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Characterization of an Antiproliferative Surface-Associated Protein from *Actinobacillus actinomycetemcomitans* Which Can Be Neutralized by Sera from a Proportion of Patients with Localized Juvenile Periodontitis

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The gentle agitation of suspensions of *Actinobacillus actinomycetemcomitans* serotype a, b, or c in saline resulted in the release of a proteinaceous surface-associated material (SAM) which produced a dose-dependent inhibition of tritiated thymidine incorporation by the osteoblast-like cell line MG63 in culture. This cell line was sensitive to low concentrations of SAM (50% inhibitory concentration, 200 ng/ml for serotype c). Immunoglobulin G antibodies to constituents of the SAM were found in the blood of patients with localized juvenile periodontitis (LJP). Sera from 9 of 16 patients with LJP significantly neutralized the antiproliferative activity of the SAM, while sera from 15 controls, with no evidence of periodontal disease, were unable to neutralize this activity. Neutralization was not directly related to the patient's antibody titer to the whole SAM. Characterization of the antiproliferative activity in the SAM demonstrated that it was not cytotoxic and was heat and trypsin sensitive. The active component separated in a well-defined peak in anion-exchange high-performance liquid chromatography (HPLC) which, when further analyzed by size exclusion HPLC, revealed a single active peak, which had an apparent molecular mass of approximately 8 kDa. The lipopolysaccharide from *A. actinomycetemcomitans* was only weakly active. SAM from *Porphyromonas gingivalis* W50 and *Eikenella corrodens* NCTC 10596 did not exhibit any antiproliferative activity with this cell line, even at concentrations as high as 10 μg/ml. This study has shown that SAM from *A. actinomycetemcomitans* contains a potent antiproliferative protein whose activity can be neutralized by antibodies in the sera from some patients with LJP.

Actinobacillus actinomycetemcomitans is a gram-negative bacterium that has been strongly implicated in the etiology of localized juvenile periodontitis (LJP) (25, 26). The loss of the alveolar bone and periodontal ligament which support the tooth is one of the hallmarks of this disease and is the result of either the removal of the tissue by some destructive process or the failure to produce sufficient tissue to keep pace with the normal rate of tissue remodelling. It is widely assumed that the loss of the extracellular matrices of these connective tissues is a result of the local gingival inflammation. However, in LJP the afflicted gingivae are only mildly inflamed (15) and it is possible that other mechanisms play a role in tissue loss.

The alveolar bone and periodontal ligament are considered to be tissues with a rapid rate of remodelling (11). Agents which inhibit cellular proliferation could therefore adversely affect these tissues, resulting in the tissue loss associated with LJP. A number of antiproliferative factors from periodontopathogenic bacteria have been documented (3, 4, 13, 16, 17, 19), yet it remains unclear exactly what role these factors play in the pathogenesis of the disease. We have previously reported that surface-associated material (SAM) from *A. actinomycetemcomitans*, isolated by gentle saline extraction, contains antiproliferative activity (5, 9, 23). We now report the identification of an immunogenic protein which is responsible for this activity. Individuals with LJP have elevated serum antibodies to *A. actinomycetemcomitans* and to its SAM (2, 7, 14); however, the role that such antibodies play in the course of this disease remains unclear. Our results demonstrate that a number of patients with LJP have serum antibodies which can neutralize the activity of this potent antiproliferative protein, and the role of such neutralizing antibodies in disease is discussed.

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MATERIALS AND METHODS

Growth of bacteria. *A. actinomycetemcomitans* (NCTC 9710 serotype c and clinical isolates 286 and 670) were cultured at 37°C in a CO_2-enriched atmosphere on brain heart infusion agar (Oxoid) supplemented with 5% (vol/vol) horse blood. *A. actinomycetemcomitans* clinical isolates 286 and 670, representing serotypes a and b, respectively, were kindly donated by Maria Saarela, University of Helsinki (12). *Porphyromonas gingivalis* W50 was grown at 37°C under anaerobic conditions on a medium consisting of (per liter) 5 g of Trypticase (BBL), 5 g of Proteose Peptone (Oxoid), 2.5 g of glucose (BDH), 2.5 g of sodium chloride (BDH), 2.5 g of yeast extract (Oxoid), 0.0375 g of cysteine HCl (BDH), 0.25 g of hemin, and 0.05 g of menadione (Oxoid). *Eikenella corrodens* NCTC 10596 was grown at 37°C under anaerobic conditions on a medium consisting of brain heart infusion (37 g/liter; Oxoid), 0.375 g of cysteine HCl (BDH), 0.25 g of hemin, and 0.05 g of menadione (Oxoid). Bacteria were grown for 48 h, harvested with saline, and centrifuged at 3,000 × *g* for 20 min, and the pellet was stored at −70°C.

Extraction of SAM and LPS. SAM was extracted by a modification of the method of Wilson et al. (24). Briefly, bacteria were thawed on ice, gently stirred in 0.15 M saline for 1 h at 4°C, and centrifuged at 3,000 × *g*. The saline extraction was repeated, and the combined supernatants were dialyzed with benzoylated dialysis tubing with a 2-kDa cutoff (Sigma) against distilled water and lyophilized. The protein concentration of the SAM was determined by the Bio-Rad (Richmond, Va.) protein assay with bovine serum albumin as the standard. The carbohydrate content was determined by the method of Dubois et al. (1), and the content of lipopolysaccharide (LPS) was determined by a commercial chromogenic *Limulus* amoebocyte assay (Pyrogent, Byk-Mallinckrodt, United Kingdom). In one experiment SAM was also extracted with methanol-chloroform (2:1) to isolate lipidic materials, and the extract was lyophilized and weighed. LPS was extracted by the method of Westphal and Jann (22). The biological activity of extracted material was related to dry weight.

PAGE. The SAM and fractions from purification procedures were suspended

in buffer (0.06 M Tris, 10% glycerol, 1% sodium dodecyl sulfate [SDS], 2.5% 2-mercaptoethanol [pH 6.8]) and boiled for 4 min. Samples were then separated by either one-dimensional or two-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a Bio-Rad mini-Protean II system (6, 10). Gels were stained with colloidal Coomassie blue (Sigma) or silver stained by using a commercial kit (Sigma). Molecular weight markers ranging from 14 to 66 kDa (Sigma) were run on all gels.

Fractionation of SAM from *A. actinomycetemcomitans*. (i) Amicon filtration. Crude SAM was separated by using Amicon YM30 membranes to isolate two fractions of <30 and >30 kDa and YM100 membranes to separate the >100-kDa from lower-molecular-mass material. Fractions were dialyzed, freeze-dried, and assayed for antiproliferative activity.

(ii) Anion-exchange chromatography. SAM was fractionated by anion-exchange high-performance liquid chromatography (HPLC) on a Bio-Rad HRLC system. An anion-exchange MA7Q (Bio-Rad) column (5 by 0.78 cm) was equilibrated with 20 mM Tris buffer, pH 8.5 (buffer A). One milliliter of a 4-mg/ml solution of SAM in buffer A was injected onto the column and eluted with 5 ml of buffer A followed by linear gradients from 0 to 50% buffer B (buffer A plus 2 M NaCl) in 20 ml and 50 to 100% buffer B in 5 ml. The flow rate was 1 ml/min, and 1-ml fractions were collected, with absorbance monitored at 280 and 205 nm. Fractions were dialyzed (Sigma dialysis tubing, 2-kDa cutoff) against distilled water for 48 h, and the protein concentration of each fraction was measured. Fractions were subsequently diluted 1,000 times to assess their antiproliferative activity on cultured MG63 cells.

Size exclusion chromatography. Bioactive fractions were further fractionated by size exclusion HPLC using a Protein Pak 125 column (Waters). The column was equilibrated with 0.1 M sodium phosphate buffer, pH 6.7, and 20-μl samples (concentration, 1 mg/ml) were injected. The flow rate was 1 ml/min, and 1-ml fractions were collected, with protein absorbance monitored at both 280 and 205 nm. Fractions were assayed for their ability to inhibit [^{3}H]thymidine incorporation by MG63 cells.

Osteoblast-like cell proliferation assay. The human osteoblast-like cell line MG63 (ATCC CRL 1427) was cultured at a density of 15,000 cells per well in 96-well plates and incubated overnight at 37°C in Dulbecco's modified Eagle's medium (DMEM) (Gibco) plus 10% fetal calf serum (FCS) (Sigma) in 5% CO_2-air. The medium was then removed, and the cells were washed twice with sterile Hanks solution (Sigma). To measure antiproliferative activity, various concentrations of the test materials were added to the cells in DMEM containing 2% FCS. To test the ability of human sera to neutralize the antiproliferative activity of the SAM from *A. actinomycetemcomitans*, 1:50 or 1:500 dilutions of sera were added to 500 ng (dry weight) of SAM per ml in DMEM containing 2% FCS and the mixtures were incubated at 37°C for 1 h before addition to the cells. The cells were incubated for 24 h at 37°C. During the last 6 h of culture, 0.05 μCi of [^{3}H]thymidine (Amersham) was added to the cells. Media were then removed, and the cells were fixed in 5% trichloroacetic acid. One hundred microliters of 0.5 M NaOH was used to lyse the cells; this was neutralized with an equal volume of 0.5 M HCl. Radioactivity was measured by scintillation spectrometry. The significance of the results was calculated by use of Student's *t* test.

Enzyme and heat treatments. SAM from *A. actinomycetemcomitans* was dissolved at 1 mg/ml in Tris buffer, pH 8.5. One hundred microliters of this solution was then mixed with 100 μl of trypsin (Sigma) dissolved at 100 μg/ml (100 BAEE units) in the same buffer and incubated for 1 or 24 h. One hundred microliters of soya bean trypsin inhibitor (Sigma) dissolved at 100 μg/ml was used to terminate the enzyme reaction (10 μg inhibits approximately 20 μg of trypsin with an activity of 100 BAEE units), and the samples were stored at 4°C. Control digestion mixtures contained no SAM but were otherwise identical. Thirty microliters from each digestion mixture was diluted into 970 μl of DMEM containing 2% FCS to a final SAM concentration of 10 μg/ml. All samples were tested for antiproliferative activity. SAM from *A. actinomycetemcomitans* was dissolved in saline at a concentration of 100 μg (dry weight) per ml and heated in water baths at various temperatures for 1 h. Samples were diluted 10 times in DMEM containing 2% FCS and assayed for antiproliferative activity.

Cytotoxicity assays. The cytotoxicity of the SAM was determined by lactate dehydrogenase (LDH) release measured by the CytoTox 96 nonradioactive cytotoxicity assay (Promega). Briefly, MG63 cells were cultured for 24 h in the presence of various concentrations of SAM ranging from 0.1 to 100 μg/ml. LDH levels in culture supernatants were measured with a 30-min coupled enzymatic assay which results in the conversion of a tetrazolium salt [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride] into a red formazan product. A_{592} was measured. The percent cell lysis was established by using a formula which correlates sample levels of LDH to the maximally induced level (by using Triton X-100 to lyse cells) and control LDH release.

Cytotoxicity was also monitored by acridine orange uptake after cells were incubated with the SAM at a concentration of 100 μg/ml for 24 h. The proportions of acridine orange-stained cells in control cultures and in those exposed to SAM were counted and compared with the incorporation of [^{3}H]thymidine into cells in parallel control cultures or cultures exposed to SAM.

Serum samples. Studies were performed with sera from 16 patients diagnosed as having LJP by standard criteria, including radiographic evidence of bone loss and first permanent molar or incisor pocket depths of 5 mm or more. Patients varied in age from 12 to 39 years. Samples were also obtained from 15 individuals judged to be periodontally normal, ranging in age from 13 to 41 years. Serum from clotted blood was harvested by centrifugation and stored at −20°C.

Enzyme-linked immunosorbent assay for serum antibody titers. Ninety-six-well microtiter plates (Immulon 4; Dynatech) were coated with *A. actinomycetemcomitans* SAM at 10 μg/ml in phosphate-buffered saline (PBS) overnight at 4°C. Wells were washed three times with PBS containing 0.05% Tween (Sigma) (PBS-T) to remove any unbound antigen and blocked with PBS-T containing 1% nonfat milk powder (Safeway) (PBS-TM) for 1 h at 37°C. Sera were then incubated in the wells at dilutions ranging from 1:100 to 1:64,000 in PBS-TM for 1 h at 37°C. Plates were washed three times with PBS-T, and the bound antibody was detected with horseradish peroxidase-conjugated goat anti-human immunoglobulin G (IgG) (γ-chain specific) (Sigma) dissolved at a 1:1,000 dilution in PBS-TM, again incubated for 1 h at 37°C. Tetramethylbenzidine dihydrochloride (0.1 mg/ml) plus hydrogen peroxide (2 μl of fresh 30% solution per 10 ml) in 0.1 M citrate buffer (pH 5.1) was used as the enzyme substrate, and the reaction was terminated after 10 min by the addition of 1 M sulfuric acid. Plates were read at 450 nm with a Titertek Multiskan plate reader.

The relative binding of each serum at each dilution was calculated with reference to a 100% control (wells coated with excess human IgG [Sigma]) and a nonspecific binding control (antigen omitted), and from these results the serum titer giving 30% binding was determined. The significance of the results was tested by Wilcoxon's rank sum test.

Western blotting (immunoblotting). SAM separated on 15 and 12% gels by SDS-PAGE was electrophoretically transferred to nitrocellulose membranes as described by Towbin et al. (20). The membranes were washed for 5 min in PBS containing 1% Triton X-100 and then for a further 25 min in PBS containing 0.1% Triton X-100. Nitrocellulose membranes were then rinsed in blocking buffer (PBS containing 0.1% Triton X-100 and 2% FCS) for 1 h and incubated in human serum diluted 1:100 in blocking buffer for 1 h. Following a further wash, membranes were incubated for 1 h in goat anti-human IgG horseradish peroxidase conjugate (Sigma), diluted 1:1,000, and washed again. Membranes were placed in 3,3′-diaminobenzidine tetrahydrochloride solution (10 mg in 15 ml of Tris-buffered saline, pH 7.6) containing 12 μl of 30% hydrogen peroxide until bands were visualized.

Depletion of serum antibody using protein A. To determine if the neutralizing activity in the sera was due to antibody, the serum with the highest neutralizing activity (from patient 10) was diluted 1:10 in PBS, and Sepharose (CL-4B)-bound protein A (Sigma) was added and mixed for 2 h at 4°C. The protein A-Sepharose was then pelleted by centrifugation. Fifty microliters of this depleted serum was removed for analysis, and the remaining material was added to fresh protein A-Sepharose, which was again mixed for 2 h at 4°C. This process was repeated one more time. To demonstrate the removal of antibody, the washed protein A-Sepharose from each step was boiled in SDS-PAGE sample buffer and run on a 12% gel. This showed a progressive decline in antibody uptake onto the beads. Undepleted-serum or serum depleted once, twice, or three times was assayed for its ability to neutralize the antiproliferative activity of SAM (500 ng/ml) at a dilution of 1:500. To determine if the protein A-Sepharose had any effect on MG-63 proliferation or on the antiproliferative activity of the SAM, it was added to cells in the presence or absence of SAM.

RESULTS

Characterization of the SAM. The range of values for the composition of the SAM obtained from six separate batches was approximately 60 to 70% protein, with the remainder being carbohydrate and lipid. The LPS content of the SAM was low, generally on the order of 0.0001 to 0.001 IU/ng. On SDS-PAGE, the SAM showed a relatively large number of protein bands, and on two-dimensional gels over 40 Coomassie blue-stained spots were visible.

Antiproliferative activity of SAM. In the human osteoblast-like cell line MG63, the SAM from *A. actinomycetemcomitans* (NCTC 9710) caused a concentration-dependent inhibition of [^{3}H]thymidine incorporation with a reproducible 50% inhibitory concentration (IC_{50}) of approximately 200 ng/ml. The SAMs from two other periodontopathogenic bacteria, *P. gingivalis* W50 and *E. corrodens* NCTC 10596 were also tested but failed to show potent antiproliferative activity with this cell line (Fig. 1). In contrast to the SAM, the LPS from *A. actinomycetemcomitans* at a concentration of 10 μg/ml produced only 13% inhibition of proliferation (results not shown). Comparison of the antiproliferative activities of the SAMs from the three major serotypes (a, b, and c) of *A. actinomycetemcomitans* demonstrated that they all had the capacity to inhibit proliferation (Fig. 2). The methanol-chloroform extract of the SAM

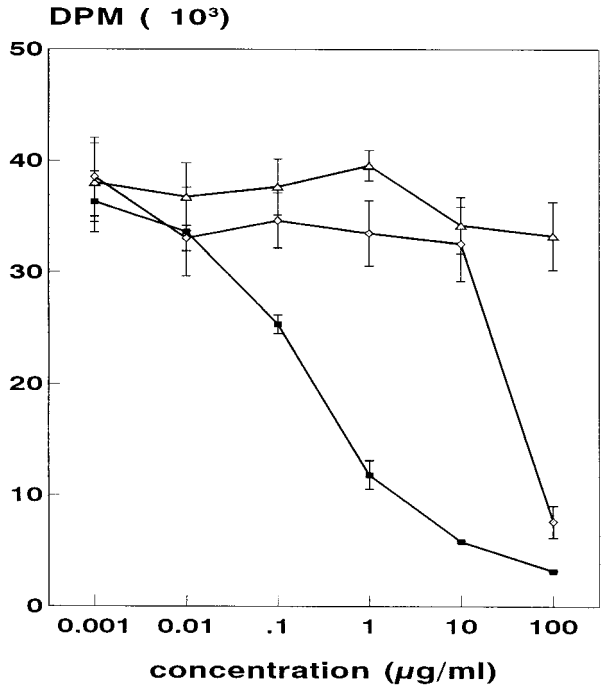

FIG. 1. Inhibitory effect of increasing concentrations of SAMs from *A. actinomycetemcomitans* (square), *P. gingivalis* (diamond), and *E. corrodens* (triangle) on DNA synthesis, measured as incorporation of [^{3}H]thymidine into DNA, by MG63 cells. The results are expressed as means and standard deviations for six replicate cultures.

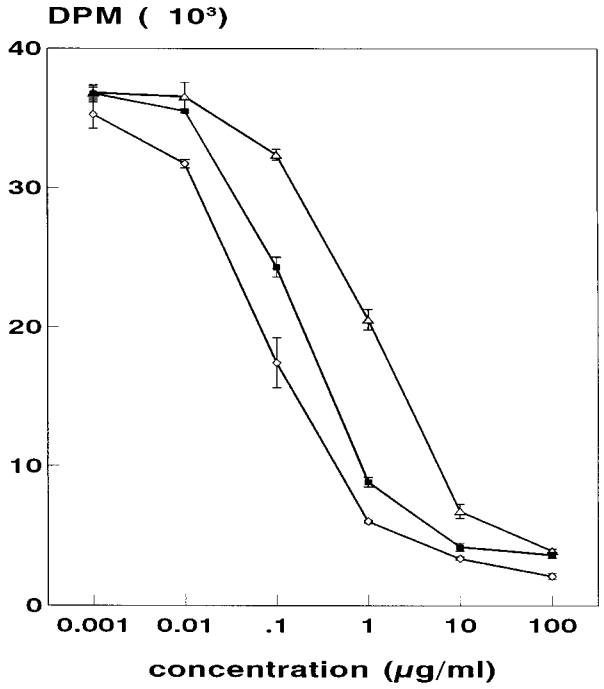

FIG. 2. Inhibitory effect of increasing concentrations of SAMs from *A. actinomycetemcomitans* serotypes a (triangle), b (diamond), and c (NCTC 9710) (square) on DNA synthesis, measured as incorporation of [^{3}H]thymidine into DNA, by MG63 cells. The results are expressed as means and standard deviations for six replicate cultures.

was also tested for antiproliferative activity but failed to show any activity at a concentration of 10 μg/ml.

Heat treatment of *A. actinomycetemcomitans* SAM demonstrated that at 56°C the SAM maintained its antiproliferative activity while at 78 and 100°C 81 and 95%, respectively, of the activity were lost (Fig. 3). Treatment with trypsin for 1 h destroyed over 70% of the antiproliferative activity, with no activity remaining after 24-h incubation (data not shown).

The SAM was not cytotoxic as assessed by a commercial assay for cytotoxicity based upon release of LDH which showed that cells exposed to the SAM released amounts of LDH (16% of maximum) that were similar to the amounts released by control cells. Use of a second measure of cytotoxicity, acridine orange uptake, also demonstrated that the SAM was not cytotoxic, as the number of nonviable cells in cultures incubated with SAM was not significantly different from that in the controls. However, at the concentration of SAM used, there was almost complete inhibition of [^{3}H]thymidine incorporation (Fig. 4).

Fractionation of the SAM. Amicon filtration of the crude SAM on YM30 membranes showed that all of the activity was in the >30-kDa fraction and that on YM100 cutoff membranes a large proportion of the antiproliferative activity was also retained. The crude SAM extract was separated by anion-exchange HPLC. The elution profile showed that the majority of the antiproliferative activity appeared in one well-defined peak (fraction 12) (Fig. 5). This bioactive material was further fractionated by HPLC gel filtration on a Protein Pak 125 column (Waters), and the antiproliferative activity was assessed (Fig. 6). Bioactivity was eluted as a broad peak and, on the basis of the retention time of standard markers, had a mean molecular mass of 8 kDa. Bioactive fractions were pooled, concentrated, and retested over a protein concentration range of 1 to 100 ng/ml. This semipurified material was 50 times more active than the crude material, with an IC_{50} of 4 ng/ml. This pooled fraction, analyzed by SDS-PAGE on a 15% gel stained with Coomassie blue, contained two proteins with molecular

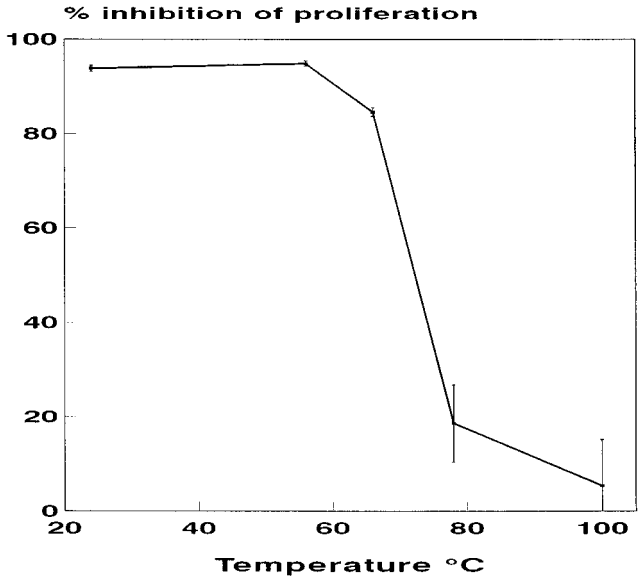

FIG. 3. Effect of temperature on the inhibitory activity of SAM from *A. actinomycetemcomitans* on DNA synthesis, measured as incorporation of [^{3}H]thymidine into DNA, by MG63 cells. Solutions of SAM were heated at various temperatures for 1 h and tested at a concentration of 10 μg/ml. The results are expressed as means and standard deviations for six replicate cultures.

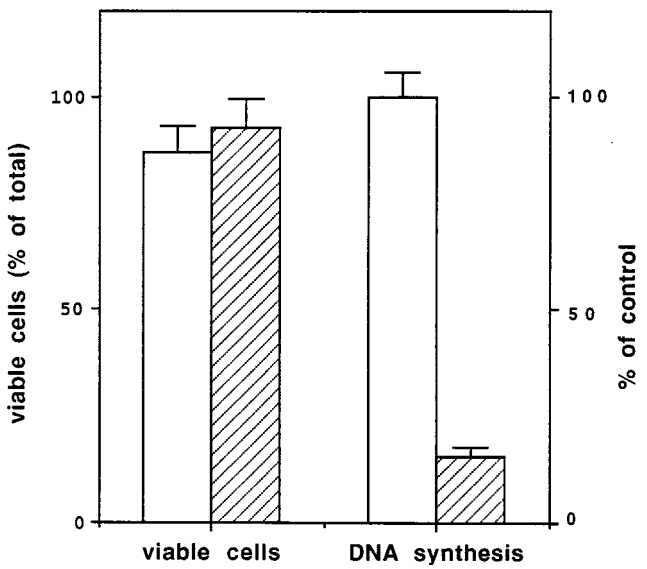

FIG. 4. Comparison of the effect of SAM on cell viability and on cellular replication, as measured by [^{3}H]thymidine incorporation into DNA. MG63 osteosarcoma cells were exposed to 100 μg of SAM per ml for 24 h. The control cultures, to which SAM was not added (open bars), and SAM-treated cultures (hatched bars) were pulsed with [^{3}H]thymidine to determine DNA synthesis (expressed as percentage of control) or tested for acridine orange uptake as a measure of cell viability (expressed as percentage of total cells). Results are expressed as means and standard deviations for three replicate cultures.

masses of less than 15 kDa and two other minor proteins of approximately 26 kDa (Fig. 7).

Serum antibody titers. Sera from 16 patients with LJP were assessed for titers of anti-SAM antibodies on microtiter plates coated with SAM. The titers of these sera and 15 representative controls are shown in Fig. 8. Sera from patients with LJP had titers that were significantly higher (median, 5,350) than those of the control sera (median, 620) ($P < 0.001$ [Wilcoxon's rank sum test]).

Serum inhibition of antiproliferative activity. Patients' sera had no effect on the proliferation of MG63 cells. Sera from 16 patients with LJP were added at a 1:50 or 1:500 dilution to MG63 cells incubated in the presence of 500 ng of SAM per ml to determine if they could neutralize the antiproliferative activity associated with the surface-associated protein. At 1:50 dilutions, 9 of the 16 sera significantly blocked the antiproliferative activity ($P < 0.001$) and sera from patients 7 and 10 almost completely neutralized this activity (Fig. 9). At 1:500 dilutions, five sera (patients 3, 7, 8, 10, and 16) were still able to significantly block the antiproliferative activity of the SAM ($P < 0.01$) (results not shown). High concentrations (1:50 dilutions) of sera from 15 individuals with no evidence of periodontal disease had no effect on the ability of SAM to inhibit cell proliferation (results not shown).

The serum which most potently blocked the antiproliferative effect of SAM (patient 10) was tested at various concentrations to establish whether the effect was concentration dependent. Figure 10 shows that sera from control, disease-free volunteers were unable to block the antiproliferative activity of 500 ng of SAM per ml. In contrast, serum from a patient with LJP (patient 10; titer, 1:9,200) was capable of inhibiting this antiproliferative activity in a concentration-dependent manner.

Removal of antibodies from the serum of patient 10 using protein A-Sepharose decreased the neutralizing ability after each treatment, reaching control levels (100% inhibition) after the third adsorption (Fig. 11). Protein A-Sepharose alone was not antiproliferative, nor did it effect the antiproliferative activity of the SAM.

Immunoblotting of sera. Immunoblot analysis of SDS-PAGE-separated SAM from *A. actinomycetemcomitans* was

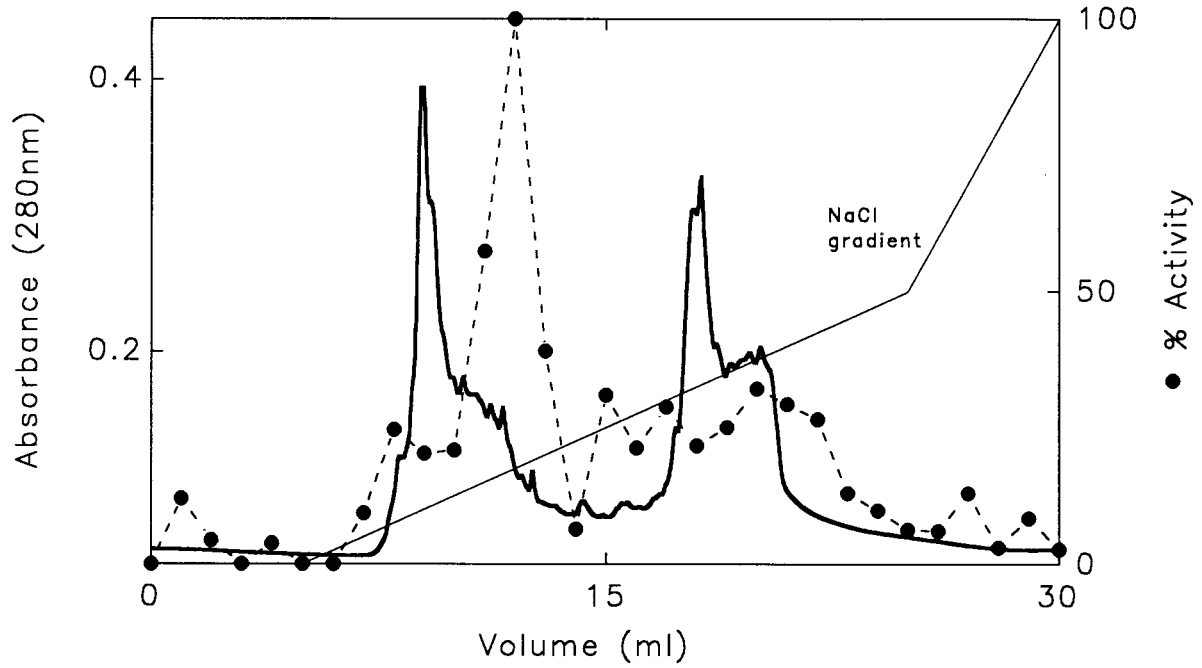

FIG. 5. Anion-exchange HPLC of SAM from *A. actinomycetemcomitans*. The protein elution profile is shown as A_{280} (solid line). Charged components were eluted with a 0 to 2 M NaCl gradient. Each fraction was assayed for inhibition of [^{3}H]thymidine incorporation into MG63 cells (dotted line), and percent activity was compared with the activity of the most active fraction (fraction 12), which was deemed 100% active.

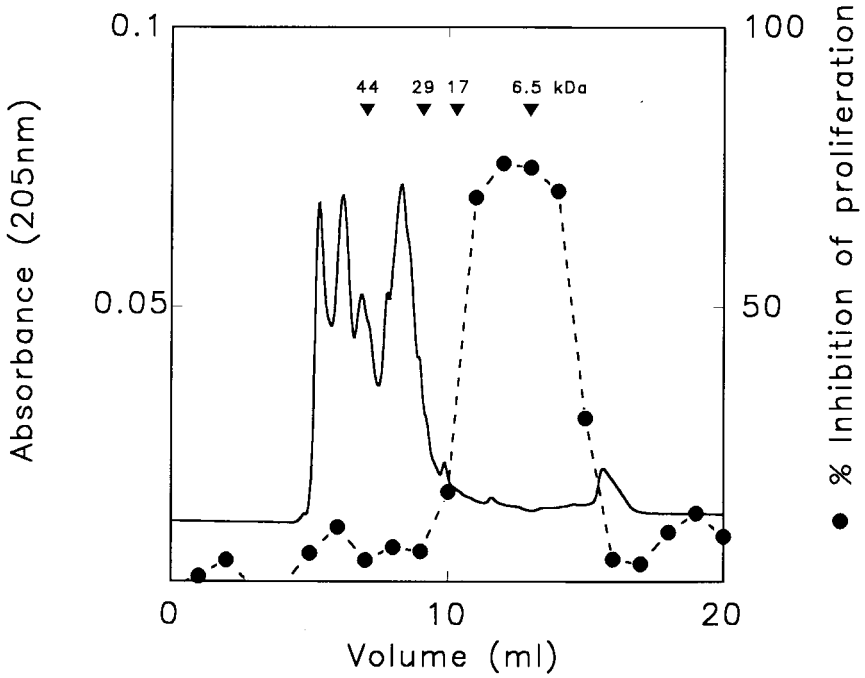

FIG. 6. Size exclusion HPLC of the most active fraction (fraction 12) from the HPLC anion-exchange column. The protein elution profile is shown as A_{205} (solid line). Each fraction was assayed for inhibition of [^{3}H]thymidine incorporation into MG63 cells (dotted line). Protein standards: aprotinin (6.5 kDa), myoglobin (17 kDa), carbonic anhydrase (29 kDa), and ovalbumin (44 kDa).

carried out with sera from all 16 LJP patients. Antibodies bound to a large number of the proteins ranging in molecular mass from >66 to <14 kDa. A representative immunoblot from patient 7 (Fig. 12) shows the pattern of antibody binding. Neutralizing and nonneutralizing sera showed similar patterns of IgG-binding antibodies, with dominant bands at 24 and 29 kDa.

DISCUSSION

Gentle saline extraction of *A. actinomycetemcomitans* (NCTC 9710) releases SAM which can potently inhibit [^{3}H]thymidine incorporation in numerous cell types including the fibroblast cell line L929, the monocyte cell line U937, keratinocytes, osteosarcoma cell line U2OS, gingival fibroblasts, periodontal fibroblasts, and primary osteoblasts (9, 11a, 22a). The component responsible may play a role in the pathogenesis of LJP by preventing the replication of cells in the alveolar bone and periodontal ligament, thus causing a de-

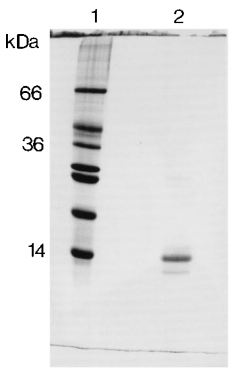

FIG. 7. SDS-PAGE of the biologically active fraction eluted from the size exclusion column, activity being found in fractions 10 to 14. Molecular weight markers are displayed in lane 1. Fractions 10 to 14 were pooled and concentrated, and the proteins present are shown in lane 2. The gel used contained 15% polyacrylamide and was stained with Coomassie blue to disclose protein bands.

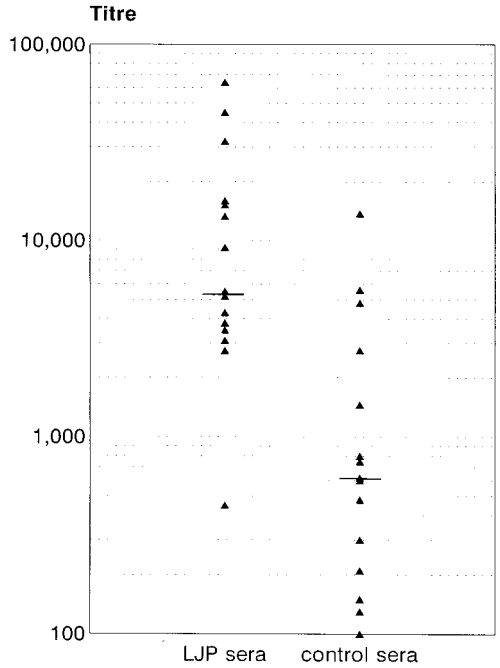

FIG. 8. Range of IgG antibody titers to *A. actinomycetemcomitans* SAM in the sera of individuals with LJP and individuals with no evidence of periodontal disease. Horizontal lines, median antibody titers. $P < 0.001$ (Wilcoxon's rank sum test).

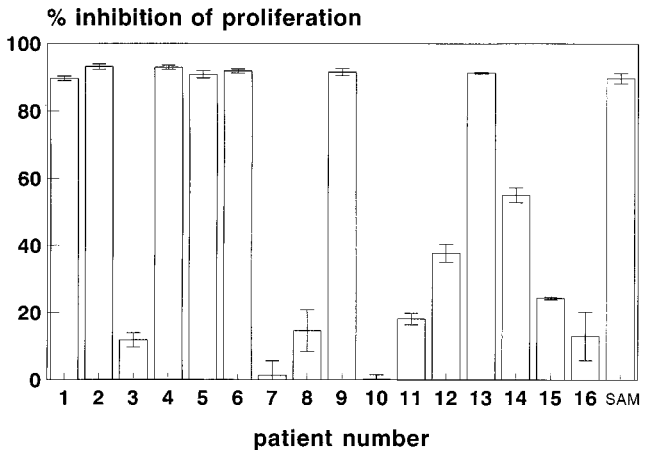

FIG. 9. Influence of LJP sera, used at a 1:50 dilution, on the antiproliferative activity of SAM from *A. actinomycetemcomitans* (incubated with cells at a concentration of 500 ng/ml). Activity is measured as percent inhibition of [^{3}H]thymidine incorporation relative to control cultures. The effect of SAM with no serum added is shown on the far right (SAM). The patient with the lowest titer of antibodies against the whole SAM is patient 1, and highest-titer patient is patient 16. Results are expressed as means and standard deviations for six replicate cultures.

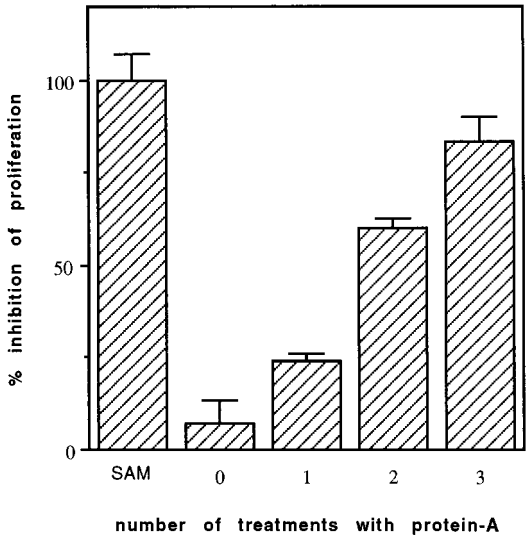

FIG. 11. Effect of sequentially adsorbing serum from patient 10 with protein A-Sepharose (to remove antibody) on the serum-mediated inhibition of the antiproliferative activity of SAM. The normalized percent inhibition of MG63 proliferation induced by SAM (500 ng/ml) is shown on the far left (SAM). 0, SAM plus unadsorbed serum; 1 to 3, inhibitory activities remaining after 1, 2, or 3 adsorptions of the serum with protein A. Results are expressed as means and standard deviations for six replicate cultures.

crease in the rate of replacement of these labile tissues. The consequence of such inhibition of cellular proliferation could be the loss of connective tissue matrices and thus be equivalent to the loss by destructive processes, as is generally assumed to occur.

We have now demonstrated that SAM from *A. actinomycetemcomitans* (NCTC 9710) is an extremely potent inhibitor of the proliferation of the human osteoblast-like cell line MG63, with an IC_{50} of approximately 200 ng/ml. SAMs from *A. actinomycetemcomitans* serotypes a and b also demonstrated antiproliferative activity. A concentration-dependent response parallel to that of serotype c was seen, although SAM from serotype a was not as active as SAMs from serotypes b and c, with an IC_{50} of approximately 1 μg/ml. The antiproliferative component is heat labile, trypsin sensitive, and noncytotoxic, elutes in a well-defined peak upon anion-exchange HPLC, and has an approximate molecular mass of 8 kDa as determined by size exclusion HPLC. Active fractions were pooled, concentrated, and separated by SDS-PAGE, revealing two major proteins of low molecular masses. It is conceivable that the smaller of these is the antiproliferative protein, although further purification is needed to confirm this. While size exclusion chromatography showed the activity to be of low molecular mass, on Amicon membrane filtration the antiproliferative activity was found exclusively in the >30-kDa fraction and approxi-

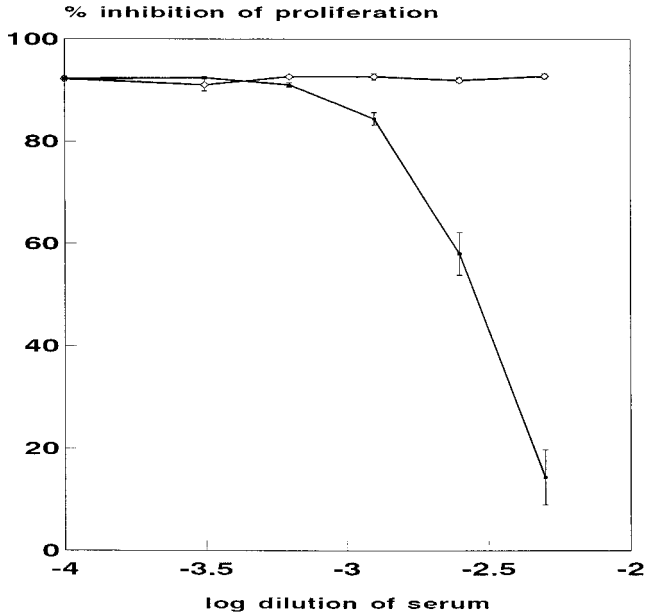

FIG. 10. Titration curves showing the effect of serum from patient 10 on the antiproliferative activity of 500 ng of SAM from *A. actinomycetemcomitans* per ml on MG63 cells. The graph shows that control serum from an individual with no evidence of periodontal disease was unable to block the antiproliferative activity (diamond). In contrast, serum from a patient with LJP was capable of inhibiting the antiproliferative activity of the SAM in a concentration-dependent manner. Results are expressed as means and standard deviations for six replicate cultures.

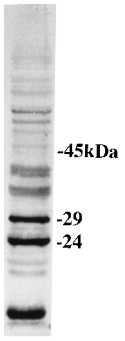

FIG. 12. Representative Western blot of SDS-PAGE-separated components of *A. actinomycetemcomitans* SAM. The blot was stained to show IgG binding of serum from a patient with LJP (patient 7). The molecular sizes of markers are indicated on the right.

mately half of the activity failed to pass through an Amicon 100-kDa cutoff membrane. Two possible explanations for these anomalous findings are (i) the active molecule is self-associating under particular circumstances and/or (ii) the active protein binds to some carrier protein. We have thus far failed to resolve this question. For example, treatment of the SAM with 6 M urea to dissociate complexes led to complete loss of antiproliferative activity. Further purification is continuing in order to isolate this potent antiproliferative protein. However, with the finding that sera from patients with LJP are able to neutralize this antiproliferative activity, we are now using an alternative strategy for isolation, namely, cloning of this protein by differential screening with nonneutralizing and neutralizing sera to identify positive clones.

The present study confirms earlier findings (7) that the SAM from *A. actinomycetemcomitans* is strongly immunogenic, and high titers of antibodies to this extract were found in the blood of patients with LJP. Antibodies in patients' sera reacted with many of the components in the SAM, and no differences in the binding pattern of neutralizing, compared with nonneutralizing, sera could be distinguished. This polyclonal response to the many components in the SAM is presumably why there was no relationship between antibody titer to SAM and neutralizing capacity of the sera. We have previously shown that the sera from a proportion of patients with LJP could block the bone-resorbing activity of the SAM from this organism (8). We now report that, in a similar manner, a proportion of patients with LJP have neutralizing antibodies to the antiproliferative component of SAM. At a dilution of 1:50 9 of the 16 sera could significantly block the antiproliferative action of the surface-associated protein, and at a 1:500 dilution 5 sera were still able to significantly block activity. The remaining sera, and sera from 15 individuals with no evidence of periodontal disease, failed to inhibit the antiproliferative activity. The neutralizing ability of sera could be removed by adsorbing the sera with protein A-bound Sepharose, indicating that the neutralizing capability of the sera was due to antibody.

The role played by high levels of circulating antibodies against periodontopathogenic bacteria is unclear, and the biological function of such antibodies has not been studied in detail. Tsai et al. (21) showed that 90% of sera from patients with juvenile periodontitis neutralized the leukotoxic activity of sonicated extracts of *A. actinomycetemcomitans* whereas most sera from periodontally healthy individuals and patients with adult periodontitis had no such activity. Taichman et al. (18) reported that a bacterial sonicate of *A. actinomycetemcomitans* was capable of inhibiting endothelial cell growth at a concentration of 10 μg/ml. In this study monoclonal antibodies which inhibited the leukotoxin from *A. actinomycetemcomitans* could not neutralize the endothelial cell inhibitor but sera from patients with juvenile periodontitis could.

Two unanswered questions remain to be elucidated: is the in vitro neutralization of the antiproliferative activity of *A. actinomycetemcomitans* repeated in vivo and do patients with the ability to block the antiproliferative activity have a less severe prognosis. These questions are now the focus of active research.

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