INFECTION AND IMMUNITY, Dec. 1993, p. 5252–5260
0019-9567/93/125252-09$02.00/0

Vol. 61, No. 12

Cytokine Induction by Extracellular Products of Oral Viridans Group Streptococci

HARUHIKO TAKADA,[1]* YOSHIHIRO KAWABATA,[1] MASATO TAMURA,[1] KENJI MATSUSHITA,[1] HIDEO IGARASHI,[2] HISASHI OHKUNI,[3] YUKO TODOME,[3] TAKEHIKO UCHIYAMA,[4] AND SHOZO KOTANI[5]

Department of Microbiology and Immunology, Kagoshima University Dental School, 8-35-1 Sakuragaoka, Kagoshima 890,[1] Department of Microbiology, Tokyo Metropolitan Research Laboratory of Public Health, Sinjuku-ku, Tokyo 169,[2] Division of Immunology, Institute of Gerontology, Nippon Medical School, Nakahara-ku, Kawasaki 211,[3] Department of Microbiology, Tokyo Women's Medical College, Sinjuku-ku, Tokyo 162,[4] and Osaka College of Medical Technology, Kita-ku, Osaka 530,[5] Japan

Received 19 April 1993/Returned for modification 21 May 1993/Accepted 20 September 1993

During an etiological study of Kawasaki disease (mucocutaneous lymph node syndrome [MCLS]), we found that dominant viridans streptococcal strains on tooth surfaces and in the throat of both MCLS patients and non-MCLS control children formed erythrogenic and biologically active, extracellular products. In this study, we demonstrated that erythrogenic culture supernatant concentrates of representative strains (two *Streptococcus mitis* and two *Streptococcus oralis*), when injected intravenously, induced serum tumor necrosis factor alpha, interleukin-6 (IL-6), and gamma interferon in muramyldipeptide- or *Propionibacterium acnes*-primed C3H/HeN mice. The concentrates also induced tumor necrosis factor alpha, IL-6, and thymocyte-activating factor (essentially IL-1) in murine peritoneal macrophage, human monocyte, and human whole-blood cultures. An erythrogenic, heat-labile extracellular protein fraction (F-1) that was concentrated from the culture supernatants of a representative *S. mitis* strain exhibited the above-mentioned cytokine-inducing activity. This partially purified F-1 fraction also induced thymocyte-activating factor and IL-6 in human umbilical vascular endothelial cell and gingival fibroblast cultures.

Beta-hemolytic *Streptococcus pyogenes* produces extracellular, biologically active proteins, such as the erythrogenic, pyrogenic, and superantigenic exotoxins SPE-A, -B, and -C (1, 7, 10). However, little is known about the exotoxin of the viridans group streptococci, which are dominant in the oral cavity, except for a preliminary report on a heat-labile permeability factor produced by *Streptococcus sanguis* (23).

We have been studying the etiology of Kawasaki disease (mucocutaneous lymph node syndrome [MCLS]) on the basis of the working hypothesis that oral or throat bacteria, which have the potency to produce exotoxins capable of stimulating immunocompetent cells to produce inflammatory cytokines, may be involved in its pathogenesis. This was in view of the fact that many investigators have demonstrated important roles of cytokines in the manifestation of clinical and pathological signs and laboratory abnormalities characteristic of MCLS (4, 13–16, 18, 22).

Ohkuni et al. (21) showed by means of DNA-DNA homology that the dominant bacterial species isolated from the tooth surfaces or throat of both MCLS patients and age-matched non-MCLS control or healthy children were *Streptococcus mitis* and *Streptococcus oralis* (beta-hemolytic *S. pyogenes* was not isolated from either MCLS or non-MCLS control children). Most of these viridans streptococci produced extracellular factors that increased capillary permeability at the early stage of the reaction and then redness with swelling and bleeding in the later stages in rabbit skin.

In this study, we examined the cytokine-inducing activity of concentrated and partially purified culture supernatants from four representative viridans streptococci isolated from the tooth surfaces of MCLS and non-MCLS control children.

MATERIALS AND METHODS

Preparation of erythrogenic toxin fractions. Two *S. mitis* and two *S. oralis* strains were selected, on the basis of a pilot study, as representatives of clinical isolates from MCLS and control children (Table 1). These four strains were grown in 4% NZ-Amine A medium (Humko Sheffield Chemical, Lyndhurst, N.J.) supplemented with 0.2% glucose at 35°C for 48 h. Culture supernatants were passed through a Millipore filter (pore size, 0.45 μm). In one representative experiment, the filtrate (4.3 liters; pH 5.7) was diluted with 15 liters of distilled water, and the pH was adjusted to 4.6. The diluted culture supernatant was mixed with 280 g (wet weight) of SP-Sephadex C-25 (Pharmacia, Uppsala, Sweden) preequilibrated with 0.01 M citrate-phosphate buffer (CPB) (pH 4.6) and agitated at 20°C for 3 h. The resin washed with CPB was poured into a column (5.0 by 9.0 cm), and ion-exchange chromatography was carried out by gradient elution with 0.01 M CPB (pH 4.6) (1.5 liters) and 0.05 M CPB (pH 6.7) (1.5 liters) at a flow rate of 100 ml/h. Coincident with an abrupt increase in the pH of the eluate, the fraction that absorbed UV at 280 nm was eluted as a peak (Fig. 1A). UV-absorbing material was similarly eluted during ion-exchange chromatography of noninoculated NZ-Amine A medium, but at a far lower concentration (Fig. 1B). Each of the pooled peak UV-absorbing fractions from the test culture supernatants and the noninoculated medium was concentrated by dialysis against Aquacide II A (a sodium salt of carboxymethyl cellulose [molecular weight, 500,000; Calbiochem Corp., La Jolla, Calif.) and passed through a Millipore filter (0.45-μm-pore size). The degrees of concentration

* Corresponding author.

TABLE 1. Background of test streptococcal strains

Strain	Subject			Source	Species, as determined by DNA-DNA homology[a]
	Age	Sex	Diagnosis		
18	1 yr 3 mo	Male	MCLS (fourth day)	Tooth surface	*S. oralis*
27	1 yr 9 mo	Male	Upper respiratory infection	Tooth surface	*S. oralis*
82	3 mo	Male	MCLS (sixth day)	Tooth surface	*S. mitis*
108	1 yr 3 mo	Male	Aseptic meningitis	Tooth surface	*S. mitis*

[a] See reference 21.

achieved were similar between the test and noninoculated medium control specimens in terms of the ratios of the volume of the starting medium to the volume of the final concentrate, but the protein content of the control specimens prepared from noninoculated medium was 3 to 5% of that of test culture supernatant specimens.

The concentrated culture supernatants of the four bacterial strains were erythrogenic in rabbit skin. Intradermal injection (0.1 ml containing 110 to 210 μg of protein) on the delipidated back skin of Japanese domestic white rabbits (male, weighing about 2.5 kg) caused increased capillary permeability after 1 to 3 h. This was followed by redness, swelling, and bleeding from a few hours to 4 days. The reference fraction (containing 6 μg of protein) prepared from noninoculated medium did not induce any notable skin reactions. A colorimetric *Limulus* test (Endospecy test; Seikagaku Co. Ltd., Tokyo, Japan) (20) showed that contamination of the culture supernatant concentrates (the starting material in the following fractionation experiments) with extraneous, endotoxic lipopolysaccharide (LPS) was at most 0.009% in terms of the reference *Escherichia coli* (O111:B4) LPS equivalent content/protein content (weight ratio).

The erythrogenic culture supernatant concentrate from one of the four test strains (strain 108; Table 1) was further fractionated. An erythrogenic concentrate derived from 15 liters of culture supernatant was desalted by chromatography on SP-Sephadex C-25 and then eluted through a Sephadex G-25 (Pharmacia) column (2.5 by 45 cm) preequilibrated with 0.025 M Tris-acetic acid (pH 8.3) by use of the same buffer. The eluate was applied to a chromatofocusing PBE94 (Pharmacia) column (1.0 by 67 cm) preequilibrated with 0.025 M Tris-acetic acid buffer and eluted with 600 ml of a mixture of Polybuffer 96 (30%) and Polybuffer 74 (70%) (both from Pharmacia) (pH 5.0) and then with a gradient of 0 to 1.0 M NaCl (400 ml). The pooled protein fractions obtained by NaCl gradient elution were applied to a Sephacryl S-200 Superfine (Pharmacia) column (1.5 by 85 cm) and eluted with 0.1 M NH_4HCO_3 at a flow rate of 20 ml/h. Two peak fractions were separated as shown in Fig. 2, and the fractions near peaks F-1 and F-2 were pooled. The protein contents of the pooled F-1 and F-2 specimens were 0.72 and 1.14 mg/ml, respectively.

Each of these pooled fractions was heated at 100°C for 3

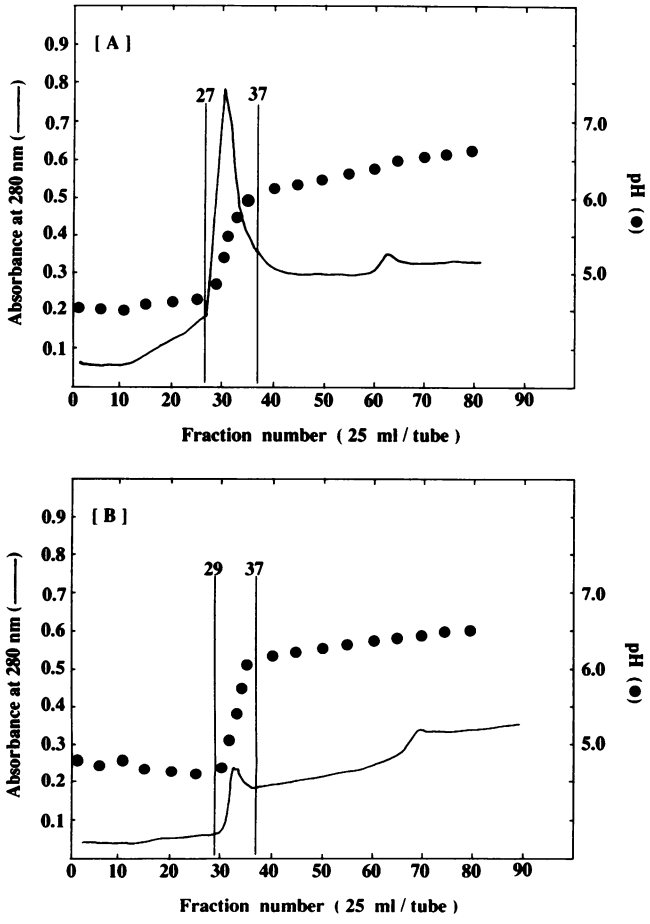

FIG. 1. Elution profiles of the culture supernatant of *S. mitis* 108 (A) and a noninoculated NZ-Amine A broth control (B) in SP-Sephadex C-25 ion-exchange chromatography.

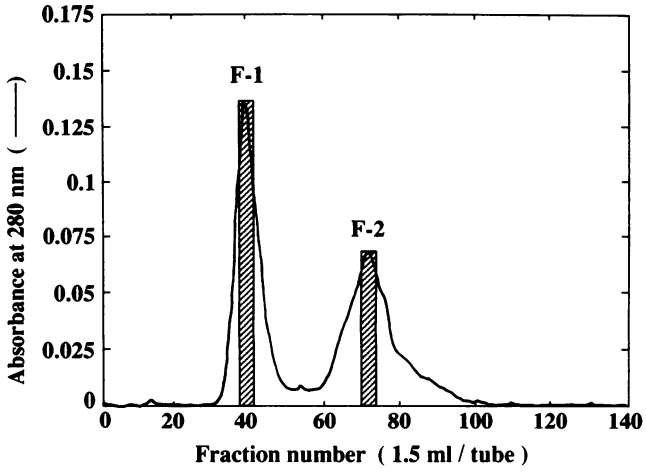

FIG. 2. Gel filtration on a Sephacryl S-200 Superfine column. Protein fractions were obtained from *S. mitis* 108 by NaCl gradient elution on a chromatofocusing PBE94 column. Hatched areas were pooled to form fractions F-1 and F-2.

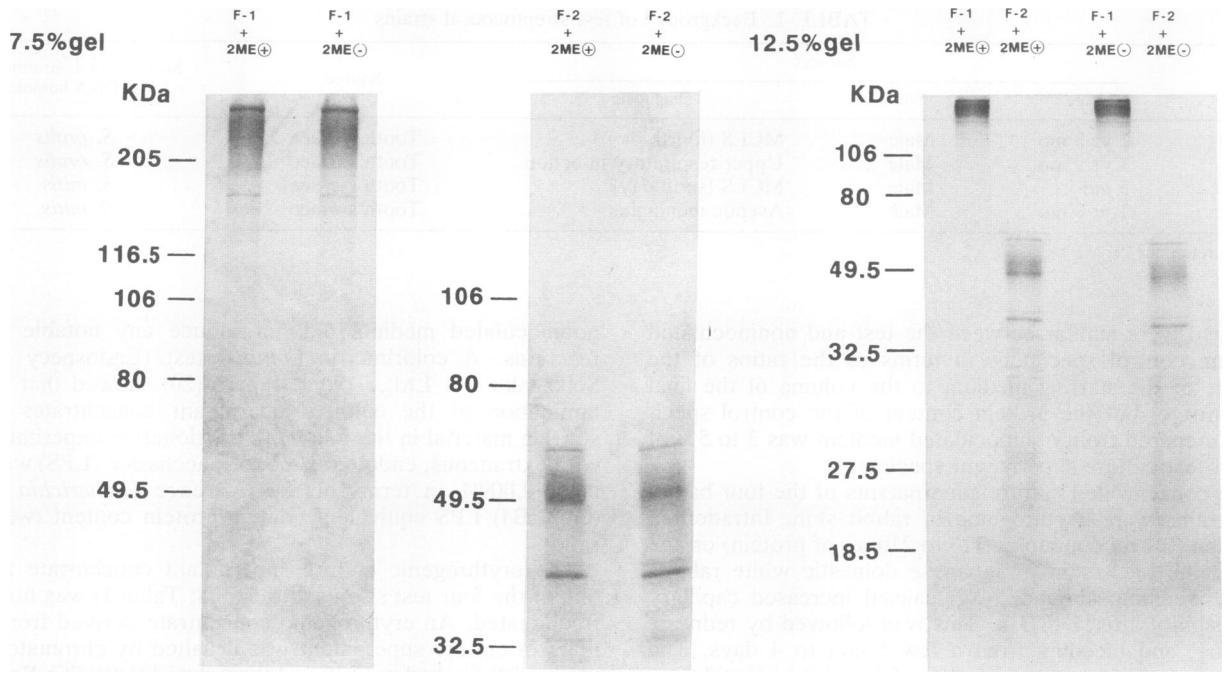

FIG. 3. SDS-PAGE profiles of fractions F-1 and F-2 from *S. mitis* 108. The fractions were mixed with an equal volume of sample buffer with or without 2-mercaptoethanol and heated at 100°C for 3 min, and then 10-μl aliquots (containing 3.6 and 5.7 μg of protein for F-1 and F-2, respectively) were subjected to SDS-PAGE with 7.5 and 12.5% acrylamide gels (1.0-mm thick) and visualized with Coomassie brilliant blue. Myosin (205 kDa), β-galactosidase (116.5 kDa), bovine serum albumin (80 kDa), ovalbumin (49.5 kDa), carbonic anhydrase (32.5 kDa), soybean trypsin inhibitor (27.5 kDa), and lysozyme (18.5 kDa) (Bio-Rad) were used as molecular mass markers.

min with or without 2-mercaptoethanol and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5 and 12.5% acrylamide gels (12). As shown in Fig. 3, the F-1 fraction showed several bands with a molecular mass above 150 kDa and a broad major band that was larger than 200 kDa. The F-2 fraction showed a profile quite different from that of F-1. It consisted of several components with molecular masses below 60 kDa. No effects of 2-mercaptoethanol were noted in the SDS-PAGE profile of either fraction. In conventional double immunodiffusion in an agar gel (Fig. 4), fraction F-1 showed one clear and two faint precipitin bands in a reaction with a rabbit antiserum raised against the concentrated culture supernatant of strain 108, while fraction F-2 showed a single distinct precipitin band. The culture concentrate used for rabbit immunization showed three or four precipitin bands in a reaction with the test antiserum. Fraction F-1 at a dose of 12 μg of protein per site caused increased capillary permeability, followed by redness with swelling and bleeding in the rabbit skin. Fraction F-2 at a dose of 7 μg of protein per site also caused increased capillary permeability but did not induce redness with swelling and bleeding, like streptococcal pyrogenic exotoxins did (Table 2).

To generally characterize the bioactive principle, fraction F-1 was first treated with KuttsuClean (Taiyo Fishery Co., Ltd., Tsukuba, Japan), which are affinity beads coated with an endotoxin-binding cationic peptide (Tachyplesin) from horseshoe crab, five times to remove any extraneous LPS. An aliquot of KuttsuClean-treated F-1 was heated in 0.1 M NH_4HCO_3 (pH 8.3) at 100°C for 30 min. Another was digested with a protease attached to carboxymethyl cellulose (P-0387; Sigma) at a concentration of 10 U/ml at 37°C overnight. The ability of the heated or digested F-1 specimen to induce tumor necrosis factor alpha (TNF-α) production in human peripheral blood cell cultures was determined in comparison with that of nontreated F-1 as described below.

Other reagents and cell lines. *N*-Acetylmuramyl-L-alanyl-D-isoglutamine, muramyl dipeptide (MDP), was a gift from the Daiichi Pharmaceutical Co. Ltd., Tokyo, Japan. Formalin-killed *Propionibacterium acnes* was supplied by the Tokyo Institute for Immunopharmacology, Tokyo, Japan.

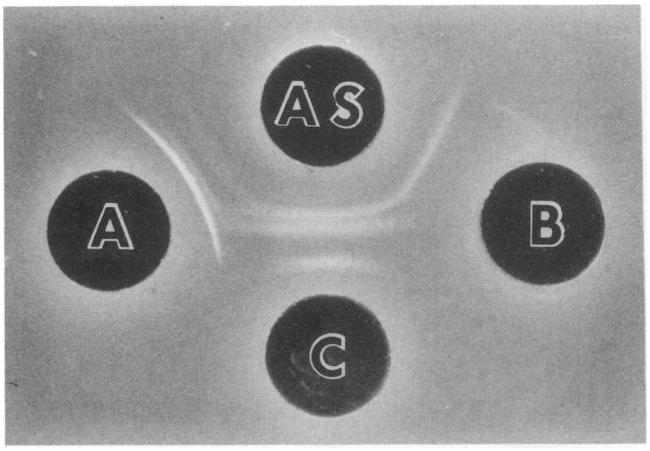

FIG. 4. Conventional double immunodiffusion in an agar gel. Wells: A, fraction F-1 (7 μg of protein equivalents per well); B, fraction F-2 (5 μg per well); C, erythrogenic concentrate of the culture supernatant from *S. mitis* 108, with which a rabbit was immunized (100 μg per well); AS, antiserum (undiluted) from a rabbit immunized with the above-mentioned concentrate.

TABLE 2. Rabbit skin reactions caused by fractions F-1 and F-2 isolated from culture supernatants of *S. mitis* 108 compared with reference specimens

Test specimen	Dose[a]	Reaction[b]	Dimension of the reaction (diam, mm × mm) at the following time after intradermal injection:						
			5 min	15 min	30 min	1 h	3 h	6 h	24 h
F-1	11.8	B	10 × 12	7 × 12	12 × 12	15 × 15	0	0	0
		R	0	0	0	0	16 × 17	18 × 18	19 × 18
F-2	6.6	B	10 × 12	9 × 9	9 × 8	19 × 13	0	0	0
		R	0	0	0	0	±[c]	0	0
0.1 M phosphate buffer		B	10 × 8	6 × 4	0	0	0	0	0
		R	0	0	0	0	0	0	0
SPE-A[d]	10.0	B	7 × 5	±	±	±	0	0	0
		R	0	0	0	0	0	0	0
SPE-B[d]	10.9	B	16 × 14	10 × 16	12 × 12	12 × 12	0	0	0
		R	0	0	0	0	±	±	0
SPE-C[d]	10.3	B	7 × 10	7 × 5	±	0	0	0	0
		R	0	0	0	0	0	0	0
0.1 M phosphate buffer		B	5 × 6	0	0	0	0	0	0
		R	0	0	0	0	0	±	0

[a] Micrograms of protein per skin site.
[b] B, blueing (leakage of i.v.-injected Evans blue) due to increased capillary permeability; R, redness (with swelling and sometimes with bleeding).
[c] ±, diameter of the reaction was less than 5 mm.
[d] Prepared as described previously (10, 11).

Ultrapurified LPS prepared from *Salmonella abortus-equi* (5) was a gift from C. Galanos, Max-Planck-Institut für Immunbiologie, Freiburg, Germany. Phytohemagglutinin (PHA; HA-16) was purchased from Wellcome Diagnostics, Temple Hill, United Kingdom. Recombinant human TNF-α (rHuTNF-α) (25) and recombinant human interleukin-6 (rHuIL-6) (8, 9) were gifts from the Dainippon Pharmaceutical Co. Ltd., Osaka, Japan, and T. Hirano, Osaka University, Osaka, Japan, respectively. The anti-murine TNF-α rabbit antibody was supplied by Suntory Inc., Osaka, Japan. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Wako Pure Chemicals, Osaka, Japan. [^{125}I]Deoxyuridine was obtained from Dupont, NEN Research Products, Boston, Mass.

Human endothelial cells isolated from umbilical blood vessels were a gift from J. Mihara, Osaka University Faculty of Dentistry. GF-5 human gingival fibroblast cells were obtained as described previously (24). L929 cells (NCTC clone 929; ATCC CCL-1) were purchased from Dainippon Pharmaceutical Co. Interleukin-6 (IL-6)-dependent cell line MH60,BSF2 (17) was a gift from T. Kishimoto, Osaka University.

Cytokine induction in mice. To prepare test sera for TNF-α and IL-6 assays, groups of three male C3H/HeN mice (7 to 12 weeks old; Clea Japan, Osaka) were primed by an intravenous (i.v.) injection with MDP (100 μg in 0.2 ml of physiological saline). Four hours thereafter, the mice were given an i.v. injection of test specimens in 0.2 ml of saline. After 90 min, the mice were bled from an axillary vein. For the induction of gamma interferon (IFN-γ), the mice were primed by an intraperitoneal injection of formalin-killed *P. acnes* whole cells (1.2 mg [dry weight] in 0.2 ml); 7 days later, the mice received an i.v. injection of test specimens. After 4 h, the mice were bled.

Cytokine induction in murine peritoneal macrophage cultures. C3H/HeN mice were injected intraperitoneally with 1.5 ml of thioglycolate medium (Nissui Pharmaceuticals, Tokyo, Japan), and peritoneal exudate cells were collected from the mice 5 days after the irritative injection. The cells, 10^5 per 0.1 ml of RPMI 1640 medium (Nissui Pharmaceuticals) supplemented with 5% fetal calf serum (FCS; GIBCO Laboratories, Grand Island, N.Y.) per well of flat-bottom microculture plates (type 25860; Corning Glass Works, Corning, N.Y.), were incubated in a CO_2 incubator at 37°C for 2 h. Monolayer cultures were washed three times with RPMI 1640 medium to remove nonadherent cells. The culture supernatant was then replaced with fresh RPMI 1640 medium with or without test material, and the samples were cultured in triplicate for 24 h. The plates were centrifuged at 800 × *g* for 10 min. The triplicate supernatants for each test point were pooled as cell-free specimens to determine the levels of TNF-α, IL-6, and cell-free thymocyte-activating factor (TAF) activities. Residual macrophages grown as a monolayer, on the other hand, were washed carefully but vigorously three times with RPMI 1640 medium. Fresh medium (100 μl) was added to each well, and the cells were disrupted by freezing (−80°C) and thawing (25°C) three times. The triplicate suspensions were pooled as cell-associated specimens for the TAF assay.

Cytokine induction in human monocyte cultures. Human mononuclear cells were obtained from the heparinized peripheral blood of a healthy male adult volunteer by Ficoll-Hypaque centrifugation. The cells were incubated at a density of 5 × 10^5 cells per 0.1 ml of RPMI 1640 medium supplemented with 5% FCS in microculture plate wells for 2 h and then washed with RPMI 1640 medium to remove nonadherent cells. Procedures similar to those described for the murine macrophage cultures resulted in cell-free specimens for TNF-α, IL-6, and cell-free TAF measurements. Cell-associated specimens could not be prepared for this assay, because human monocytes easily detached from the culture plates during washing.

Cytokine induction in human peripheral whole-blood cultures. Heparinized peripheral blood (25 μl) was directly (without fractionation) incubated with test specimens in RPMI 1640 medium (100 μl) for 24 h. Triplicate culture supernatants were pooled as cell-free specimens for TNF-α, IL-6, IFN-γ, and TAF assays.

Cytokine induction in HUVEC and fibroblast cultures. Cell-free fractions were prepared from human umbilical vascular endothelial cell (HUVEC) and fibroblast cultures by the same procedures, except for the culture media. HUVECs were grown in medium 199 (GIBCO) containing heparin sodium salt (0.1 mg/ml; Nakarai Chemicals, Kyoto, Japan) and endothelial cell growth supplement (Sigma). Fibroblasts were cultured in α-MEM (Flow Laboratories, McLean, Va.). Suspensions (5×10^4/100 μl) of HUVECs and fibroblasts in their respective media supplemented with 10% FCS were seeded in microculture plate wells. After 2 days at 37°C in a CO_2 incubator, monolayers were washed with media three times, and then test stimulants in 100 μl of their respective media containing 1% FCS were added. Incubation proceeded for a further 24 h. Triplicate culture supernatants were pooled as cell-free specimens for TAF, IL-6, and TNF-α assays.

TNF-α assay. TNF-α activity in test sera and cell-free specimens of the cell cultures was determined by measuring cytotoxic activity against L929 cells. These cells were cultured for 24 h at a density of 5×10^4 cells per 100 μl of RPMI 1640 medium containing 10% FCS per well and then incubated with 2.0 μg of actinomycin D (A-1410; Sigma) per ml (final concentration after the addition of a test specimen). Diluted test sera or cell-free specimens (25 μl) were added to the wells. After a further 24 h of incubation, the supernatant containing detached dead cells was discarded, and the remaining viable cells were fixed and stained with 2% formaldehyde containing 0.2% crystal violet. The intensity of staining was measured with a microplate reader (Wellreader SK601; Seikagaku Co.) at an optical density of 595 nm. The dilution of test specimens that caused 50% cytotoxicity against L929 cells was determined on the basis of a titration curve. TNF-α activity in a test specimen was calculated as nanograms per milliliter on the basis of the ratio of a 50% cytotoxic dose of the test specimen to that of standard rHuTNF-α. Thus, strictly speaking, the values given for murine sera and macrophage culture specimens are the values for rHuTNF-α. To confirm that the cytocidal activity of test specimens for L929 cells was due to TNF-α, a neutralization test was performed with representative test murine sera and an anti-murine TNF-α rabbit antibody.

IL-6 assay. The IL-6 activity of test sera and cell-free specimens was determined by measuring the in vitro growth of IL-6-dependent cell line MH60.BSF2 as described by Matsuda et al. (17), with minor modifications, by use of MTT (19). In brief, MH60.BSF2 cells (10^4/100 μl of RPMI 1640 medium with 10% FCS per well) were cultured with serially diluted test specimens (25 μl) for 48 h. During the last 4 h of culturing, 100 μg of MTT was added. The plates were centrifuged at $800 \times g$ for 10 min, and the supernatants were removed by gentle aspiration so as not to lose any cells. The cells in the culture plates were solubilized with 100 μl of dimethyl sulfoxide, and the intensity of developing color was measured with a microplate reader at an optical density of 595 nm. IL-6 activity in a test specimen was determined on the basis of a comparison with the titration curve for standard rHuIL-6 and expressed as units per milliliter.

IFN-γ assay. IFN-γ levels in murine sera and human whole-blood cultures were determined by use of mouse and human IFN-γ enzyme-linked immunosorbent assay kits (Genzyme, Boston, Mass.) and expressed as nanograms per milliliter.

TAF assay. TAF activity in test cell-free and cell-associated specimens was determined by the conventional comitogenic assay for IL-1 measurement with thymocytes from 2- to 4-week-old C3H/HeJ mice, which were originally obtained from Clea Japan and bred in the Animal Center of Kagoshima University Dental School. In brief, thymocytes (1.5×10^6/150 μl of RPMI 1640 medium containing 10% FCS in microculture plates) were cultured for 48 h with 25 μl each of test specimen and PHA to yield an eightfold-diluted test specimen and 1.0 μg of PHA per ml in the final culture. During the final 6 h of incubation, cultures were pulsed with 18.5 kBq (0.5 μCi)/ml of [^{125}I]deoxyuridine. The cells were harvested and washed, and the incorporated radioactivity was measured with a gamma counter (Auto Well Gamma System ARC-300; Aloka Co., Tokyo, Japan). Assays were performed in triplicate, and the results were expressed as the stimulation index (SI), where SI = [^{125}I]deoxyuridine uptake (counts per minute) in the presence of test stimulant and PHA/[^{125}I]deoxyuridine uptake in medium (stimulated with PHA alone).

Miscellaneous methods. All test specimens for cytokine assays were stored at −80°C until use. In some assays, on the basis of the mean ± standard error of the mean (SEM), the statistical significance of the differences between each test and its respective control was examined by Student's *t* test. In other experiments, in which calculation of the SEM was not practical, the range of fluctuations of induced cytokine levels in triplicate assays was within 20% of the respective mean.

RESULTS

Cytokine induction by concentrated erythrogenic culture supernatants. (i) Serum cytokine induction in MDP-primed mice. All four test preparations from viridans streptococcal strains induced serum TNF and IL-6 in MDP-primed C3H/HeN mice under our experimental conditions. The noninoculated culture medium, which was obtained by essentially the same chromatography and concentration procedures as the test culture supernatants, was virtually inactive (Table 3). The levels of TNF induced in serum by the four test preparations were essentially the same, while for IL-6 induction, the concentrate from strain 27 was more active. The induced TNF was found to be of the alpha type, because the cytotoxic activity of serum from the mouse that received the representative preparation of *S. mitis* 108 (160 μg of protein) for L929 cells was completely neutralized by incubation with the anti-murine TNF-α antibody, as was that of serum from the mouse injected with LPS of *S. abortus-equi* (0.8 μg) (data not shown).

(ii) Cytokine induction in murine peritoneal macrophage cultures. The test preparations stimulated murine peritoneal macrophage cultures to generate TNF-α and IL-6 activities in supernatants of cell cultures and cell-associated TAF (Table 4). TAF activity was also found in cell-free specimens, but at levels far lower than those of cell-associated activity (data not shown). The control, i.e., the noninoculated medium concentrate, exhibited only slight IL-6 activity and cell-associated TAF induction at the lowest test dilution.

(iii) Cytokine induction in human monocyte cultures. Table 5 shows that the concentrated erythrogenic culture supernatants from four test viridans streptococcal strains induced TNF-α, IL-6, and TAF (cell free) by stimulating human

TABLE 3. Induction of serum TNF and IL-6 in MDP-primed C3H/HeN mice by concentrated erythrogenic culture supernatants from viridans streptococci[a]

Source of concentrate (strain)	Dose (μg of protein/mouse)	TNF (ng/ml)	IL-6 (U/ml)
18	120	16 ± 16	31 ± 9[b]
27	110	46 ± 9	375 ± 145[c]
82	210	26 ± 6	23 ± 18
108	160	24 ± 13	24 ± 2[b]
Control[d]	6	<1	5 ± 3

[a] Groups of three C3H/HeN mice were primed with an i.v. injection of MDP (100 μg) and, 4 h later, given an i.v. injection of test materials (in 200 μl of saline). TNF and IL-6 levels in sera collected 4 h later were determined as described in the text. The results are means ± SEMs.
[b] Significantly different from the control, as determined by Student's *t* test ($P \leq 0.01$).
[c] Significantly different from the control, as determined by Student's *t* test ($P \leq 0.05$).
[d] The control specimen used here and in the experiments reported in Tables 4 to 6 was obtained from noninoculated culture medium by essentially the same procedures as those described in the text for test culture supernatants.

peripheral blood monocyte cultures. There were no significant differences among the potencies of the four strains. The control, i.e., the noninoculated medium concentrate, exhibited only slight IL-6 activity and cell-free TAF induction at the lowest test dilution.

(iv) Cytokine induction in human whole-blood cultures. Separation of the monocyte fraction from peripheral blood requires 10 to 20 ml of whole blood, but it is impractical to collect this volume of blood from children of the age at which MCLS is prevalent. We therefore examined whether cytokine induction by test preparations could be assayed with peripheral whole blood without separating the mononuclear cells. Definite induction of cell-free TNF-α, IL-6, and TAF was noted for whole-blood cultures stimulated with all four test preparations under assay conditions in which the control prepared from noninoculated medium was scarcely induced (Table 6). The same individual donated the blood for the assays presented in Tables 5 and 6, and there were no essential differences in the IL-6 responses to test preparations between the cultured fractionated monocytes and whole blood, although the former showed higher levels of TNF-α and TAF induction than the latter, probably reflecting differences in the density of target cells.

TABLE 4. Induction of cytokines in murine peritoneal macrophage cultures by concentrated erythrogenic culture supernatants from viridans streptococci

Source of concentrate (strain)	Dose (mg of protein/ml)	Dilution	CF specimen[a]		CA specimen[b] TAF (SI, mean ± SEM)[c]
			TNF-α (ng/ml)	IL-6 (U/ml)	
18	1.2	1:20	0.70	2.0	40.8 ± 1.9
		1:100	0.25	3.3	39.0 ± 2.4
		1:500	<0.01	0.5	22.0 ± 1.4
27	1.1	1:20	0.20	4.8	36.4 ± 0.7
		1:100	0.09	1.9	36.0 ± 1.2
		1:500	<0.01	0.4	9.0 ± 2.6
82	2.1	1:20	0.62	4.8	57.0 ± 0.7
		1:100	0.51	4.2	43.1 ± 1.9
		1:500	0.02	3.1	39.7 ± 1.5
108	1.6	1:20	0.62	4.8	57.0 ± 0.7
		1:100	0.51	4.2	43.1 ± 1.9
		1:500	0.02	3.1	39.7 ± 1.5
Control	0.06	1:20	<0.01	0.5	7.4 ± 0.9
		1:100	<0.01	0.4	2.0 ± 0.3
		1:500	<0.01	0.4	1.0 ± 0.2

[a] Cell culture supernatant.
[b] Suspension of disrupted adherent cells.
[c] All the test specimen SI values were significantly different from those for the control at the corresponding dilution, as determined by Student's *t* test ($P \leq 0.01$).

TABLE 5. Induction of cytokines in human monocyte cultures by concentrated erythrogenic culture supernatants from viridans streptococci

Source of concentrate (strain or LPS)	Dose (mg of protein/ml)[a]	Dilution	TNF-α (ng/ml)	IL-6 (U/ml)	TAF (SI, mean ± SEM)[b]
18	1.2	1:20	7.5	135	27.0 ± 3.1
		1:100	6.6	75	15.9 ± 1.0
		1:500	5.5	47	13.4 ± 1.5
27	1.1	1:20	13.2	150	14.3 ± 2.1
		1:100	22.8	70	12.4 ± 0.7
		1:500	13.2	45	13.7 ± 0.2
82	2.1	1:20	30.0	105	10.6 ± 1.4
		1:100	14.4	85	11.6 ± 1.5
		1:500	20.4	21	12.1 ± 1.1
108	1.6	1:20	10.8	80	20.1 ± 1.0
		1:100	7.5	125	13.3 ± 0.2
		1:500	7.2	46	12.0 ± 0.4
Control	0.06	1:20	3.1	18	5.9 ± 0.3
		1:100	0.2	<1	0.8 ± 0.0
		1:500	<0.1	<1	0.9 ± 0.2
LPS	10		14.4	30	12.2 ± 1.1
	1		14.4	25	13.6 ± 1.5
	0.1		20.4	20	8.1 ± 0.9
None			<0.1	<1	1.0 ± 0.1

[a] The dose of LPS (from *S. abortus-equi*; see also Tables 6 to 8) was given in micrograms per milliliter (net weight per milliliter).
[b] All the test specimen and reference LPS SI values were significantly different from those for the noninoculated medium control at the corresponding dilution and the cell culture medium (none) control, respectively, as determined by Student's *t* test ($P \leq 0.01$).

Cytokine induction by partially purified fractions F-1 and F-2. (i) Cytokine induction in primed mice. As shown in Table 7, the erythrogenic F-1 fraction induced high levels of serum TNF-α and IFN-γ in MDP- and *P. acnes*-primed C3H/HeN mice, respectively. The potency of F-1 in TNF-α and IFN-γ induction was not very different from that of LPS in terms of the levels of cytokines induced, though the test dose of F-1 was more than 10 times higher than that of LPS. Fraction F-2, which increased the capillary permeability of rabbit skin but was devoid of erythrogenicity, was only weakly active in the TNF-α and IFN-γ assays. Both fraction F-1 and fraction F-2 induced much less IL-6 than reference LPS.

(ii) Cytokine induction in human whole-blood cultures. Fraction F-1 definitely induced TNF-α, IL-6, IFN-γ, and TAF in supernatants of human whole-blood cultures (Table 8). The potency of fraction F-2 in inducing cytokines other

TABLE 6. Induction of cytokines in human whole-blood cultures by concentrated erythrogenic culture supernatants from viridans streptococci

Source of concentrate (strain or LPS)	Dose (mg of protein/ml)[a]	Dilution	TNF-α (ng/ml)	IL-6 (U/ml)	TAF (SI, mean ± SEM)[b]
18	1.2	1:20	1.4	106	7.1 ± 0.5
		1:100	0.6	133	12.3 ± 0.4
		1:500	<0.07	69	2.4 ± 0.4
27	1.1	1:20	8.7	125	8.0 ± 0.3
		1:100	2.7	135	10.2 ± 0.7
		1:500	<0.07	73	3.7 ± 0.4
82	2.1	1:20	4.2	156	7.9 ± 0.4
		1:100	1.3	94	11.1 ± 0.4
		1:500	<0.07	39	3.3 ± 0.2
108	1.6	1:20	1.5	138	4.6 ± 0.3
		1:100	1.3	150	7.9 ± 0.4
		1:500	<0.07	94	6.4 ± 0.2
Control	0.06	1:20	<0.07	12	1.6 ± 0.1
		1:100	<0.07	0.09	0.9 ± 0.0
		1:500	<0.07	0.08	1.0 ± 0.1
LPS	10		2.1	136	9.5 ± 0.6
	1		1.5	125	11.0 ± 1.1
	0.1		1.9	98	10.0 ± 0.4
None			<0.07	<0.05	1.0 ± 0.1

[a] See Table 5, footnote *a*.
[b] See Table 5, footnote *b*.

than TAF seemed to be generally lower than that of fraction F-1 in these assays. No difference was noted for cell-free TAF induction between these two test fractions.

Treatment of fraction F-1 with KuttsuClean to exclude the possible involvement of extraneous endotoxin did not affect the TNF-α-inducing ability of the fraction in human whole-blood cultures (Table 9). Heating at 100°C for 30 min and protease treatment, on the other hand, almost completely abolished the TNF-α-inducing activity of fraction F-1, indicating that the active component is a heat-labile protein.

(iii) Cytokine induction in HUVEC and human gingival fibroblast cultures. As shown in Fig. 5, fraction F-1 induced significant TAF activity in culture supernatants of HUVEC and human gingival fibroblast cultures, whereas fraction F-2 was scarcely active. The potency of fraction F-1 in HUVEC was comparable to that of *S. abortus-equi* LPS with regard to the extent of TAF induction. Figure 6 shows that both fraction F-1 and fraction F-2 significantly stimulated both HUVEC and fibroblast cultures to produce IL-6. Fraction F-2 seemed to be more active than fraction F-1 in this assay.

TABLE 7. Induction of serum cytokines in MDP- and *P. acnes*-primed C3H/HeN mice by partially purified fractions F-1 and F-2 from *S. mitis* 108

Fraction or LPS	Dose (μg)[a]	Cytokine level (mean ± SEM) in mice primed with: MDP TNF-α (ng/ml)	MDP IL-6 (U/ml)	*P. acnes* (IFN-γ, ng/ml)
F-1	10	305 ± 57	9.3 ± 0.4	71.0 ± 45.1
F-2	10	11 ± 4.1	7.1 ± 0.6	6.1 ± 1.3
LPS	0.8	629 ± 51	2,584 ± 451	120.7 ± 9.7

[a] Protein for F-1 and F-2; net weight for LPS.

TABLE 8. Induction of cytokines in human whole-blood cultures by fractions F-1 and F-2 from *S. mitis* 108

Fraction or LPS	Dose (μg/ml)[a]	TNF-α (ng/ml)	IL-6 (U/ml)	IFN-γ (ng/ml)	TAF (SI, mean ± SEM)[b]
F-1	5	11.0	156.3	1.0	18.6 ± 3.2
	1	2.0	18.8	6.2	20.5 ± 0.4
	0.2	0.5	13.8	4.3	20.0 ± 0.7
F-2	5	0.2	6.9	0.8	12.5 ± 0.7
	1	0.2	4.6	0.5	20.2 ± 2.7
	0.2	0.01	2.6	1.0	11.4 ± 0.8
LPS	10	2.5	27.5	NT[c]	22.2 ± 1.5
	1	1.6	25.0	NT	24.0 ± 1.3
	0.1	1.7	53.8	NT	22.4 ± 2.9
	0.01	0.5	14.4	NT	23.4 ± 2.9
	0.001	0.6	12.5	NT	22.4 ± 2.9
	0.0001	0.6	11.9	NT	25.3 ± 2.8
None		<0.01	0.7	0.5	1.0 ± 0.1

[a] Protein for F-1 and F-2; net weight for LPS.
[b] All the test specimen and reference LPS SI values were significantly different from those for the cell culture medium (none) control, as determined by Student's *t* test ($P \leq 0.01$).
[c] NT, not tested.

DISCUSSION

The present study revealed that culture supernatant concentrates from two *S. mitis* and two *S. oralis* strains and a heat-labile, extracellular protein produced by a representative of these strains induced various inflammatory cytokines

TABLE 9. Abolition of TNF-α-inducing activity in human whole-blood cultures by heating and protease digestion of fraction F-1

Treatment of fraction F-1	Dose (μg of protein/ml)	TNF-α (ng/ml)
None	5	54.2
	1	14.2
	0.2	5.8
KuttsuClean[a]	5	54.0
	1	13.0
	0.2	4.2
KuttsuClean + heating[b]	5	6.6
	1	5.0
	0.2	<0.1
KuttsuClean + protease digestion[c]	5	<0.1
	1	2.0
	0.2	<0.1
Medium alone	0	<0.1

[a] A stock solution of fraction F-1 in 0.1 M ammonium bicarbonate buffer (pH 8.3) was treated with KuttsuClean five times to exhaust possible LPS contamination.
[b] KuttsuClean-treated F-1 was boiled for 30 min at 100°C.
[c] KuttsuClean-treated F-1 was digested with protease fixed on carboxylmethyl cellulose.

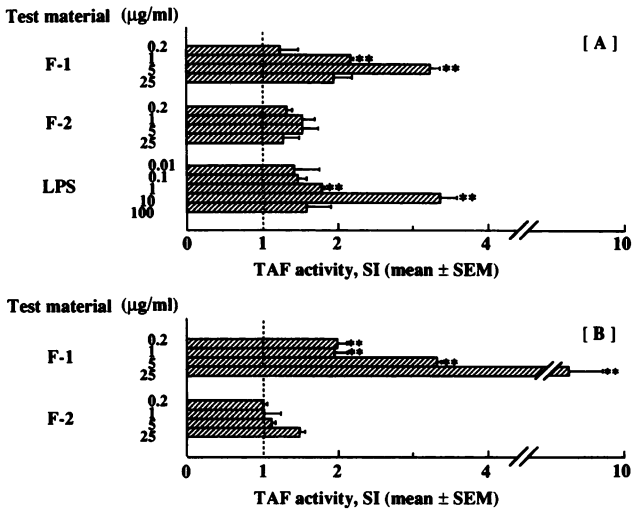

FIG. 5. Induction of TAF activity in HUVEC (A) and human gingival fibroblast (B) cultures by fractions F-1 and F-2 from *S. mitis* 108. Confluent cell monolayers were stimulated with test materials for 24 h. Triplicate culture supernatants were pooled, and their TAF activity was determined as described in the text. LPS was highly purified LPS from *S. abortus-equi*. **, Significantly different from the control (PHA alone), as determined by Student's *t* test ($P \leq 0.01$).

after administration to primed mice and by stimulating murine peritoneal macrophage, human peripheral mononuclear cell, HUVEC, and fibroblast cultures. This is the first report to describe an extracellular cytokine inducer(s) of viridans streptococci, although for beta-hemolytic streptococci there are reports that TNF and IL-1 were induced by SPE-A and streptolysin O (2, 6). On the other hand, Fujimaki et al. (3), who have focused upon fraction F-2, demonstrated that it functioned as a superantigen for human T cells, by selectively activating T cells bearing Vβ1, Vβ2, Vβ5.1, amd Vβ5.2, particularly Vβ2.

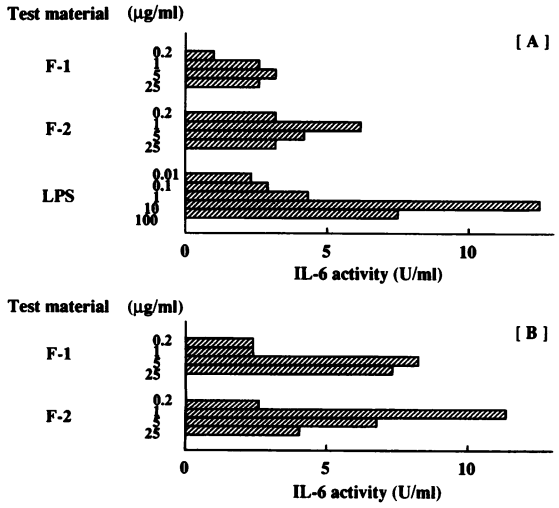

FIG. 6. Induction of IL-6 activity in HUVEC (A) and human gingival fibroblast (B) cultures by fractions F-1 and F-2 from *S. mitis* 108. The IL-6 activity in the cell-free specimens assayed for TAF as described in the legend to Fig. 5 was determined as described in the text.

The viridans streptococcal extracellular proteins capable of inducing various cytokines, like other biological response modifiers of bacterial origin, may have both beneficial and harmful effects on the host. This is especially true with viridans streptococcal products, since viridans streptococci are one of the dominant normal flora of the human oral and intestinal cavities. If the host has normal susceptibility, cytokine-inducing fraction F-1 (and superantigenic fraction F-2) may serve as useful immunomodulators. However, if the host is in a hypereactive state, either through innate or acquired causes, these products might trigger pathological processes that culminate in the manifestation of clinical signs in some diseases.

Several reports have suggested that inflammatory cytokines, such as TNF-α, IL-1, IL-6, and IFN-γ, are associated with the pathogenesis of MCLS (4, 13–16, 18, 22). Our findings suggest that cytokine production induced by extracellular proteins of oral viridans group streptococci may be involved in the development of MCLS. However, none of the present data support our hypothesis on the pathological roles of oral viridans streptococci and their exotoxins in MCLS. Particularly the finding (21) that there were no differences in the rates of recovery of toxicogenic viridans streptococci between MCLS and age-matched control children raises various issues. To examine the possibility that, despite these findings, these streptococci and their bioactive extracellular products have anything to do with MCLS, a clinical study is under way to determine whether are there any significant differences in susceptibility to the toxic and immunobiologically active components produced by viridans streptococci between MCLS and control children. These include, for example, the cytokine levels generated in peripheral whole-blood cultures for MCLS and control children in response to fractions F-1 and F-2.

Analysis by SDS-PAGE and double immunodiffusion revealed that fraction F-1 is not homogeneous. Consequently, little is known about the chemical and physicochemical properties of the bioactive component in fraction F-1, except that it is a heat-labile, protease-sensitive extracellular protein with a molecular mass above 150 kDa. The possibility that the observed bioactivity of fraction F-1 was influenced by contamination with extraneous LPS could be excluded, since there was no reduction in the TNF-α-inducing ability of fraction F-1 by treatment with KuttsuClean. Further studies are required to isolate, purify, and characterize the bioactive component.

ACKNOWLEDGMENTS

We thank R. Arakaki and K. Iki (Kagoshima University Dental School) for help in the bioassays of cytokines. We also thank H. Tamura and J. Aketagawa (Seikagaku Co., Tokyo, Japan) for the Endospecy test.

This work was supported in part by grants from the Japan Heart Association, Tokyo.

REFERENCES

1. **Abe, J., J. Forrester, T. Nakahara, J. A. Lafferty, B. L. Kotzin, and D. Y. M. Leung.** 1991. Selective stimulation of human T cells with streptococcal erythrogenic toxins A and B. J. Immunol. **146**:3747–3750.
2. **Fast, D. J., P. M. Schlievert, and R. D. Nelson.** 1989. Toxic shock syndrome-associated staphylococcal and streptococcal pyrogenic toxins are potent inducers of tumor necrosis factor production. Infect. Immun. **57**:291–294.
3. **Fujimaki, W., H. Kato, H. Igarashi, H. Ohkuni, Y. Todome, T.**

Uchiyama, and S. Kotani. Unpublished data.

4. **Furukawa, S., T. Matsubara, K. Jujoh, K. Yone, T. Sugawara, K. Sasai, H. Kato, and K. Yabuta.** 1988. Peripheral blood monocyte/macrophages and serum tumor necrosis factor in Kawasaki disease. Clin. Immunol. Immunopathol. **48:**247–251.
5. **Galanos, C., O. Lüderitz, and O. Westphal.** 1979. Preparation and properties of a standardized lipopolysaccharide from *Salmonella abortus equi* (Novo-Pyrexal). Zentralbl. Bakteriol. Mikrobiol. Hyg. Abt. 1 Orig. Reihe A **243:**226–244.
6. **Hackett, S. P., and D. L. Stevens.** 1992. Streptococcal toxic shock syndrome: synthesis of tumor necrosis factor and interleukin-1 by monocytes stimulated with pyrogenic exotoxin A and streptolysin O. J. Infect. Dis. **165:**879–885.
7. **Hauser, A. R., S. C. Goshorn, E. L. Kaplan, D. L. Stevens, and P. M. Schlievert.** 1991. Molecular analysis of the streptococcal pyrogenic exotoxins, p. 195–200. *In* G. M. Dunny, P. P. Cleary, and L. L. McKay (ed.), Genetics and molecular biology of streptococci, lactococci, and enterococci. American Society for Microbiology, Washington, D.C.
8. **Hirano, T., T. Taga, N. Nakano, K. Yasukawa, S. Kashiwamura, K. Shimizu, K. Nakajima, K. H. Pyun, and T. Kishimoto.** 1985. Purification to homogeneity and characterization of human B-cell differentiation factor (BCDF or BSFp-2). Proc. Natl. Acad. Sci. USA **82:**5490–5494.
9. **Hirano, T., K. Yasukawa, H. Harada, T. Taga, Y. Watanabe, T. Matsuda, S. Kashiwamura, K. Nakajima, K. Koyama, A. Iwamatsu, S. Tsunasawa, F. Sakiyama, H. Matsui, Y. Takahara, T. Taniguchi, and T. Kishimoto.** 1986. Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. Nature (London) **324:**73–76.
10. **Imanishi, K., H. Igarashi, and T. Uchiyama.** 1990. Activation of murine T cells by streptococcal pyrogenic exotoxin type A. Requirement for MHC class II molecules on accessory cells and identification of Vβ elements in T cell receptor of toxin-reactive T cells. J. Immunol. **145:**3170–3176.
11. **Imanishi, K., H. Igarashi, and T. Uchiyama.** 1992. Relative abilities of distinct isotypes of human major histocompatibility complex class II molecules to bind streptococcal pyrogenic exotoxin types A and B. Infect. Immun. **60:**5025–5029.
12. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
13. **Leung, D. Y. M., T. Collins, L. A. Lapierre, R. S. Geha, and J. S. Pober.** 1986. Immunoglobulin M antibodies present in the acute phase of Kawasaki syndrome lyse cultured vascular endothelial cells stimulated by gamma interferon. J. Clin. Invest. **77:**1428–1435.
14. **Leung, D. Y. M., R. S. Cotran, E. Kurt-Jones, J. C. Burns, J. W. Newburger, and J. S. Pober.** 1989. Endothelial cell activation and high interleukin-1 secretion in the pathogenesis of acute Kawasaki disease. Lancet **ii:**1298–1302.
15. **Leung, D. Y. M., R. S. Geha, J. W. Newburger, J. C. Burns, W. Fiers, L. A. Lapierre, and J. S. Pober.** 1986. Two monokines, interleukin 1 and tumor necrosis factor, render cultured vascular endothelial cells susceptible to lysis by antibodies circulating during Kawasaki syndrome. J. Exp. Med. **164:**1958–1972.
16. **Matsubara, T., S. Furukawa, and K. Yabuta.** 1990. Serum levels of tumor necrosis factor, interleukin 2 receptor, and interferon-γ in Kawasaki disease involved coronary-artery lesions. Clin. Immunol. Immunopathol. **56:**29–36.
17. **Matsuda, T., T. Hirano, and T. Kishimoto.** 1988. Establishment of an interleukin 6 (IL 6)/B cell stimulatory factor 2-dependent cell line and preparation of anti-IL 6 monoclonal antibodies. Eur. J. Immunol. **18:**951–956.
18. **Maury, C. P. J., E. Salo, and P. Pelkonen.** 1988. Circulating interleukin-1β in patients with Kawasaki disease. N. Engl. J. Med. **319:**1670–1671.
19. **Mosmann, T.** 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods **65:**55–63.
20. **Obayashi, T., H. Tamura, S. Tanaka, M. Ohki, S. Takahashi, M. Arai, M. Masuda, and T. Kawai.** 1985. A new chromogenic endotoxin-specific assay using recombined limulus coagulation enzymes and its clinical applications. Clin. Chim. Acta **149:**55–65.
21. **Ohkuni, H., Y. Todome, M. Mizuse, S. Ohtani, H. Suzuki, H. Igarashi, Y. Hashimoto, T. Ezaki, K. Harada, Y. Imada, S. Ohkawa, and S. Kotani.** 1993. Biologically active, extracellular products of oral viridans streptococci—etiology of Kawasaki disease. J. Med. Microbiol. **39:**352–362.
22. **Rowley, A. H., S. T. Shulman, O. T. Preble, B. J. Poiesz, G. D. Ehrlich, and J. R. Sullivan.** 1988. Serum interferon concentrations and retroviral serology in Kawasaki syndrome. Pediatr. Infect. Dis. J. **7:**663–666.
23. **Takeda, T., K. Sakagami, and N. Kobayashi.** 1988. Toxin study of *Streptococcus sanguis*, abstr. E-11, p. 100–101. Proc. 3rd Int. Kawasaki Disease Symp., Tokyo, Japan.
24. **Tamura, M., M. Tokuda, S. Nagaoka, and H. Takada.** 1992. Lipopolysaccharides of *Bacteroides intermedius* (*Prevotella intermedia*) and *Bacteroides* (*Porphyromonas*) *gingivalis* induce interleukin-8 gene expression in human gingival fibroblast cultures. Infect. Immun. **60:**4932–4937.
25. **Yamada, M., Y. Furutani, M. Notake, J. Yamagishi, M. Yamayoshi, T. Fukui, H. Nomura, M. Komiya, J. Kuwashima, K. Nakano, Y. Sohmura, and S. Nakamura.** 1985. Efficient production of human tumour necrosis factor in *Escherichia coli*. J. Biotechnol. **3:**141–153.