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Immunosuppressive Factor from *Actinobacillus actinomycetemcomitans* Down Regulates Cytokine Production

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A cytoplasmic soluble fraction of *Actinobacillus actinomycetemcomitans* Y4 was isolated and characterized as suppressing mitogen-stimulated proliferation of and cytokine production by C3H/HeN mouse splenic T cells. This factor, designated suppressive factor 1 (SF1), was isolated from the supernatant of sonicated whole bacteria and purified by Q-Sepharose Fast Flow column chromatography, DEAE-Sepharose Fast Flow column chromatography, hydroxyapatite high-pressure liquid chromatography (HPLC), and Protein Pack 300 & 125 gel filtration HPLC. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis revealed that the purified SF1 migrated as a single band corresponding to a molecular mass of 14 kDa. This molecule was protease labile, heat resistant, and noncytotoxic. N′-terminal sequence analysis revealed no homology with any known peptides of periodontopathic bacteria or with any host-derived growth factors. Purified SF1 suppressed the proliferation of mouse splenic T cells which had been stimulated with concanavalin A, as well as suppressing the production of interleukin-2 (IL-2), gamma interferon, IL-4, and IL-5 from $CD4^+$ T cells at 0.1 μg/ml or more. These data suggest that SF1 produced by the periodontal pathogen *A. actinomycetemcomitans* functions as a virulence factor by down regulating T-cell proliferation and cytokine production at local defense sites.

Host defense mechanisms play an important role in keeping the numbers of bacteria in subgingival plaque down and in preventing some of the resident species from causing tissue pathology (19). Although the exact role of the host immune system in the pathogenesis of periodontal disease is still not understood, it is generally accepted that the immune system plays an important role in protection from bacterial infection in the gingival region (3, 13). This is supported by evidence that a deficiency or defect in cellular and/or humoral immune responses to periodontopathogens contributes to the progress of periodontal disease (14, 20, 21).

Actinobacillus actinomycetemcomitans produces a number of virulence factors which may be involved in the pathogenesis of periodontitis and which include the bacterial capsules and fimbriae, which permit the microorganism to adhere to teeth or gingiva, as well as factors such as leukotoxin and immunosuppressive agents, which help the bacteria evade host defense mechanisms (25). Leukotoxin has been shown to affect not only polymorphonuclear leukocytes and monocytes (1) but also to kill mature T- and B-lymphocyte cell lines (18) and to facilitate a nonlethal suppression of immune cells (12). *A. actinomycetemcomitans* also produces a 60-kDa protein which down regulates both T- and B-cell responsiveness through the activation of a subpopulation of B lymphocytes (17).

Our previous studies (7, 11) have shown that a soluble sonicated extract (SE) from *A. actinomycetemcomitans* had an immunosuppressive effect on immunoglobulin (Ig) production, CD4/CD8 ratios, and concanavalin A (ConA)-induced proliferation of murine spleen cells. The mechanism of inhibition involved suppression of interleukin 2 (IL-2) synthesis, IL-2 receptor expression, and IL-6 secretion (7). Furthermore, the adoptive transfer of T cells treated with the immunosuppressive fraction resulted in the complete inhibition of antigen-specific immune responses (8). In the present study, we purified the immunosuppressive factor (ISF) from the cytoplasmic soluble fraction of *A. actinomycetemcomitans* by extensive column chromatography. We found that the bacteria produce a novel 14-kDa substance (designated suppressive factor 1 [SF1]) which suppresses cell proliferation and cytokine production by mouse splenic T cells.

MATERIALS AND METHODS

Mice. C3H/HeN mice (female, 8 to 9 weeks old) were obtained from Charles River Breeding Laboratories (Kanagawa, Japan). The mice were maintained in the Animal Facility of Nihon University School of Dentistry at Matsudo under standard care and given food and water ad libitum. Mice were used at 9 to 10 weeks of age. In individual experiments, age-matched mice were used.

SF1 purification. *A. actinomycetemcomitans* Y4 was kindly provided by T. Koga, Kyushu University, Japan. The cells were grown in 50 liters of Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) supplemented with yeast extract (Difco) at 37°C for 3 days in a 5% CO_2 atmosphere. Microorganisms were harvested by centrifugation (10,000 × *g*), washed three times with phosphate-buffered saline (PBS [pH 7.2]), and lyophilized. Soluble SE from *A. actinomycetemcomitans* Y4 were prepared as previously described (11). Briefly, lyophilized cells (4.1 g) were resuspended in PBS and sonicated for 40 min in ice with a sonicator (Ohtake Works, Tokyo, Japan). The cell supernatant was collected by centrifugation at 25,000 × *g* for 30 min. To prepare the cytoplasmic soluble fraction, the supernatants were further centrifuged at 100,000 × *g* for 60 min. The supernatants which contained cytoplasm were precipitated by addition of saturated ammonium sulfate to 60%. After extensive dialysis against 20 mM Tris buffer (pH 8.2), the sample was applied to a Q-Sepharose Fast Flow column (1.5 by 20 cm; Pharmacia-LKB, Uppsala, Sweden) equilibrated in the same buffer. The column was then extensively washed and eluted with a linear NaCl gradient (0 to 0.8 M). Ten-milliliter fractions were collected and monitored for UV A_{280}. The fractions were assayed for mitogen inhibitory activity as described below. Fractions which suppressed mitogen stimulation were pooled, dialyzed against 20 mM Tris buffer (pH 8.2), and then applied to a DEAE-Sepharose Fast Flow column (1.5 by 20 cm; Pharmacia-LKB) preequilibrated in 20 mM Tris buffer. The column was then extensively washed and eluted with a linear NaCl gradient (0 to 0.6 M). Suppressive fractions were pooled, dialyzed against distilled water, and lyophilized. The lyophilized materials were dissolved in 10 mM phosphate buffer (KH_2PO_4-Na_2HPO_4 [pH 7.4]) and applied to a hydroxyapatite high-pressure liquid chromatography (HPLC) (Tosoh Co., Ltd., Tokyo, Japan) column preequilibrated in 10 mM phosphate buffer. The column was extensively washed and eluted with a linear K_2PO_4 gradient (0 to 0.5 M). Suppressive fractions were pooled, dialyzed against distilled water, and lyophilized. The lyophilized material was dissolved in 0.1 M KH_2PO_4 (pH 7.0) and then fraction-

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ated by gel filtration chromatography on a Protein Pack 300 & 125 HPLC (Waters-Millipore, Tokyo, Japan) column preequilibrated in 0.1 M KH_2PO_4 (pH 7.0). Suppressive fractions were pooled, concentrated by ultrafiltration (Ultra free C3LCC; Millipore Corp., Bedford, Mass.), and then assessed for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentration was determined with the Bio-Rad protein assay system (Nippon Bio-Rad Laboratories, Tokyo, Japan).

SDS-PAGE analysis. Samples were loaded at concentrations of 10 to 50 μg of protein per lane of a 10% (wt/vol) polyacrylamide gel that contained SDS and were run at a constant current of 15 mA. One gel lane was stained with silver stain reagents (Wako, Pure Chemical Industries, Tokyo, Japan). SDS was removed from the rest of the unstained gel lanes by overnight treatment in 75% ethanol at 25°C. These gels were rehydrated in PBS at 25°C until they expanded to the length of the original gels and were then serially sectioned at 3-mm intervals. Gel slices were individually incubated in PBS at 4°C overnight, and the extracts were tested in a mitogen-induced proliferative assay with mouse spleen cells.

Amino acid sequencing. N′-terminal sequencing of the pure proteins was performed after transfer to Immobilon-P^{SQ} membrane (Millipore) as described previously (9). Pure SF1 (30 μg), after treatment with a sample buffer at 60°C for 5 min, was loaded onto a 10% acrylamide gel. After electrophoresis, the protein was transferred onto an Immobilon-P^{SQ} membrane according to the manufacturer's instructions. The membrane was stained with Coomassie brilliant blue R250 and destained with 50% ethanol and 10% acetic acid. The target SF1 protein band was excised from the dried membrane, and its amino acid sequences were determined at the laboratories of Takara Shuzo (Tokyo, Japan). Samples were subjected to Edman degradation with an Applied Biosystems 470A gas-phase sequencer.

Splenic cell preparations. Spleens were aseptically removed, and single-cell suspensions were prepared by gently teasing the cells through sterile stainless steel screens as described previously (2).

T-cell preparation. Preparation of T cells of mouse spleen cells was described previously (8). Briefly, T-cell-enriched fractions were obtained by using a Sephadex G-10 (Pharmacia, Piscataway, N.J.) column followed by panning (24) on plastic Petri dishes (15 by 100 mm) coated with rabbit IgG anti-mouse $F(ab')_2$ (Organon Teknika Co., West Chester, Pa.). After incubation for 90 min at 4°C, the non-adherent T-cell-enriched population was recovered by gentle swirling and was usually more than 95% Thy 1.2^+ and less than 5% Ig^+, as detected by indirect immunofluorescence with a FACScan fluorescence-activated cell sorter (Becton Dickinson and Co., Sunnyvale, Calif.).

Proliferation assay. Single-spleen-cell suspensions or purified splenic T cells from C3H/HeN mice were washed and resuspended in RPMI 1640 supplemented with 5% fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.), L-Glutamine (2.0 mM), kanamycin (0.2 mg/ml), and *N*-2-hydroxyethylpiperazine-*N*′-2-ethansulfonic acid (HEPES) buffer (pH 7.2; 15 mM) and then were adjusted to 3.5×10^6 viable cells per ml prior to the experiment. Purified splenic T cells (7.0×10^5 per well) were cultured in triplicate in a 96-well microculture plate in a total volume of 0.2 ml of the supplemented RPMI 1640 medium containing 2.5 μg of ConA (Sigma) per ml either in the presence or absence of column and gel fractions or purified SF1. The cells were incubated for 72 h at 37°C in humidified air containing 5% CO_2. Cultures were pulsed with 0.1 mCi (3.7 kBq) of [^{3}H]thymidine ([^{3}H]TdR; Amersham Corp., Little Chalfont, Buckinghamshire, United Kingdom) during the final 6-h incubation. Cells were then harvested onto glass fiber filters with a multiple-cell harvester. The filters were dried, placed in vials with scintillation fluid, and analyzed with an Aloka scintillation counter, type LSC-673 (Aloka, Tokyo, Japan). The data are presented as the averages for triplicate cultures and standard errors of counts per minute.

Enzyme and heat treatments. SF1 (3.7 μg/ml) in 50 mM potassium phosphate buffer (pH 7.2) was heated for 10 min at 100°C. Trypsin sensitivity was tested by incubation of SF1 (3.7 μg/ml) with trypsin (10,000 U/ml; Sigma, St. Louis, Mo.) at 37°C for 2 h. The enzyme was inactivated by addition of trypsin inhibitor from soybeans (1 mg/ml). Pronase sensitivity was tested by incubation of SF1 with pronase K (1 μg/ml; Kaken Kogyo, Ltd., Tokyo, Japan) at 37°C for 2 h. The enzyme was inactivated by being heated for 10 min at 100°C. After treatment, the preparations were dialyzed against distilled water at 4°C. These samples were lyophilized and dissolved in PBS and stored at −80°C until used. The samples were tested with a mitogen-induced proliferative assay with mouse spleen cells.

Cell viability in the presence of SF1. To evaluate cell viability, splenic T cells were incubated for up to 21 h with 3.7 μg of SF1 per ml. Subsequently, cells were washed twice and resuspended in RPMI 1640 without FCS. One volume of trypan blue (0.4%; Gibco) was then added to 5 volumes of cell suspension. After incubation at 23°C for 5 min, the cells were examined by light microscopy (type CK2-TRC; Olympus, Tokyo, Japan).

Cytokine production. Splenic T cells were suspended in RPMI 1640 containing 2-mercaptoethanol (0.05 mM) and 10% FCS. Cells (4.0×10^6 cells per ml) were cultured with 2.5 μg of ConA in 24-well tissue culture plates (Falcon, Becton-Dickinson Labware, Lincoln Park, N.J.) in the presence or absence of various amounts of SF1. After 24 or 48 h of incubation at 37°C, culture supernatants were obtained by centrifugation at 3,000 × *g*, filtered through a 0.22-μm-pore-size Millipore filter, and stored at −20°C until assayed for IL-2, gamma interferon (IFN-γ), IL-4, and IL-5 as described below.

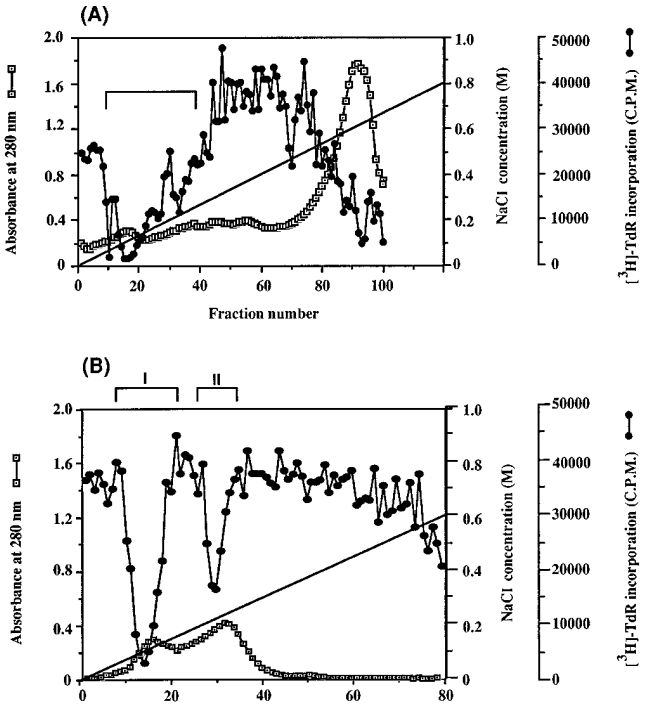

FIG. 1. Anion-exchange chromatography of SF. (A) Cytoplasmic soluble fraction was applied to a Q-Sepharose Fast Flow column. Absorbances of individual fractions corresponding to the A_{280} trace are shown. The protein was eluted with a 0 to 0.8 M linear NaCl gradient. The starting buffer used was 20 mM Tris buffer (pH 8.2). (B) Fractions (fractions 7 to 40) from the Q-Sepharose Fast Flow column containing suppressor activity were collected, concentrated, dialyzed against the starting buffer, and applied to a DEAE-Sepharose Fast Flow column. The protein was eluted on a 0 to 0.6 M linear NaCl gradient.

Cytokine assays. Enzyme-linked immunosorbent assays were used to quantitate levels of murine IL-2 (Becton Dickinson Labware, Bedford, Mass.) IFN-γ (Endogen, Boston, Mass.), IL-4 (Endogen), and IL-5 (Endogen) according to the manufacturer's instructions. Results are expressed as mean international units (IL-2) or picograms (IFN-γ, IL-4, and IL-5) per milliliter from the triplicate assays.

Statistics. Statistical analysis was performed with Student's *t* test.

RESULTS

Purification of SF1. ISF was isolated from the cytoplasmic soluble fraction of *A. actinomycetemcomitans* by precipitation with saturated ammonium sulfate to 60%. The precipitate was chromatographed on a Q-Sepharose Fast Flow column, and fractions which suppressed ConA-induced mitogenic activity were eluted with 0.1 to 0.3 M NaCl in a broad range (Fig. 1A). These fractions were pooled, dialyzed, and applied to a DEAE-Sepharose Fast Flow column (Fig. 1B). Two suppressive peaks (I and II) were eluted at 0.13 and 0.22 M NaCl, respectively (Fig. 1B). The first suppressive peak, which had the stronger inhibitory activity, was used for further purification. The fractions in peak I were pooled, lyophilized, dissolved in 10 mM phosphate buffer (pH 7.4), and applied to a hydroxyapatite HPLC column. SF1 factor was eluted in a major peak corresponding to a position of 0.31 M K_2PO_4 (Fig. 2). The active fractions were pooled, dialyzed against distilled water, and lyophilized. The lyophilized material was dissolved in 0.1 M KH_2PO_4 (pH 7.0) and applied to a Protein Pack 300 & 125 gel filtration HPLC column. The third protein peak eluted corresponding to a molecular mass of 13.7 kDa and contained SF1 (Fig. 3). The gel filtration HPLC column-purified fractions were then subjected to the SDS-PAGE analysis. When the pro-

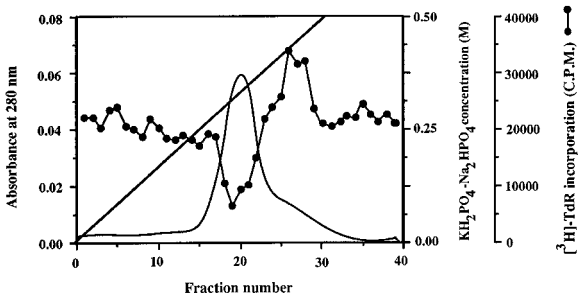

FIG. 2. Hydroxyapatite chromatography of SF1. Active fractions from the DEAE-Sepharose column were pooled, concentrated, and applied to a hydroxyapatite HPLC column equilibrated in 10 mM phosphate buffer (pH 7.4). Activity was eluted with a linear K_2PO_4 gradient (0.01 to 0.5 M). The profile of optical density at 280 nm (line) is indicated. Alternate fractions were assayed for their ability to inhibit lymphocyte proliferation in response to ConA (●).

tein was eluted from the gel and examined for inhibitory activity, suppression of ConA-induced lymphocyte blastogenesis was only seen with the fraction containing a 14-kDa protein (Fig. 4). No suppressor activity was evident in other fractions. SDS-PAGE analysis of the fraction containing the suppressive factor revealed a single band corresponding to a molecular mass of 14 kDa (Fig. 4) that was designated SF1. The purification procedure is shown in Table 1 and demonstrates a 133-fold purification.

Characterization of SF1. With heat treatment of SF1 at 100°C for 10 min, SF1 still maintained 95% of its suppressive activity. On the other hand, treatment with trypsin (10,000 U/ml) and pronase K (1 μg/ml) at 37°C for 2 h destroyed over 85% of the suppressive activity. After incubation of splenic T cells with SF1 for 21 h, 85% of the cells maintained viability. Repeated (three or four times) freezing and thawing of SF1 in PBS diminished the suppressive activity (data not shown). The N′-terminal amino acid sequence of the 14-kDa polypeptide is shown in Fig. 5. This sequence was compared by the Genetics Computer Group program with all known protein sequences in the PIR43 and Swiss-Prot databases. Preliminary homology analysis revealed that proteins containing an identical or similar sequence have never been reported.

Inhibitory effects of SF1 on T-cell proliferation responses. As shown in Fig. 6, SF1 was capable of inhibiting ConA-induced T-cell [^{3}H]TdR incorporation. The response to ConA was inhibited in a dose-dependent fashion. When a different concentration of SF1 (0.06 to 8.5 μg/ml) was examined for suppressor activity, inhibition of ConA-induced T-cell proliferation was noted at as little as 0.2 μg of SF1 per ml. Complete inhibition of ConA-induced T-cell responses was observed at the dose of 3.7 μg of SF1 per ml. A dose of approximately 0.4 μg of SF1 per ml resulted in 50% inhibition of [^{3}H]TdR incorporation of ConA-induced T cells.

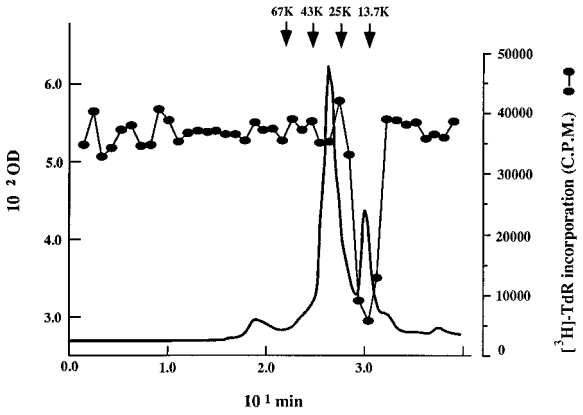

FIG. 3. Gel filtration chromatography of SF1. Peak fractions from the hydroxyapatite HPLC column were pooled, concentrated, and applied to a Protein Pack 300 & 125 column equilibrated in 0.1 M KH_2PO_4 buffer (pH 7.0). Molecular mass was calibrated by using standard markers (bovine serum albumin as 67 kDa [67K], ovalbumin as 43 kDa [43K], chymotrypsin as 24.5 kDa [25K], and RNase A as 13.7 kDa [13.7K]). OD, optical density.

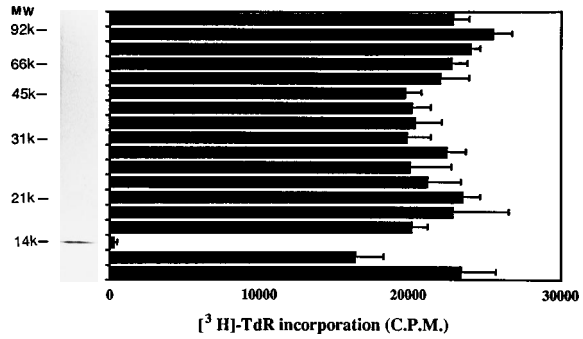

FIG. 4. Lymphoproliferative response of fractions that had been separated by SDS-PAGE. Gel filtration chromatography-purified SF1 was subjected to SDS-PAGE in a 10% polyacrylamide gel and electrophoresed in duplicate. One gel lane was stained by the silver staining method. The other gel lane, after being soaked in 75% ethanol at 25°C overnight and rehydrated in PBS for 1 h, was then sliced into 18 sections. Fractions of SF1 were extracted from gel sections and assayed for their activity to inhibit lymphocyte proliferation of C3H/HeN splenic T-cell cultures incubated with 0.5 μg of ConA. Significant suppression was observed only when the fraction with a molecular mass (MW) near 14 kDa was employed ($P < 0.01$).

Effect of SF1 on cytokine production. Since SF1 inhibited ConA-induced T-cell proliferation, it was of interest to determine if SF1 affected cytokine production by helper T cells. Splenic $CD4^+$ T cells were stimulated in vitro with ConA in the presence or absence of various doses of SF1 and examined for IL-2, IFN-γ, IL-4, and IL-5 production. SF1 caused a dose-dependent reduction of IL-2, IFN-γ, IL-4, and IL-5 synthesis (Table 2). IL-2 production and IL-5 production were inhibited at 0.1 μg or more of SF1 per ml, whereas IFN-γ production and IL-4 production were inhibited at 0.5 μg or more of SF1 per ml. Over 50% inhibition of cytokine production was seen in cultures incubated with 1.0 μg of SF1 per ml. These results indicated that SF1 suppresses cytokine production from mouse splenic Th1 and Th2 cell populations.

DISCUSSION

We have previously shown that SE from *A. actinomycetemcomitans* inhibited T-cell-dependent in vivo antibody responses from C3H/HeN mouse spleen cells in a dose-dependent manner (11). This inhibitory mechanism for Ig synthesis was, in

TABLE 1. Purification of SF1 from *A. actinomycetemcomitans* Y4

Fraction	Protein (mg)	Total SF1 activity (U)[a]	Sp act (U/mg)	Purification (fold)
Cytoplasmic soluble fraction	1,400	28,000	20	1
Q-Sepharose	45	13,000	289	14.5
DEAE-Sepharose	15	6,438	429	21.5
Hydroxyapatite column	0.135	120	889	44.5
Protein 300 & 125 column	0.003	8	2,667	133.3

[a] One unit is defined as the reciprocal of the highest dilution resulting in 50% inhibition of ConA-induced spleen cell proliferation.

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1     5     10    15    20
S E V L H S S D A T F V A (D) V L N S E V P V -

S: Ser,  E: Glu,  V: Val,  L: Leu,  H: His,
D: Asp,  A: Ala,  T: Thr,  F: Phe,  P: Pro
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FIG. 5. N′-terminal amino acid sequence of 14-kDa protein purified from *A. actinomycetemcomitans* Y4.

part, explained by a suppression of cytokine production by the splenic T cells stimulated with ConA (8). To further investigate the inhibitory activity of *A. actinomycetemcomitans*-derived SE at both biochemical and immunological levels, the suppressive factor was further purified from the cytoplasmic soluble fraction of crude SE by extensive column chromatography. The suppressive molecule eluted as a broad peak from Q-Sepharose (Fig. 1A) and was further separated into two fractions, I and II, by a DEAE-Sepharose Fast Flow column (Fig. 1B). Although both fractions inhibited ConA-stimulated T-cell proliferation, the suppressive potential of peak I was stronger than that of peak II. Peak I showed no mitogen activities for splenic T and B cells, while peak II exhibited B-cell mitogenic activity (data not shown). These results indicate that *A. actinomycetemcomitans* produces at least two substances with different immune modulating characteristics.

SF1 was further purified through hydroxyapatite HPLC and protein pack 300 & 125 gel filtration HPLC from fraction I on a DEAE-Sepharose Fast Flow column. The SDS-PAGE analysis of SF1 obtained after gel filtration HPLC resulted in a single band with a molecular mass of 14 kDa, where the antiproliferative activity could be detected. Biochemical characterization of SF1 indicates that it is heat-resistant, noncytotoxic, and sensitive to protease treatments. However, it is resistant to treatment with either neuraminidase (4 U/ml), phospholipase A_2 (10 U/ml), or phospholipase C (4 U/ml). Furthermore, it does not contain a carbohydrate molecule (data not shown). Finally, only a small amount of protein, a recovery of 0.03% of the total SF1 activity in our starting sample with only a 133-fold increase in specific activity, was obtained because of the instability of SF1 under repeated freezing and thawing conditions and during dialysis. For these reasons, it is possible that SF1 itself being labile self-degrades or that SF1 is degraded to a small molecule by the coexisting protease during the purification steps, which then results in SF1 passing through the membrane in dialysis. Homology searches of N′-terminal amino acid sequence data revealed that the SF1 molecule does not contain a sequence identical to or similar to any that has ever been reported, suggesting that SF1 is a novel bacterial product.

Other studies have described a component of *A. actinomycetemcomitans* which exhibited immunosuppression against lymphocytes (15). This ISF inhibited mitogen-induced T-cell proliferation and Ig production, and SDS-PAGE analysis revealed a single protein band corresponding to approximately 60 kDa (17). Although SF1 possesses similar properties to the ISF, such as the ability to inhibit the growth of T lymphocytes and the ability to suppress Ig production, these molecules differ in molecular weight, effect on cytokine production by mouse splenic T cells, and biochemical properties. Besides SF1 and ISF, leukotoxin and lipopolysaccharide (LPS) of *A. actinomycetemcomitans* have also been shown to be immunosuppressive agents. Leukotoxin, with a molecular mass of 115 kDa, generally kills human polymorphonuclear cells and monocytes but not lymphocytes. However, by subverting monocytes, it inhibits human peripheral blood lymphocyte responsiveness to mitogens and antigens (12). LPS suppresses the primary immune response to T-dependent antigen by activating suppressor B cells, not inducing suppressor macrophages or suppressor T cells (6), and it also suppresses antigen- and mitogen-induced human T-cell proliferation by inducing the release of prostaglandin E_2 from activated macrophages (4). So far, whether SF1 also directly affects monocytes, macrophages, or B lymphocytes besides T lymphocytes remains unknown. In any case, SF1 is a novel product distinct from leukotoxin and LPS. We did not recognize any contaminants such as leukotoxin or LPS in the purified 14-kDa SF1 as shown in Fig. 4 or by sensitive silver staining for LPS (22). Furthermore, when we calculated the content of LPS in the purified SF1 with a chromogenic *Limulus* amoebocyte lysate assay kit, the LPS content in the protein was negligible (less than 10^{-6} [data not shown]). Therefore, *A. actinomycetemcomitans* produces multiple ISFs, including SF1, and it is likely that the immunosuppressive activities of *A. actinomycetemcomitans* are increased by the synergistic action of these factors.

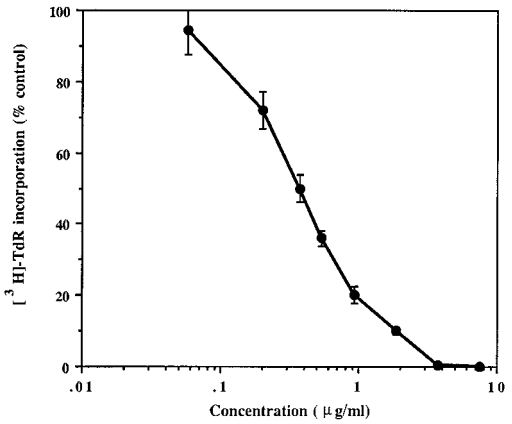

FIG. 6. Effect of SF1 on mitogenic activity. C3H/HeN splenic T cells (7.0×10^5 per well) were cultured with SF1 (●) at the indicated concentrations and 0.5 μg of ConA. [^{3}H]TdR incorporation was measured after incubation for 72 h. The results are plotted as the percentage of [^{3}H]TdR incorporation in cultures receiving ConA alone. The values are expressed as means ± standard errors with triplicate cultures. Net incorporation of [^{3}H]TdR in cells receiving ConA alone averaged 28,450 cpm; incorporation in control cultures (no mitogen) averaged 196 cpm.

A. actinomycetemcomitans is also known to produce virulence factors which affect the growth and/or metabolism of cell types other than lymphocytes (19). Shenker et al. (16) reported the 150-kDa inhibitory factor which suppresses human and murine fibroblast proliferation in vitro. Preliminary study indicated that it is heat labile and distinct from leukotoxin, en-

TABLE 2. Effect of SF1 on cytokine production by CD4$^+$ T cells[a]

SF1 concn (μg/ml)	Production of:			
	IL-2 (U/ml)	IFN-γ (pg/ml)	IL-4 (pg/ml)	IL-5 (pg/ml)
0	47 ± 6	2,900 ± 100	220 ± 35	200 ± 24
0.1	32 ± 3	2,800 ± 120	180 ± 22	110 ± 18[b]
0.5	18 ± 4[b]	1,050 ± 95[c]	130 ± 18	80 ± 12[b]
1.0	14 ± 2[c]	530 ± 60[c]	44 ± 5[c]	60 ± 5[c]

[a] Splenic CD4$^+$ T cells (4.0×10^6 per well) were cultured with various concentrations of SF1 in the presence of 2.5 μg of ConA for 24 h (IL-2, IFN-γ, and IL-4) or 48 h (IL-5), and the supernatants were assayed for cytokine activity as described in Materials and Methods. The data represent the means ± standard errors from three different experiments with triplicate cultures. In the absence of ConA and SF1, each cytokine activity was 2.5 U/ml for IL-2, 0 pg/ml for IL-4, and 10 pg/ml for IL-5.

[b] Significantly different from control at $P < 0.05$.

[c] Significantly different from control at $P < 0.01$.

dotoxin, and ISF. Kataoka et al. (5) also demonstrated that a factor (65 kDa) showing inhibitory activity against human gingival fibroblasts was extracted from the cytosol fraction of *A. actinomycetemcomitans* Y4. These factors had the ability to suppress DNA synthesis without affecting cell viability. Most recently, White et al. (23) described an 8-kDa antiproliferative component from *A. actinomycetemcomitans* which potently inhibits [^{3}H]TdR incorporation in numerous cell types, including fibroblasts, monocytes, and osteoblasts (10). This component is heat labile, trypsin sensitive, and noncytotoxic. The 8-kDa active fractions reveal two major proteins with low molecular masses and two other minor proteins when separated by SDS-PAGE. It is uncertain whether our 14-kDa SF1 also affects the growth and/or metabolism of other cell types such as fibroblasts, osteoblasts, and endothelial cells. So far, it is difficult to determine if we have identified a new factor or if we have characterized some previously reported suppressive factor, because we could obtain no information about the precise chemical and molecular nature of those factors. It is not considered that SF1 is a degradation product of the inhibitory factors as described above, because when we subjected the SE from *A. actinomycetemcomitans* to the SDS-PAGE analysis and examined it for inhibitory activity, we could see suppressive activity with the fraction containing the 14-kDa protein (data not shown). Although SF1 is fairly similar to the antiproliferative component described by White et al. in their biochemical properties such as trypsin sensitivity, cytotoxicity, and SDS-PAGE profile, but not for heat sensitivity, it is difficult to conclude that SF1 is identical to the component, because it was not completely purified, and it is uncertain whether antiproliferative activity exists among the protein bands separated by SDS-PAGE. It is conceivable that they have isolated a small fraction of the total SF1 activity because they likely monitored a degradation product that remained biologically active. Therefore, SF1 is a unique factor which we have purified chemically and molecularly. Actually, whether the suppressive factors described above contribute to periodontal disease remains unknown. However, the ability of these factors to modulate immunological responsiveness by means of inhibition of cell growth and cytokine production is an established phenomenon. Therefore, it can be speculated that impaired host defense mechanisms may contribute to the disease process.

In summary, an ISF, SF1, has been purified from *A. actinomycetemcomitans* Y4. SF1 is a 14-kDa protein which inhibits T-cell proliferation and production of Th1 (IL-2 and IFN-γ) and Th2 (IL-4 and IL-5) cytokines by ConA-stimulated splenic T cells. Therefore, this molecule could affect the induction of humoral and/or cell-mediated immune responses through the modulation of the T-cell responses, including cytokine production. Studies are currently under way to elucidate the exact nature of another ISF, SF2. The exact role of SF-induced suppression of cytokine production by $CD4^+$ and $CD8^+$ T cells at periodontal disease sites requires further investigation. The physicochemical and biological characteristics of SFs should be further explored to see whether SF is widely distributed in periodontopathic bacteria.

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