specific activity, 5 μCi/mmol; Amersham) was added to the cultures for the last 2 h of incubation. After incubation, the medium was 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. The cells were incubated for 10 min in the presence of the trypsin-EDTA solution and then harvested with an automatic cell harvester (model 24 V; Brandel, Rockville, Md.). Radiolabeled precursor incorporation was determined by counting in a Packard Tri-carb Prias liquid scintillation spectrometer. The net incorporation of labeled precursor into cells exposed to bacterial sonic extracts was expressed as a percentage of net incorporation observed in control cultures (exposed to medium alone); means and standard errors were then calculated.

In other experiments, direct cell counts were employed to assess cell proliferation. Cultures were established in 24-well Linbro culture plates (Linbro, Hamden, Conn.) with 1.0 ml of fibroblasts (10^5 cells per ml) in medium as described above. After 24 h, 1.0 ml of sonic extract diluted in medium, or medium alone, was added to the cultures. The cells were then incubated for an additional 48 h, and the medium was removed and replaced by 1.0 ml of trypsin-EDTA solution. After 10 min, the cells were placed into plastic tubes (12 by 75 mm), centrifuged at 250 × *g* for 10 min, and resuspended in 0.5 ml of medium, and the cell number was determined with a Neubauer hemacytometer. Cell viability was assessed by Trypan blue exclusion.

Gel filtration chromatography. Sonic extracts prepared from *A. actinomycetemcomitans* 652 (nonleukotoxic) were concentrated on Diaflo PM-10 membranes (Amicon, Lexington, Mass.) and applied to a Sephadex G-150 column (Pharmacia Fine Chemicals, Uppsala, Sweden). The column (2.5 by 85 cm) was equilibrated in 50 mM Tris containing 100 mM NaCl buffer (pH 7.5) at 4°C. The column was eluted at 15 ml/h; the rate was controlled by a peristaltic pump (Buchler, Fort Lee, N.J.). Fractions were then assayed for their ability to inhibit fibroblast proliferation (see above) and lymphocyte activation (see below).

Assay for immunosuppressive activity. Column fractions were tested for the presence of the *A. actinomycetemcomitans* immunosuppressive factor as previously described (16). Briefly, human peripheral blood lymphocytes were isolated by buoyant density centrifugation on Ficoll-Hypaque from 100 to 200 ml of heparinized venous blood from healthy donors. The lymphocytes were washed and suspended to 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 glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. A 0.1-ml volume of lymphocyte suspension was placed into each well of flat-bottomed microculture plates (Costar), and various dilutions (diluted in medium) of

TABLE 1. Kinetics of *A. actinomycetemcomitans*-induced inhibition of fibroblast proliferation

Time (h) of extract[a] addition to cultures	[^{3}H]TdR incorporation[b] (% of control)
−24	10.8 ± 0.6
−20	15.4 ± 1.6
−8	48.5 ± 1.2
−4	61.6 ± 6.4
−2	64.9 ± 3.3
−1	67.8 ± 4.6
−0.5	77.6 ± 3.3
0[c]	80.5 ± 3.3

[a] Sonic extracts prepared from strain Y4 (50 μg per culture) were added to murine fibroblast cultures (see the text) at the indicated times. The time is with respect to the addition of [^{3}H]TdR (time 0).

[b] The data represent the mean ± standard error of quadruplicate cultures for each of two experiments.

[c] Sonic extract was added simultaneously with [^{3}H]TdR.

column fractions were added. After 90 min of incubation, an optimal mitogenic dose of concanavalin A (2 μg per culture; Calbiochem, LaJolla, Calif.) was added. The cells were incubated for 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 TdR incorporation was determined as described above. The immune inhibitory activity is plotted as ID_{80} units per milliliter, that is, the volume of sample required to reduce TdR incorporation by 80% of the control values (cultures receiving mitogen alone).

RESULTS

A. actinomycetemcomitans Y4 was used in this study as a prototype because its biological properties have been more fully characterized than those of other strains (2, 16, 19). Sonic extracts of strain Y4 caused a significant dose-dependent suppression of [^{3}H]TdR incorporation in murine fibroblasts (Fig. 1), with inhibition ranging from 15% (1.3 μg) to greater than 90% (100 μg). The inhibitory effect was not due to the culture medium or to the addition of excessive amounts of cold TdR, since the simultaneous addition of sonic extract with [^{3}H]TdR (Table 1) failed to have a significant effect. Furthermore, the sonic extracts had been extensively dialyzed to remove salts and other low-molecular-weight substances that might interfere with the assays. Since [^{3}H]TdR incorporation measures only one aspect of cell proliferation, we also assessed strain Y4 sonic extract for its effect on RNA synthesis ([^{3}H]uridine incorporation) and cell concentration per culture. Cell proliferation was suppressed in terms of RNA synthesis and cell number; both were inhibited

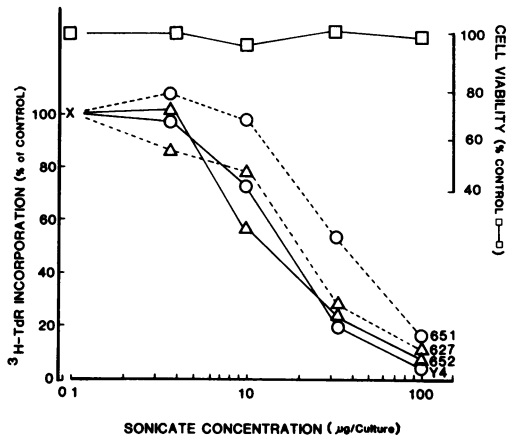

FIG. 2. Representative effects of different *A. actinomycetemcomitans* strains on murine fibroblast proliferation. Sonic extracts from four different *A. actinomycetemcomitans* strains were examined for their effect on the proliferation of the fibroblast cell line L929: leukotoxic strains Y4 (○—○) and 651 (○---○) and nonleukotoxic strains 652 (△—△) and 627 (△---△). Results are plotted as a percentage of [^{3}H]TdR incorporation observed in control cultures (72,580 cpm). Each point represents the mean of triplicate cultures of each of two experiments and the standard errors were within 4% of the mean. Fibroblast viability (□) was determined by trypan blue exclusion after a 2-h exposure to strain Y4.

in a dose-dependent fashion (Fig. 1). Table 1 also demonstrates the kinetics of this *A. actinomycetemcomitans*-induced suppression. In these studies, strain Y4 extract was added to fibroblast cultures at various times. Maximal suppression was observed when the cells were exposed for the entire 24-h period; decreasing the period of exposure resulted in less inhibition.

Certain *A. actinomycetemcomitans* strains (including Y4) produce a heat-labile leukotoxin capable of killing both human polymorphonuclear leukocytes and monocytes; the leukotoxin has been reported not to affect lymphocyte or fibroblast viability (2). To demonstrate that the observed inhibition of fibroblast proliferation was not due to the leukotoxin, we tested sonic extracts from both non-leukotoxin- and leukotoxin-producing strains for their ability to alter fibroblast proliferation (Fig. 2). Both leukotoxic strains Y4 and 651, as well as nonleukotoxic strains 652 and 627, suppressed proliferation in a dose-dependent fashion, although potency varied among strains as demonstrated by different ID_{50} values (doses that cause 50% inhibition) (Table 2). There was no effect on cell viability, as evidenced by trypan blue exclusion (Fig. 2). Furthermore, microscopic examination of the cultures failed to demonstrate the presence of

TABLE 2. Comparison of human and murine fibroblast sensitivity to *A. actinomycetemcomitans* sonic extracts

Strain	Inhibitory effects (ID_{50})[a]	
	Human	Murine
Y4[b]	14.5	17
651[b]	<2.0	36
627	6.0	19
652	12.5	12.5

[a] ID_{50}, Concentration of sonic extract (microgram per culture) needed to reduce [^{3}H]TdR incorporation to 50% of the control values.
[b] Leukotoxic strains.

detached, nonadherent cells or any other evidence of cytopathic effects.

We have also examined *A. actinomycetemcomitans* sonic extracts for their ability to alter normal human skin fibroblast proliferation. Extracts, from all four strains tested (Y4, 651, 652, and 627) inhibited proliferation in a dose-dependent fashion (Fig. 3). Table 2 compares typical ID_{50} values for the four strains; in all instances except strain 652, the ID_{50} was lower for human fibroblasts. As with the murine fibroblasts, there was no effect on cell viability and no evidence of cytopathic effects.

Having established that sonic extracts of *A. actinomycetemcomitans* are capable of inhibiting fibroblast proliferation, we next wanted to determine whether this effect could be reversed by washing the cells after various periods of exposure. The results of these experiments indicate that the suppressive effect could be totally reversed when the cells were washed within 3 h (Fig. 4) and partially reversed after 24 h of exposure to the sonic extract. The effect was no longer reversible by washing after 48 h. These results suggest that the effect(s) of the *A. actinomycetemcomitans* inhibitory factor is not immediate and most likely occurs somewhere between 3 and 24 h after exposure; this is in agreement with the kinetic data (Table 1).

Since *A. actinomycetemcomitans* sonic extracts have been shown to contain several biologically active mediators, we wanted to determine whether the fibroblast inhibitory activity represented a distinct factor or was just another biological effect of these other mediators (2, 8, 16, 19). First, fibroblast inhibition is due to an entity distinct from the leukotoxin, since the former is present in both leukotoxic and nonleukotoxic *A. actinomycetemcomitans* strains. Furthermore, the fibroblast inhibitory activity is probably not due to endotoxin, since it is heat labile. Figure 5 shows the heat sensitivity of the fibroblast inhibitory activity in two *A. actinomycetemcomitans* strains, Y4 (leukotoxin producing) and 652 (nonleukotoxic). The activity from both strains was stable at 5°C and 37°C but was partially destroyed at 56°C and totally destroyed at 75°C. Furthermore, gel filtration chromatography (Fig. 6) demonstrated that the fibroblast inhibitory activity and the previously reported immunosuppressive factor (16) are also distinct entities. After fractionation of a sample of strain 652 sonic extract on Sephadex G-150, the fractions were tested for their ability to inhibit both fibroblast proliferation and lymphocyte activation. The immunosuppressive activity appeared in fractions corresponding to a molecular weight of approximately 50,000, and the fibroblast inhibitory activity appeared in the void volume. The possibility that the fibroblast factor represents an aggregate of the lymphocyte factor cannot be excluded at this time. We are currently attempting to further purify and characterize the fibroblast inhibitory factor(s).

DISCUSSION

The present paper reports on the ability of *A. actinomycetemcomitans* to suppress human and murine fibroblast proliferation in vitro. Both human fibroblasts and the murine fibroblast cell line L929 were inhibited in a dose-dependent fashion, although the human fibroblasts were more sensitive to the suppressive effects. Furthermore, preliminary experiments demonstrat-

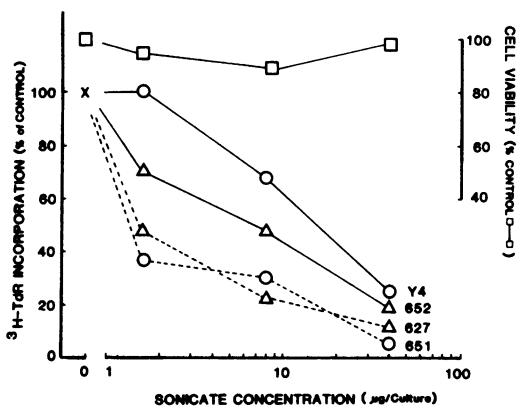

FIG. 3. Representative effects of different *A. actinomycetemcomitans* strains on human fibroblast proliferation. Sonic extracts from four different *A. actinomycetemcomitans* strains were examined for their effect on the proliferation of human fibroblasts: leukotoxic strains Y4 (○—○) and 651 (○---○) and nonleukotoxic strains 652 (△—△) and 627 (△---△). Results are plotted as a percentage of [^{3}H]TdR incorporation in control cultures (2,027 cpm). Each point represents the mean of triplicate cultures of each of two experiments, and the standard errors were within 7% of the mean. Fibroblast viability (□) was determined by trypan blue exclusion after a 2-h exposure to strain Y4.

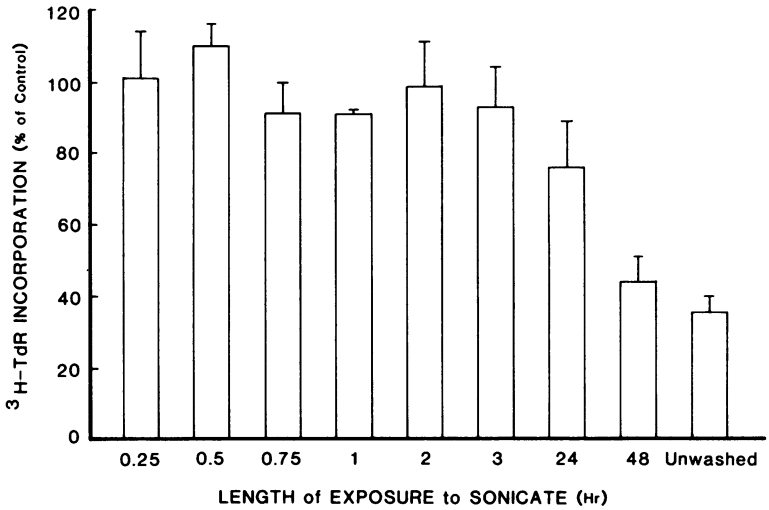

FIG. 4. Reversibility of inhibitory effects. The reversibility of the *A. actinomycetemcomitans* inhibition was assessed by adding strain Y4 sonic extracts (25 μg per culture) to murine fibroblasts for various periods. The cultures were then washed with warm medium and incubated for a total of 48 h in 0.2 ml of medium. DNA synthesis was assessed by [^{3}H]TdR incorporation as described in the text and is expressed as a percentage of the incorporation observed in control cultures (40,590 cpm). Each point represents the mean ± standard error of triplicate cultures in each of three experiments.

ed similar inhibition of human gingival fibroblasts (data not shown). The fibroblast inhibitory activity was present in all *A. actinomycetemcomitans* strains examined including nonleukotoxic organisms. We are currently assessing other oral bacteria for the presence of similar activity. Although the mechanism of action of this factor(s) is not yet known, it does suppress DNA and RNA synthesis with no effect on cell viability; its effects are not as immediate as those previously reported for the *A. actinomycetemcomitans*-derived immunosuppressive factor (16) based on reversal (washing) studies. Preliminary characterization of the fibroblast factor indicates that it is temperature sensitive, suggesting that the activity is not due to endotoxin. We are currently purifying and characterizing this factor; preliminary results indicate that it has a molecular weight of approximately 150,000. Finally, the fibroblast inhibitory factor appears to be immunogenic, based on the finding that serum obtained from some JP patients, as well as rabbit antisera made to crude *A. actinomycetemcomitans* sonic extracts, can neutralize this inhibitory activity.

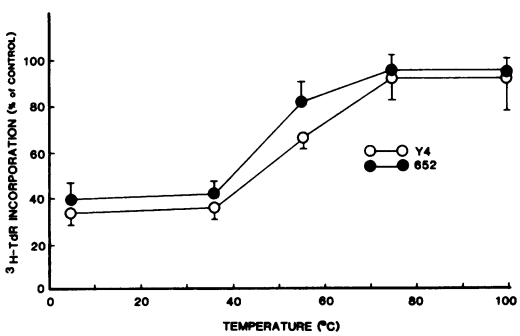

FIG. 5. Effect of temperature on *A. actinomycetemcomitans* fibroblast inhibitory activity. Temperature sensitivity of the fibroblast inhibitory activity was determined by exposing sonic extracts of strains Y4 and 652 (500 μg/ml) to different temperatures for 10 min, followed by re-equilibration to 37°C. The samples were then added to 24-h cultures of murine fibroblasts (25 μg per culture) and incubated for 48 h with the addition of [^{3}H]TdR for the last 2 h. Results are plotted as a percentage of [^{3}H]TdR incorporation in control cultures (53,405 cpm). Each point represents the mean ± standard error of triplicate cultures in a typical experiment.

These findings are particularly relevant for two reasons: (i) *A. actinomycetemcomitans* is a suspected etiological agent in JP, and (ii) one of the more prominent features of this disease is the early and extensive loss of collagen in diseased sites (12, 13). Although the mechanism responsible for collagen loss is not known, it is most likely due to a combination of several factors. First, degradation of collagen, both intra- and extracellularly, may occur at an altered rate. This could be the result of the presence of increased levels of bacterial-, polymorphonuclear leukocyte-, or macrophage-derived collagenase in the diseased tissue (5, 20). Second, a

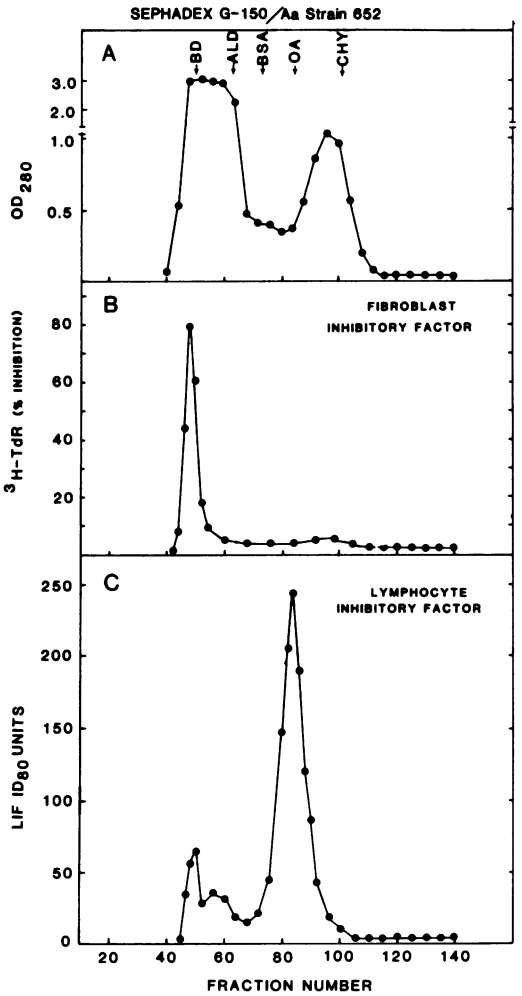

FIG. 6. Sephadex G-150 gel filtration of strain 652 sonic extract. Sonic extract was prepared from 10 liters of strain 652 and chromatographed as described in the text. The profile of optical density at 280 nm (OD_{280}), along with molecular weight markers, is shown in (A): BD, blue dextran; ALD, aldolase; BSA, bovine serum albumin; OA, ovalbumin; CHY, chymotrypsinogen A. Alternate fractions were assayed for their ability to inhibit fibroblast proliferation (B) and lymphocyte activation (C).

change in the type of collagen normally synthesized could occur. For example, Narayanan and Page (10) have shown that gingival fibroblasts from healthy tissue produce types I and III collagen, whereas fibroblasts obtained from periodontally diseased sites produce an altered type I collagen and insignificant amounts of type III collagen. Although the direct implication of these changes is not clear, it is reasonable to assume that altered collagen structure could lead to altered tissue structure. Finally, collagen loss could be due to decreased synthesis of collagen. This could be due to an absolute decrease in synthesis by individual cells or a net decrease as a result of depressed fibroblast proliferation.

Modulation of fibroblast proliferation can be caused by several different factors, all of which are probably present in diseased gingival tissue. (i) Substances released by activated polymorphonuclear leukocytes and macrophages are capable of activating fibroblast proliferation (1, 4, 15). (ii) Supernatants obtained from cultures of mitogen (and antigen)-activated lymphocytes contain factors capable of both stimulating and suppressing fibroblast proliferation as well as suppressing collagen synthesis (7, 11, 21). (iii) Several microorganisms isolated from both plaque and periodontal lesions have been shown to inhibit fibroblast proliferation, including *Streptococcus intermedius* (6), *Actinomyces viscosus* (6), spirochetes (B. J. Shenker and N. S. Taichman, unpublished data), and capnocytophaga (17)—and, as we report here, *A. actinomycetemcomitans* is a very potent inhibitor. On the basis of our observations, we feel that if bacterial-induced inhibition of fibroblast proliferation occurs in vivo, it could play an important role in the collagen loss associated with periodontal disease. In addition, these studies provide further support for the pathogenic potential of *A. actinomycetemcomitans*. It now appears that this organism, which has already been associated with JP (18), is capable of producing four distinct biologically active mediators: the fibroblast inhibitory factor(s), the lymphocyte inhibitory factor, a leukotoxin, and an endotoxin capable of bone resorption. We propose that these factors contribute to the pathogenesis of JP and that inhibition of fibroblast growth contributes to the collagen loss associated with this disease.

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