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Immunopathological Activities of Extracellular Products of *Streptococcus mitis*, Particularly a Superantigenic Fraction

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Previously, we prepared extracellular products, fractions F-1 and F-2 of *Streptococcus mitis* 108, an isolate from the tooth surface of an infant, and showed that F-1 exhibited inflammatory cytokine-inducing activities. In the present study, we present evidence that fraction F-2 induced human T-cell proliferation in the presence of irradiated human peripheral blood mononuclear cells and selectively activated T cells bearing Vβ2 and Vβ5.1 in the T-cell receptor. F-1, on the other hand, stimulated human gingival fibroblasts to support the T-cell proliferation in the same way as human gamma interferon or *Prevotella intermedia* lipopolysaccharide (LPS). Fraction F-1 also primed gingival fibroblasts to support the production of interleukin-2 and gamma interferon by the T cells upon stimulation with F-2. Human gingival fibroblasts stimulated with fraction F-1, like those stimulated by *P. intermedia* LPS and human gamma interferon, exhibited human leukocyte antigen (HLA)-DR mRNA expression and cell surface HLA-DR molecules as detected by enzyme-linked immunosorbent assay. An anti-HLA-DR monoclonal antibody inhibited T-cell proliferation in response to F-2, probably through inactivating the accessory function of HLA-DR-bearing fibroblasts. T cells activated with F-2 in the presence of irradiated peripheral blood mononuclear cells exhibited definite cytotoxic effects against fibroblasts and squamous carcinoma cells originating from human oral tissues. These findings are strongly suggestive of an association of extracellular products of viridans streptococci with pathogenesis of oral mucosal diseases, particularly those disorders in gingiva which are accompanied by heavy infiltration of T cells.

Viridans and nonhemolytic streptococci are predominant bacterial flora of the human oral cavity (9). Colonization of the tooth surfaces in the subgingival area by these bacteria is possibly responsible for the induction of gingivitis, an initial stage of periodontitis (21). However, little is known about the virulence factors of viridans streptococci which are responsible for their pathogenic potency.

Previously, we (12) found that extracellular products of *Streptococcus oralis* and *Streptococcus mitis* isolated from the tooth surface of either the infants with Kawasaki syndrome or the age-matched diseased or healthy control children were capable of inducing rabbit skin lesions such as increases in capillary permeability in the early stage and redness sometimes accompanied by bleeding in the later stage. The concentrated culture supernatant of a strain (no. 108) of *S. mitis* as a representative was fractionated to give two fractions, F-1 and F-2. We found that fraction F-1 induced inflammatory cytokine production in murine and human cell cultures, including human fibroblasts, and provoked production of serum cytokines on intravenous administration into appropriately primed mice (18). On the other hand, the study of Fujimaki et al. (7) suggested the superantigenic activities of fraction F-2.

In the present study, we present for the first time the experimental evidence on the superantigenicity of F-2 and then show that F-2 induced the proliferative response and cytokine production in human T-cell cultures with assistance from human gingival fibroblasts which were stimulated with fraction F-1, human gamma interferon (HuIFN-γ), or lipopolysaccharide (LPS) from *Prevotella intermedia* (*Bacteroides intermedius*), a periodontitis-related black-pigmented bacterium (15). Furthermore, we reveal that human peripheral blood T cells stimulated with F-2 exerted cytocidal effects on human gingival fibroblasts and other cells.

MATERIALS AND METHODS

Extracellular products of *S. mitis*. Partially purified fractions F-1 and F-2 were prepared from the culture supernatants of *S. mitis* 108, an isolate from the tooth surface of a male infant with aseptic meningitis, as described previously (18). Briefly, the bacteria 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. The culture filtrate was subjected to ion-exchange chromatography on SP-Sephadex C-25 (Pharmacia, Uppsala, Sweden). The peak fraction was concentrated by dialysis against Aquacide II A (a sodium salt of carboxymethyl cellulose [molecular weight, 500,000]; Calbiochem Corp., La Jolla, Calif.), rechromatographed on SP-Sephadex C-25 (Pharmacia), and submitted to gel filtration with a Sephadex G-25 (Pharmacia) column. Protein fractions in the filtrate were pooled and applied to a PEB94 column. The peak protein fraction obtained by a NaCl gradient elution of the PEB94 column was applied to a Sephacryl S-200 Superfine (Pharmacia) column, elution of which with 0.1 M NH_4HCO_3 gave two peak fractions, F-1 and F-2, which were well separated.

Other reagents and cell lines. An LPS specimen was prepared from *P. intermedia* ATCC 25611 by the hot phenol-water extraction method as described previously (17). Staphylococcal enterotoxin B (SEB) was purified from the culture supernatant of *Staphylococcus aureus* 243 by a combination of ion-exchange chromatography and gel filtration as described by Oda (11). HuIFN-γ and recombinant human interleukin-2 (rHuIL-2) were supplied by Hayashibara Bioscience Institute (Okayama, Japan) and Otsuka Pharmaceutical Co. (Tokushima, Japan), respectively. The anti-human leukocyte antigen (HLA)-DR monoclonal antibody (MAb) IOT2a was purchased from Cosmo Bio Co., Ltd. (Tokyo, Ja-

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pan). Biotin-conjugated goat anti-human immunoglobulin G was obtained from Zymed Laboratories (South San Francisco, Calif.). A labeled streptavidin-biotin (LSAB) kit for detecting HLA-DR molecules on cell surfaces was obtained from DAKO Japan Co. (Kyoto, Japan). Anti-CD3 MAb (OKT3) was supplied by Janssen-Kyowa Co. (Tokyo, Japan). Enzyme-linked immunosorbent assay (ELISA) kits for measuring IL-2 and IFN-γ were purchased from R&D Systems (Minneapolis, Minn.) and Genzyme Co. (Boston, Mass.), respectively. ^{125}I-deoxyuridine was obtained from Dupont, NEN Research Products (Boston, Mass.). Phytohemagglutinin (HA-16) was purchased from Wellcome Diagnostics (Temple Hill, United Kingdom). Bovine serum albumin (BSA) and *O*-phenylenediamine were purchased from Nakarai Chemicals (Kyoto, Japan). A reverse transcriptase RNA PCR (RT-PCR) kit and NuSieve 3:1 agarose were obtained from Takara (Otsu, Japan). HLA-DRB-specific primers GH46 and GH50 and a DNA thermal cycler were purchased from the Perkin-Elmer Corp. (Norwalk, Conn.). Human gingival fibroblasts, GF-5, were obtained as described previously (20). The human squamous carcinoma cell lines HSC-2 (JCRB0622), Ca9-22 (JCRB0625), and HO-1-N-1 (JCRB0831), which were originally isolated from human mouth, gingiva, and buccal mucosa, respectively, were supplied by Japanese Cancer Research Resources Bank (Tokyo, Japan).

Preparation of accessory cells and T cells. Irradiated human peripheral blood mononuclear cells (PBMC) and human gingival fibroblasts (GF-5) were used as accessory cells. PBMC were isolated from a peripheral blood specimen from healthy adult volunteers by Ficoll-Hypaque centrifugation. Suspensions (2×10^5 cells per 50 μl) of PBMC in RPMI 1640 medium (Nissui Seiyaku Co., Tokyo, Japan) supplemented with 10% fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.) (complete RPMI medium) were seeded into 96-well flat-bottomed microculture plates (model 25860; Corning Glass Works, Corning, N.Y.), irradiated at 3,400 rads with an X-ray irradiator (Hitachi Medical Co., Tokyo, Japan) to inhibit proliferation, and used as accessory cells in T-cell proliferation assays. GF-5 cells (5×10^4 cells per 100 μl) in microculture plates were cultured with F-1 (5 μg/ml), *P. intermedia* LPS (5 μg/ml), or a reference HuIFN-γ (200 U/ml) in alpha-modified Eagle's medium (αMEM) (Flow Laboratories, Inc., McLean, Va.) supplemented with 10% fetal calf serum (complete α-MEM) in a CO_2 incubator at 37°C for 3 days. The cell monolayers were then irradiated at 6,000 rads and used as accessory cells in most of the following experiments. In some experiments, the activated and irradiated GF-5 cells were further preincubated with anti-HLA-DR MAb at 37°C for 1 h and then used as accessory cells.

Purified T cells were prepared as follows: human PBMC suspension in complete RPMI 1640 medium was passed through a nylon wool column to deplete B cells and macrophages. The fraction enriched with T cells was treated with anti-HLA-DR MAb and guinea pig fresh serum as a source of complement to remove cells bearing HLA-DR antigens. Cells which escaped complement-mediated lysis were collected and used in the proliferation and cytokine induction assays. Superantigen-activated T cells were obtained according to the method of Yan et al. (22) with modifications. Briefly, purified T cells (2×10^6 cells per ml) were cultured with F-2 (25 μg/ml), SEB (25 μg/ml), or medium (complete RPMI 1640 medium) alone or in the presence of irradiated PBMC as accessory cells in tissue culture dishes (35 mm; Corning) for 3 days. These activated T cells were again passed through a nylon wool column, and the eluted cells were grown for 2 days in the presence of rHuIL-2 (200 U/ml) and used as effector cells in cytotoxic assays.

Proliferation assay. Purified T cells (2×10^5 cells per well) in the presence of pretreated PBMC or GF-5 cells in 96-well microculture plates were cultured with test stimulants in 0.2 ml of the complete RPMI 1640 medium for 72 h. Plates were pulsed with 0.5 μCi of ^{125}I-deoxyuridine during the last 8 h of culture, and the radioactivity incorporated into cells was measured with a gamma counter (Auto Well Gamma System ARC-300; Aloka Co., Tokyo, Japan). The results were expressed as a stimulation index, where stimulation index = ^{125}I-deoxyuridine uptake (counts per minute) in the test culture divided by that in the control culture (medium alone).

Determination of Vβ gene expression. To determine the proportion of T cells bearing particular T-cell-receptor (TCR) Vβ elements, the method of Choi et al. (2) was used with some modifications. To prepare T-cell blasts, human PBMC (2×10^6/ml) were cultured with F-2 or anti-CD3 for 3 days in a 24-well culture plate. Recovered cells were subjected to Percoll (Pharmacia) density gradient centrifugation. Large T-lymphoblast-enriched cells collected at a density of 1.068 were then cultured at 2×10^5/ml for 2 days with 100 U of rHuIL-2 per ml. Cultured cells were further fractionated by Percoll centrifugation to collect purified large lymphoblasts in which the proportion of $CD3^+$ T cells was higher than 95%. T-cell blasts (10^6) thus obtained were treated with 100 μg of a lysing buffer (pH 7.5; 10 mM Tris-HCl, 0.14 M NaCl, 5 mM KCl, 5 mM dithiothreitol, 1% Triton X-100) on ice for 1 min. Cell lysates were centrifuged at 13,000 × *g* for 30 s. The aqueous phase was collected and then treated for 5 min with 350 mg of magnetic beads [Dynabeads Oligo (dT)25; Dynal A.S., Oslo, Norway] per 10^6 cells in 100 μl of 2× binding buffer (pH 7.5; 20 mM Tris-HCl, 1 M LiCl, 2 mM EDTA). The beads that were bound by mRNA were collected and washed twice in the washing buffer by using a magnet. For cDNA syntheses, the following reagents were added to the beads: 0.7 U of Rous-associated virus 2 RT per μl (Takara), 0.1 μg of random primer per μl [hexadeoxynucleotide mixture; $pd(N)_6$; Takara), 0.04 U of RNase inhibitor per μl (Inhibit-Ace; 5 Prime-3 Prime Inc., Boulder, Colo.), 1 mM 2′-deoxyribonucleotide 5′-triphosphates (Pharmacia), 30 mM KCl, 50 mM Tris-HCl, and 8 mM $MgCl_2$ in a total volume of 25 μl. The reaction was allowed to proceed for 60 min at 42°C.

The 22 kinds of 5′ Vβ primers specific for 22 Vβ families, one 3′ Cβ-specific primer to be used for Vβ amplification, and 3′ and 5′ Cα primers for use as an internal standard were synthesized at Kitasato University with a DNA synthesizer. The Vβ primers had the same sequence as those described by Choi et al. (2). Each cDNA sample was coamplified with one of the 22 Vβ family primers and Cβ primers as one pair and two Cα primers as the other pair by using a Program Temp Control System PC-700 (ASTEC Japan, Shizuoka, Japan) in the presence of 1.25 U of Amplitaq polymerase (Perkin-Elmer) in the final volume of 20 μl per amplification tube. The 3′ Cα and 3′ Cβ primers were labeled by [γ-^{32}P]ATP for estimation of amplified products. The amplification schedule was 30 s of denaturation at 95°C, 30 s of annealing at 55°C, and 60 s of extension at 72°C in each cycle. Amplification was done for 25 cycles and was chosen to ensure that the amounts of Vβ products were proportional to the amounts of Cα control products. The amplified products were separated on 2% agarose gels. The gels were dried and analyzed by Bio-Image Analyzer BAS 2000 (Fuji Photo Film Co., Kanagawa, Japan). To compare the expressions of different Vβ regions, a PCR value, which was 1,000× a ratio of Vβ counts per minute and Cα counts per minute for each Vβ region, was calculated.

Cytokine induction in T-cell cultures. Purified human T cells (2×10^5 cells per well) along with the pretreated GF-5 cells in monolayer were cultured in the presence of F-2 in complete RPMI 1640 medium in microculture plates for 3 days. Triplicate culture supernatants were pooled and stored at −80°C until used in cytokine assays. IL-2 and IFN-γ levels in the culture supernatants were determined with commercial ELISA kits according to the manufacturer's instructions, and the cytokine levels in test specimens were estimated by referring to the standard curve prepared for each assay.

HLA-DR expression. GF-5 cells (5×10^4 cells per well) were cultured with F-1, *P. intermedia* LPS, or reference HuIFN-γ in complete α-MEM in the microculture plates for 3 days. After removal of the culture supernatants and two washes with 50 mM phosphate-buffered saline (PBS; pH 7.4), the GF-5 cells on the culture plates were fixed with 0.5% glutaraldehyde in PBS at 25°C for 20 min and washed three times with PBS, 3% H_2O_2 in PBS was added, and the cells were incubated at 25°C for 5 min. After three washes with PBS, 1% BSA in PBS was added, and the cells were further incubated at 37°C for 1 h to block nonspecific reaction and then washed three times with PBS to remove excess BSA. To the cell cultures was added the anti-HLA-DR MAb IOT2a (1 μg of protein equivalent per 100 μl), and the cultures were incubated at 4°C overnight. Unbound antibody was removed by washing in PBS, and the washed cells were incubated with biotin-conjugated goat anti-human immunoglobulin G (100 μl) at 37°C for 1 h and then washed with PBS. Peroxidase-conjugated streptavidin (100 μl) was then added, and the cells were incubated at 37°C for 30 min. *O*-Phenylenediamine (1.6 mg/ml) in 0.1 M citrate-phosphate buffer, pH 5.0 (100 μl), was then added. After incubation at 37°C for 1 h, the optical density of the reaction mixture at 490 nm (OD_{490}) was measured with a microplate reader (Wellreader SK601; Seikagaku Co., Tokyo, Japan). Localization of HLA-DR molecules on the cells was also determined cytochemically with an LSAB kit (DAKO Japan Co.) according to the manufacturer's instructions.

RT-PCR was carried out to detect the HLA-DR mRNA in GF-5 cells. Cells (1.5×10^6/ml) were stimulated with F-1, *P. intermedia* LPS, or HuIFN-γ in complete α-MEM in tissue culture dishes for 16 h. From these cells, RNA was extracted by the method of Chomczynski and Sacchi (3), and 1 μg of the RNA was then subjected to RT-PCR with the HLA-DRB-specific primers GH46 and GH50. Amplification was performed with a DNA thermal cycler as follows: denaturation at 96°C for 10 min and amplification for 35 cycles at 96°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by extension at 72°C for 10 min. Ten-microliter aliquots of the products were analyzed by electrophoresis on a 4% NuSieve 3:1 agarose gel and visualized by UV fluorescence after being stained with ethidium bromide.

Cytotoxicity assay. GF-5 fibroblasts (5×10^4 per well) were cultured with or without the stimulants (F-1, *P. intermedia* LPS, or HuIFN-γ) in complete α-MEM in microculture plates for 3 days and used as target cells. In an experiment, GF-5 cells precultured in the medium alone were further preincubated with anti-HLA-DR MAb at 37°C for 1 h and then used as target cells. The human carcinoma cell lines (5×10^4 per well) HSC-2, Ca9-22, and HO-1-N-1 were also used as target cells precultured in complete α-MEM in microculture plates for 2 days. Monolayers of target cells cultured with or without stimulants were washed three times with RPMI 1640 medium, then added with the activated T cells (2×10^6 per well) which were preactivated with F-2 or SEB along with irradiated PBMC for 3 days, and then grown for 2 days in the presence of rHuIL-2 in complete RPMI 1640 medium. In control experiments, the T cells precultured in medium alone for 3 days and with rHuIL-2 for 2 days and those precultured in medium alone for 5 days were used. The reaction mixtures were centrifuged at 18 × *g* for 5 min and incubated at 37°C for 4 h in the presence of graded doses of the same superantigen as that used for T-cell reactivation. After T cells and detached target cells were removed by washing, the target cells retained on the culture plates were fixed and stained with 2% formaldehyde containing 0.2% crystal violet. The intensity of staining was measured with a microplate reader at OD_{595}. The degree of cytotoxicity was expressed as percent cytotoxicity, where % cytotoxicity = OD_{595} of the control culture (target cells cultured in medium alone) − OD_{595} of the test culture/OD_{595} of the control culture.

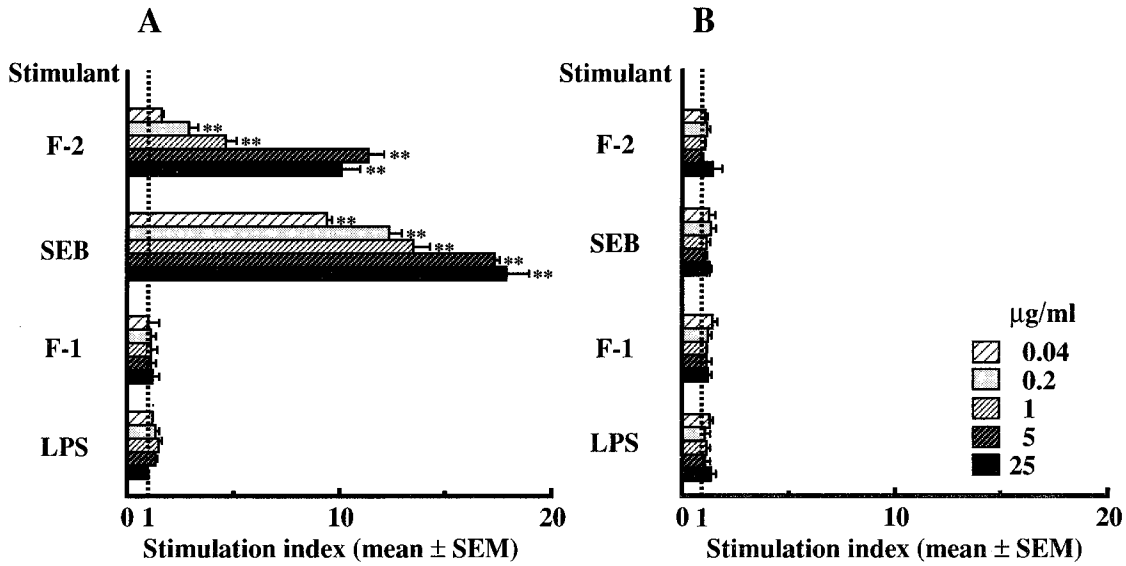


FIG. 1. Proliferative responses of purified human T cells upon stimulation with F-2 and SEB in the presence of irradiated PBMC. Freshly isolated human peripheral blood T cells (2×10^5 cells per well) were cultured with graded doses of test materials in complete RPMI 1640 medium in microculture plates in a CO_2 incubator for 72 h with (A) or without (B) the irradiated PBMC (2×10^5 cells per well). Proliferative responses were estimated in terms of ^{125}I-deoxyuridine incorporation. Stimulation index = radioactivity (counts per minute) incorporated in the test culture divided by that in the control culture without stimulants (mean ± standard error of the mean [SEM]). **, significantly different from respective control by Student's *t* test ($P \leq 0.01$).

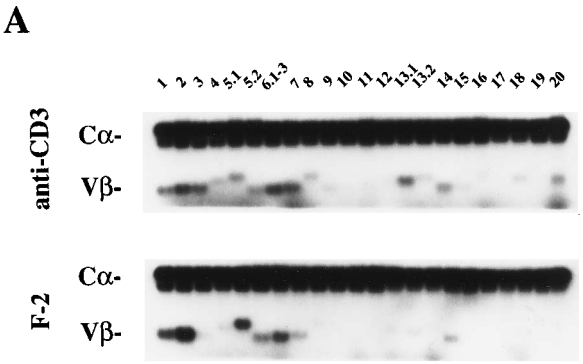
Miscellaneous. Most assays were carried out in triplicate. On the basis of the mean value and standard error of the mean (SEM), the statistical significance of differences between each test and respective control was determined by Student's *t* test.

RESULTS

Superantigenicity of the F-2 fraction. (i) Major histocompatibility complex (MHC) class II requirements. Figure 1A shows the results of the assay of the mitogenic effects of F-2 on purified human T cells in the presence and absence of irradiated PBMC. The fraction F-2, like the reference SEB, showed a powerful inductive effect on human T-cell proliferation in the presence of irradiated PBMC. In sharp contrast, both F-1 and *P. intermedia* LPS were totally inactive. T cells responded dose dependently to F-2 within a wide range of concentrations from 0.2 to 25 μg/ml, although the extent of the response to F-2 was lower than that to SEB at each corresponding concentration. F-2, like SEB, exhibited no mitogenicity on human T cells in the absence of PBMC (Fig. 1B).

(ii) TCR Vβ usage. To demonstrate that F-2 selectively activates T-cell populations bearing specified Vβ elements in TCR, PBMC from five volunteers were stimulated with F-2 and T-cell blasts obtained were examined for TCR Vβ usage by the PCR method. Anti-CD3-induced T-cell blasts were used as a control in place of nonsensitized T cells, since preliminary experiments revealed that the respective Vβ/Cα ratio is similar between these two preparations of T-cell blasts. Figure 2 shows a representative result of autoradiography and Vβ/Cα ratios of amplified TCR transcripts from T-cell blasts from a single donor. A definite increase in the amounts of Vβ1, Vβ2, and Vβ5.1 cDNA was observed in the F-2-induced T-cell blasts over those in the anti-CD3-induced T-cell blasts as controls. The amount of Vβ5.2 was unchanged by stimulation with F-2. The amounts of Vβ cDNA other than the Vβ elements described above were lower in this test sample. Figure 3 shows the results of the assay on the Vβ usage (in terms of Vβ/Cα ratio) of F-2 stimulation with five donors. Upon stimulation with F-2, the increase in the Vβ/Cα ratio in Vβ2 and Vβ5.1 was consistently observed for all of the five donors. However, the ratios of Vβ1, Vβ5.2, and Vβ6 to Cα increased with some donors but decreased with other donors by not-yet-identified mechanisms.

All in all, the above findings indicate that T-cell populations bearing Vβ2 and Vβ5.1 were certainly reactive with the F-2



B

Vβs	1	2	3	4	5.1	5.2	6	7	8	9	10	11	12	13.1	13.2	14	15	16	17	18	19	20
anti-CD3	113	149	166	87	63	97	160	110	56	32	15	16	15	95	29	80	11	10	0	21	10	56
F-2	146	243	50	39	135	94	130	65	22	7	14	13	10	16	18	65	7	5	0	12	2	12

FIG. 2. Autoradiograms of amplified TCR transcripts of human T cells reactive with F-2. Human PBMC from a healthy adult donor were stimulated with 500 ng of F-2 or 50 ng of anti-CD3 MAb per ml for 3 days. The resultant T-cell blasts were grown in the presence of 100 U of rHuIL-2 per ml for 2 days. mRNA was extracted and subjected to the TCR Vβ analysis by the PCR method. (A) Autoradiograms of amplified TCR transcripts. (B) Calculated Vβ/Cα ratio of autoradiograms of amplified TCR transcripts.

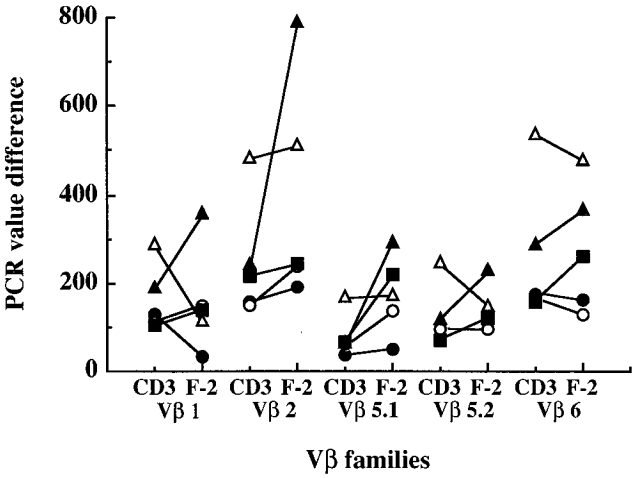


FIG. 3. Changes in human T-cell population bearing TCR Vβ1, -2, -5.1, -5.2, and -6 upon stimulation with F-2. T-cell blasts from five healthy adult donors were prepared according to the procedures described in the legend to Fig. 2 and analyzed for TCR Vβ usage. Each symbol (open and filled triangles, open and filled circles, and filled square) represents an individual donor.

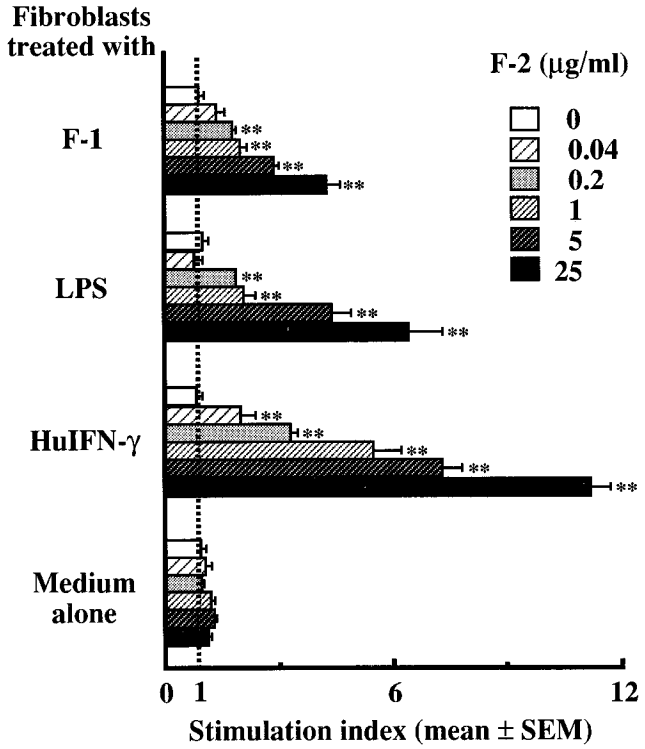


FIG. 4. Proliferative responses of human T cells upon stimulation with F-2 in the presence of irradiated human gingival fibroblasts (GF-5). Freshly isolated human peripheral blood T cells (2×10^5 cells per well) were cultured with graded doses of F-2 in complete RPMI 1640 medium in microculture plates in a CO_2 incubator for 72 h with irradiated GF-5 cells (5×10^4 cells per well) precultured with F-1 (5 μg/ml), *P. intermedia* LPS (5 μg/ml), HuIFN-γ (200 U/ml), or complete α-MEM alone for 3 days and assayed for proliferative responses in terms of ^{125}I-deoxyuridine incorporation. The stimulation index was calculated, and statistical analysis was performed as described in the legend to Fig. 1. **, significantly different from respective control, in which fibroblasts precultured in medium alone were used as accessory cells, by Student's *t* test ($P \leq 0.01$).

fraction and support the conclusion that F-2 can work on human T cells as a superantigen.

T-cell activation by F-2 in the presence of activated fibroblasts as accessory cells. We next examined whether irradiated human gingival fibroblasts (GF-5 cells) could replace PBMC as accessory cells. Under the experimental conditions in which no proliferative response of T cells to F-2 was recognized (medium-alone control), GF-5 cells pretreated with F-1, like those treated with *P. intermedia* LPS or HuIFN-γ as a positive control, served as accessory cells and significantly stimulated the proliferation response of T cells to F-2, although the potencies of F-1 and the test LPS were less than that of HuIFN-γ (Fig. 4). Acquisition of the ability to function as accessory cells by GF-5 fibroblasts pretreated with F-1, LPS, or HuIFN-γ was further demonstrated by the finding that the ability of F-2 to stimulate production of IL-2 and IFN-γ by T cells was markedly increased by coculture with irradiated GF-5 cells pretreated with F-1, *P. intermedia* LPS, or HuIFN-γ (Fig. 5).

HLA-DR expression by human gingival fibroblasts stimulated with F-1. The mechanism by which the GF-5 cells pretreated with F-1 or other stimulants supported the T-cell activation in response to F-2 was analyzed by examining whether the treated GF-5 fibroblasts expressed class II antigens on their surfaces. GF-5 monolayers were cultured for 3 days with F-1, *P. intermedia* LPS, or HuIFN-γ, and then HLA-DR expression was determined by ELISA. As shown in Fig. 6, under the experimental conditions in which HuIFN-γ powerfully induced HLA-DR expression in a dose-dependent manner, GF-5 cells treated with F-1 or *P. intermedia* LPS increased the HLA-DR expression over the baseline determined for the untreated cells. The marked increase in HLA-DR expression by GF-5 fibroblasts under stimulation with HuIFN-γ was confirmed microscopically with an LSAB kit (Fig. 7). However, the increase in HLA-DR expression by GF-5 fibroblasts in response to stimulation with F-1 and *P. intermedia* LPS was not as clear microscopically (data not shown). Induction of HLA-DR gene expression by the test stimulants was examined. RNA was extracted from GF-5 cells which had been stimulated with F-1, *P. intermedia* LPS, or HuIFN-γ and cultivated for 16 h and was amplified by RT-PCR. F-1 and *P. intermedia* LPS as well as HuIFN-γ caused strong expression of the HLA-DR transcript, although untreated cells also expressed the gene at low levels (Fig. 8).

MAb against HLA-DR inhibited F-2-induced T-cell proliferation. To determine whether T-cell proliferation induced by F-2 is dependent upon HLA-DR molecules expressed on the surfaces of the accessory cells, the effects of treatment of accessory cells with the anti-HLA-DR MAb IOT2a on T-cell responses were examined. The T-cell-proliferative response induced by F-2 in the presence of HuIFN-γ-treated GF-5 cells was strongly inhibited by preincubation of the accessory cells with the anti-HLA-DR MAb (Fig. 9). Similar results were obtained when the GF-5 cells pretreated with F-1 or *P. intermedia* LPS were used as accessory cells. In contrast, T-cell proliferation induced by phytohemagglutinin as a control was not inhibited by this MAb.

Cytotoxic activity of T cells activated with F-2. Human T cells which were precultured with F-2 or SEB and grown by stimulation with rHuIL-2 were mixed with the GF-5 fibroblasts pretreated with F-1, *P. intermedia* LPS, HuIFN-γ, or medium alone. The cell mixtures were cultured in the presence of graded doses of the same stimulant as that used for pretreatment of the T cells. T cells preactivated with F-2 exhibited distinct cytotoxicity against GF-5 fibroblasts, dependent on the dose of F-2 added to the cell mixtures (Fig. 10). No differences were noted in the susceptibility to the cytotoxic effects of F-2-activated T cells among the variously pretreated GF-5 cells,

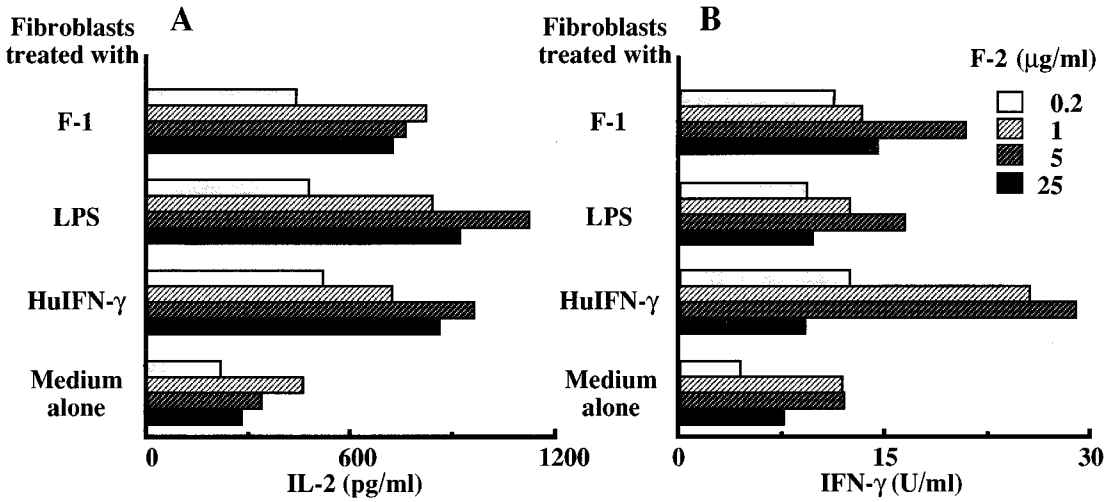


FIG. 5. IL-2 and IFN-γ production in human T-cell cultures upon stimulation with F-2 in the presence of irradiated human gingival fibroblasts. Human T cells (2×10^5 cells per well) were cultured with graded doses of F-2 in complete RPMI 1640 medium in microculture plates for 48 h in the presence of irradiated GF-5 cells (5×10^4 cells per well) precultured with F-1 (5 μg/ml), *P. intermedia* LPS (5 μg/ml), HuIFN-γ (200 U/ml), or complete α-MEM alone for 3 days. After incubation, triplicate culture supernatants were pooled, and IL-2 (A) and IFN-γ (B) levels in the supernatants were determined with ELISA kits.

including those pretreated with medium alone. The cytotoxic activity of T cells precultured in medium alone and grown by stimulation with rHuIL-2 did not increase upon stimulation with F-2; cytotoxic activity was around 50%, irrespective of the dose of F-2 added to cell mixtures. The ability of F-2 to render T cells cytotoxic to GF-5 cells by preculture and coculture seemed to be roughly comparable to that of SEB, although higher concentrations of F-2 than SEB were required to exert the same level of activity. In a control experiment, T cells precultured with neither superantigens nor rHuIL-2 (in medium alone) showed no cytotoxic activity against variously pretreated GF-5 cells, even in the presence of the highest concentration (25 μg/ml) of F-2 (data not shown). To determine whether the cytotoxicity depends upon HLA-DR molecules on the target cells, the following experiments were carried out. GF-5 cells precultured in medium alone for 3 days were treated with graded doses of an anti-HLA-DR MAb for 1 h and mixed with T cells precultured with F-2 (25 μg/ml) or medium alone for 3 days, stimulated with rHuIL-2 (200 U/ml) for 2 days, and then further incubated in the presence of F-2 (25 μg/ml) for 4 h. Only slight inhibition (less than 10%) and no inhibition of cytotoxicity were observed with T cells preactivated with F-2 followed by rHuIL-2 and those stimulated with rHuIL-2 alone, respectively (data not shown).

Figure 11 shows the cytotoxic effects of T cells activated with F-2 and grown by stimulation with rHuIL-2 on three lines of squamous carcinoma cells (HSC-2, Ca9-22, and HO-1-N-1) which originated from the human oral cavity. The activated T cells exhibited strong cytotoxicity against all of the above-mentioned carcinoma cells, dependent on the dose of F-2 added to the cell cultures (Fig. 11).

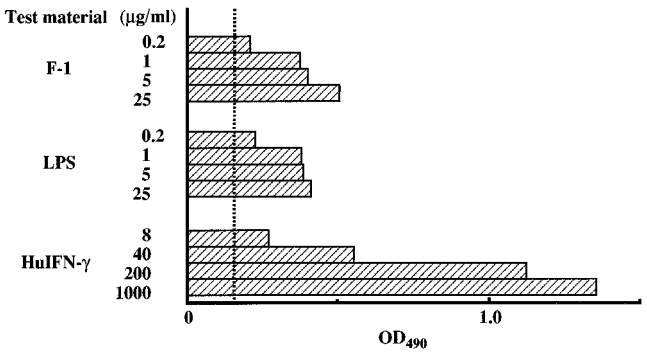


FIG. 6. Expression of HLA-DR antigens in human gingival fibroblasts cultured with F-1, *P. intermedia* LPS, or HuIFN-γ. Human gingival fibroblasts, GF-5 cells (5×10^4 cells per well), were cultured with graded doses of F-1, *P. intermedia* LPS, or HuIFN-γ in complete α-MEM for 3 days. HLA-DR antigen expression was then measured by modified ELISA as described in the text. The dotted line indicates the level obtained with GF-5 cell cultures without stimulants (medium-alone control).

DISCUSSION

In the present study, we first demonstrated that one of the extracellular products (fraction F-2) from the culture supernatant of *S. mitis* 108 (an isolate from an infant) caused the proliferation of human T cells in the presence of irradiated PBMC as accessory cells (Fig. 1) and that F-2 selectively activated T cells bearing Vβ2 and Vβ5.1 in TCR (Fig. 2 and 3). Thus, we verified the superantigenicity of F-2, which was reported by Fujimaki et al. (7). We then examined whether human gingival fibroblasts (GF-5 cells) function as accessory cells for T-cell activation by F-2. Untreated GF-5 fibroblasts did not support T-cell proliferation by F-2 (Fig. 4), probably because of the lack of MHC class II antigens on their cell surfaces. Therefore, we attempted to induce MHC class II antigen expression on GF-5 fibroblast cells by treatment with HuIFN-γ, which is known to cause fibroblasts to express MHC class II antigens (4, 14), with success. We proceeded to examine the ability of fraction F-1, another extracellular product of *S. mitis* 108, and an LPS preparation from *P. intermedia*, a periodontitis-related anaerobe, to induce MHC class II antigen expression on GF-5 fibroblasts, because these products are known to cause human gingival fibroblasts to generate inflammatory cytokines (19, 20). As expected, both F-1 and *P. intermedia* LPS induced expression of HLA-DR mRNA in GF-5 fibroblasts (Fig. 8) and that of HLA-DR antigens on their cell surfaces detectable by ELISA (Fig. 6). It must be noted that the intensity of antigen expression induced by F-1 or *P. inter-*

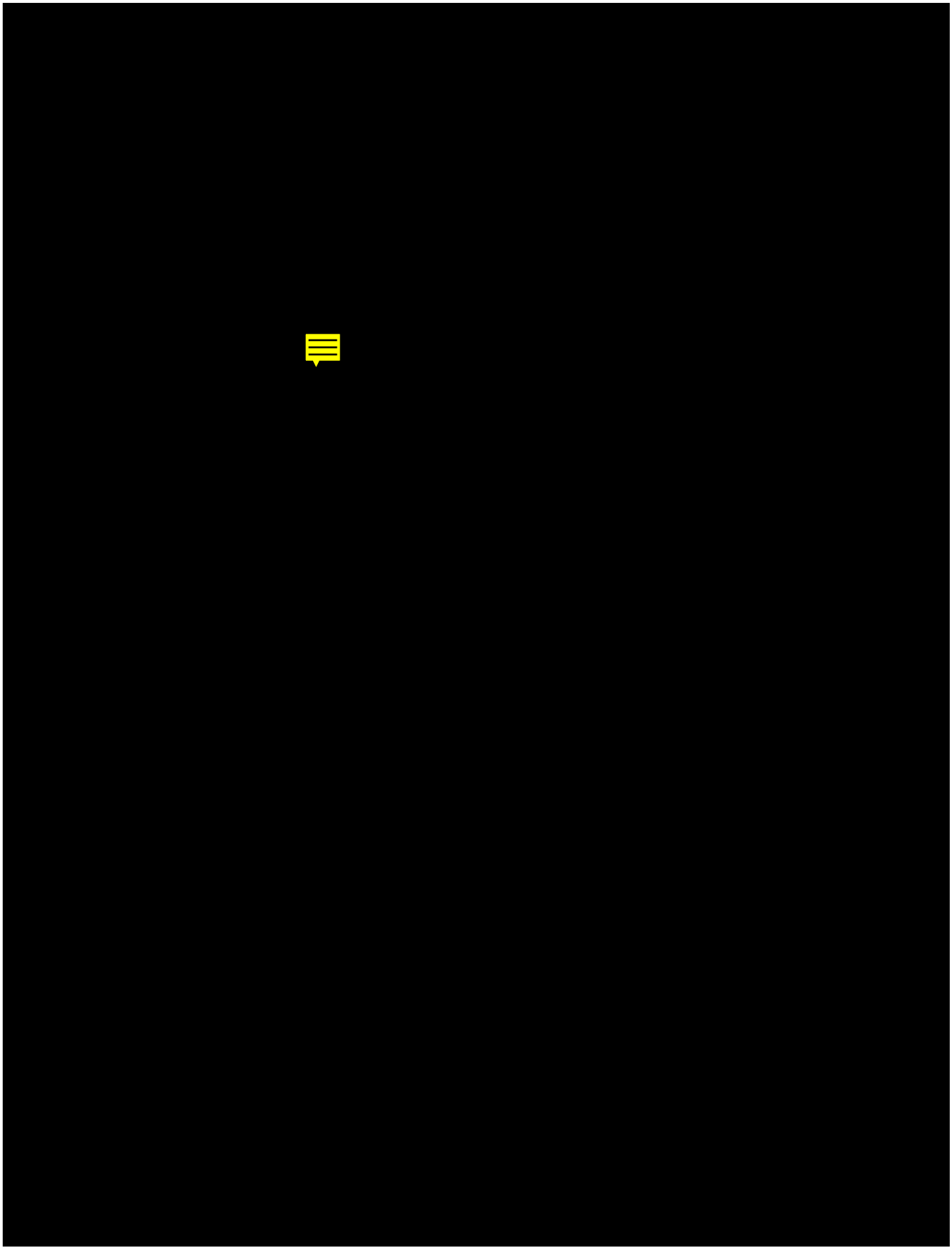


FIG. 7. Microscopic determination of HLA-DR antigen expression by human gingival fibroblasts stimulated with HuIFN-γ. GF-5 cells (5×10^4 cells) were cultured with (A) or without (B) HuIFN-γ (200 U/ml) in complete α-MEM in culture dishes for 3 days and then stained for HLA-DR antigens with the LSAB kit as described in the text. Original magnification, ×400.

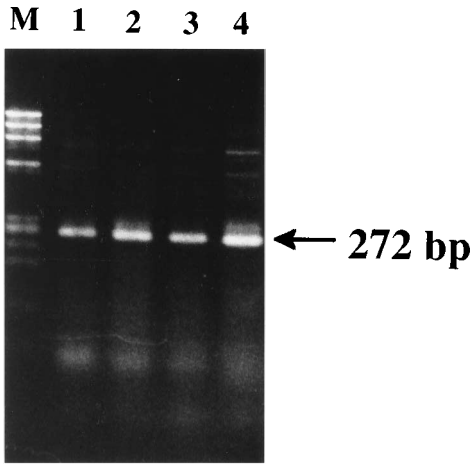


FIG. 8. HLA-DR mRNA expression in stimulated human gingival fibroblasts. GF-5 cells (1.5×10^6 cells) were cultured with F-1 (5 μg/ml), *P. intermedia* LPS (5 μg/ml), HuIFN-γ (200 U/ml), or complete α-MEM alone for 16 h. Total RNA was extracted from the test and control cultured cells and amplified by RT-PCR with HLA-DR-specific primers as described in the text. Lane M, φX174 *Hae*III-cut size marker; lanes 1 to 4, GF-5 cells stimulated with medium alone (lane 1), F-1 (lane 2), *P. intermedia* LPS (lane 3), and reference HuIFN-γ (lane 4).

media LPS was considerably lower than that induced by HuIFN-γ (Fig. 6), and the antigen expression by F-1 and the LPS was not detected by a less sensitive microscopic method. In accordance with these findings, however, GF-5 fibroblasts treated with F-1 or *P. intermedia* LPS, like those primed with HuIFN-γ, supported T-cell proliferation and enhanced IL-2

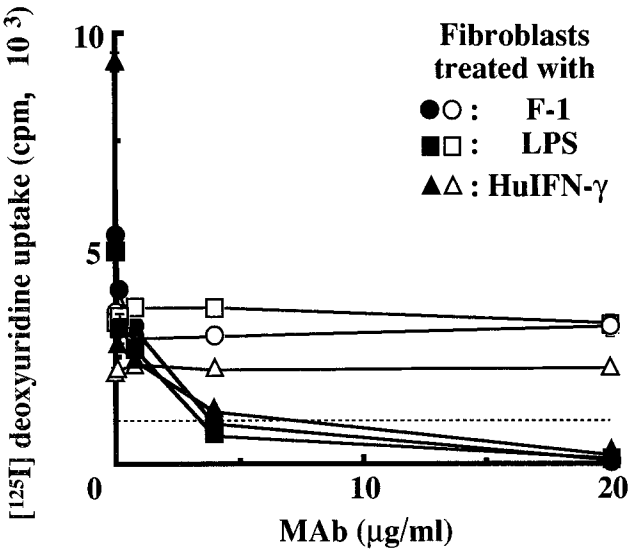


FIG. 9. Inhibitory effects of anti-HLA-DR MAb on F-2-induced T-cell proliferation. Human gingival fibroblasts (GF-5) (5×10^4 cells per well) which were precultured with F-1 (5 μg/ml), *P. intermedia* LPS (5 μg/ml), or HuIFN-γ (200 U/ml) in complete α-MEM for 3 days were further incubated with graded doses of anti-HLA-DR MAb (IOT2a) at 37°C for 1 h. After washing, the treated GF-5 cell monolayers were added to purified human T cells (2×10^5 cells per well) and cultured with F-2 (25 μg/ml; closed symbols) or phytohemagglutinin (5 μg/ml; open symbols) in complete RPMI 1640 medium for 3 days. T-cell proliferation was measured in terms of ^{125}I-deoxyuridine incorporation as described in the text. Results were expressed as mean counts per minute. The dotted line indicates the level of radioactivity incorporated into T cells cultured in the presence of nontreated GF-5 cells.

and IFN-γ production by T cells upon stimulation with F-2 (Fig. 4 and 5). Furthermore, the T-cell-proliferative responses caused by the treated GF-5 fibroblasts were completely inhibited by pretreatment of the fibroblasts with an anti-HLA-DR MAb prior to T-cell activation (Fig. 9).

We also demonstrated that human T cells activated by F-2 exhibited definite cytotoxic effects on GF-5 fibroblasts and squamous carcinoma cells of human mouth origin (Fig. 10 and 11). It may be added here that, in the assay system adopted here, nonpretreated GF-5 fibroblasts showed a susceptibility to activated T cells similar to that of those cells pretreated with F-1, *P. intermedia* LPS, or HuIFN-γ (Fig. 10). This suggests that the cytotoxicity of T cells activated with F-2 does not necessarily depend on the MHC class II antigens on the target cells. In fact, the anti-HLA-DR MAb used scarcely inhibited the cytotoxic effects of F-2-activated T cells on untreated GF-5 fibroblasts (data not shown). Dohlsten et al. (5) reported that the staphylococcal enterotoxins, including SEB, induced cytotoxic T cells which exhibited cytotoxic effects against MHC class II-expressing cells and that anti-HLA-DR antibodies blocked the cytotoxicity of these T cells. The reason for the discrepancies between these previous results and our present results is not clear at present. However, intercellular adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), like MHC class II antigens, may play an important role in superantigen-dependent cell-mediated cytotoxicity of T cells, since ICAM-1 and HLA-DR on antigen-presenting cells have been proposed to function cooperatively to induce T-cell responses (1). In this context, it may be pertinent to point out that IL-1 and IFN-γ increased ICAM-1 expression by fibroblasts in culture (6) and that both F-1 and *P. intermedia* LPS were capable of inducing IL-1 production in gingival fibroblast cultures (18–20). Furthermore, F-1 has been shown to increase the expression of the adhesion molecules ICAM-1 and endothelium-lymphocyte adhesion molecule-1 on human umbilical vein endothelial cells (8). Definite cytotoxicity by T-cell fractions prestimulated with rHuIL-2 alone was observed (Fig. 10). The effector cells may be a small number of activated natural killer (NK) or lymphokine-activated killer cells contained in the L-2-activated T-cell fraction, and their activities increased with neither pretreatment nor coincubation with superantigen (Fig. 10).

The findings described here lead us to speculation on hitherto-undescribed immunopathological effects of oral viridans streptococci, which are derived from the extracellular production of F-2-like superantigen(s) and F-1-like activator(s) on accessory cells such as macrophages, fibroblasts, and endothelial cells. The combined action of these two immunobiologically active extracellular products of oral viridans streptococci could induce the cytotoxic T cells that exert the injurious effects on mucosal epithelial cells, endothelial cells, and fibroblasts and may result in tissue destruction in oral mucosa. These mechanisms may cause a variety of oral mucosal disorders such as lichen planus, the initial stage of periodontitis, and chronic recidivous aphthosis in Behçet's disease by endogenous infection, in which definite T-cell infiltration has been noted (10, 13, 16). Searches for F-2-like superantigens in other oral bacterial species or strains are currently in progress in our laboratories.

In addition, viridans group streptococci are one of the predominant components of normal flora of the human oral cavity and intestinal canal. Immunobiologically active extracellular products of these cocci such as F-1 and F-2 fractions are not cytotoxic by themselves, unlike classical exotoxins such as diphtheria and tetanus toxins, and exert their bioactivities through stimulative effects on general immunological mechanisms of

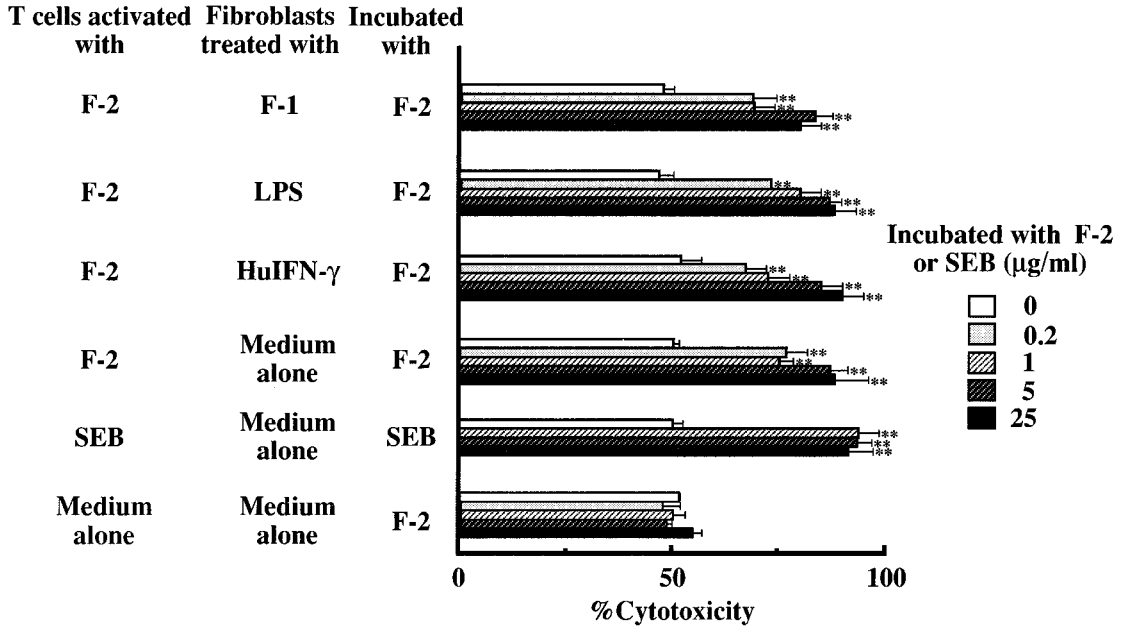


FIG. 10. Cytotoxic activity of F-2- or SEB-activated T cells against human fibroblasts. GF-5 cells (5×10^4 cells per well) were precultured with F-1 (5 μg/ml), *P. intermedia* LPS (5 μg/ml), HuIFN-γ (200 U/ml), or complete α-MEM alone for 3 days. Human T cells (2×10^6 cells per ml) were precultured with F-2 (25 μg/ml), SEB (25 μg/ml), or medium (complete RPMI 1640 medium) alone in the presence of irradiated PBMC in tissue culture dishes for 3 days, passed through a nylon wool column, and grown in the presence of rHuIL-2 (200 U/ml) for an additional 2 days. After being washed with RPMI 1640 medium, the precultured GF-5 cells in monolayer were added with activated T cells (2×10^6 cells per well) in complete RPMI 1640 medium along with graded doses of the same superantigen as that used for pretreatment of the T cells. The reaction mixtures were further incubated for 4 h. After the T cells and detached GF-5 cells were removed by gentle washing, the remaining GF-5 cells were fixed and stained with 2% formaldehyde containing 0.2% crystal violet. Percent cytotoxicity was calculated from the OD_{595} as described in the text. **, significantly different from respective control, in which T cells prestimulated with rHuIL-2 alone were used as effector cells, by Student's *t* test ($P \leq 0.01$).

the host. Furthermore, these extracellular products of viridans streptococci seem to be less potent than the corresponding products of staphylococci and beta-hemolytic streptococci, at least in terms of the activity of culture supernatants by themselves, because no activities of viridans streptococcus culture supernatants were detected unless they were fairly concentrated. It is tempting to speculate that immunobiologically active principles of normal bacterial flora such as viridans streptococci with the least direct toxicity exert continuous stimulatory or modulatory effects upon various functions of the hosts, particularly those relevant to natural and acquired host defense mechanisms.

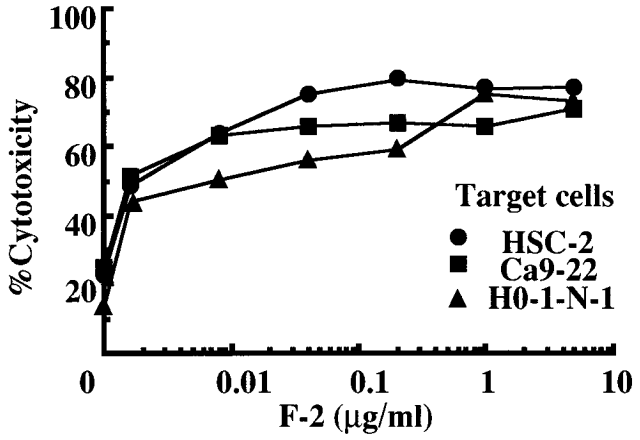


FIG. 11. Cytotoxic activity of T cells activated with F-2 against squamous carcinoma cells. The human squamous carcinoma cell lines (5×10^4 cells per well) HSC-2, Ca9-22, and HO-1-N-1 were precultured in complete α-MEM for 2 days and used as target cells. Other procedures were the same as those described for Fig. 8, except that only F-2 was used as a T-cell stimulant.

ACKNOWLEDGMENTS

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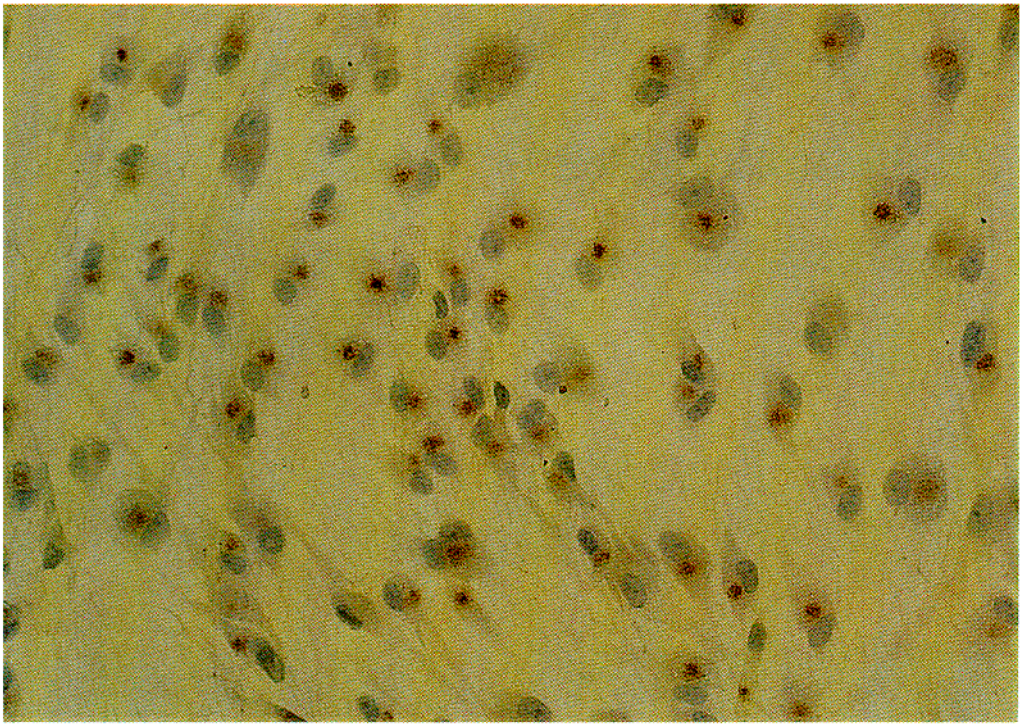

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