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# Human T-cell responses to oral streptococci in human PBMC-NOD/SCID mice

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We investigated cellular and humoral immune responses to oral biofilm bacteria, including *Streptococcus mutans*, *Streptococcus anginosus*, *Streptococcus sobrinus*, and *Streptococcus sanguinis*, in NOD/SCID mice immunized with human peripheral blood mononuclear cells (hu-PBMC-NOD/SCID mice) to explore the pathogenicity of each of those organisms in dental and oral inflammatory diseases. hu-PBMC-NOD/SCID mice were immunized by intraperitoneal injections with the whole cells of the streptococci once a week for 3 weeks. FACS analyses were used to determine the percentages of various hu-T cell types, as well as intracellular cytokine production of interleukin-4 and interferon- $\gamma$ . Serum IgG and IgM antibody levels in response to the streptococci were also determined by enzyme-linked immunosorbent assay. *S. anginosus* induced a significant amount of the proinflammatory cytokine interferon- $\gamma$  in CD4<sup>+</sup> and CD8<sup>+</sup> T cells in comparison with the other streptococci. However, there was no significant differences between the streptococci in interleukin-4 production by CD4<sup>+</sup> and CD8<sup>+</sup> T cells after inoculation. Further, *S. mutans* significantly induced human anti-*S. mutans* IgG, IgG<sub>1</sub>, IgG<sub>2</sub>, and IgM antibodies in comparison with the other organisms. In conclusion, *S. anginosus* up-regulated Th1 and Tc1 cells, and *S. mutans* led to increasing levels of their antibodies, which was associated with the induction of Th2 cells. These results may contribute to a better understanding of human lymphocyte interactions to biofilm bacteria, along with their impact on dental and mucosal inflammatory diseases, as well as endocarditis.

Key words: biofilm; cytokines; interferon- $\gamma$ ; NOD/SCID mice; oral streptococci; T cells

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Generation of effective and efficient cellular immune responses to foreign antigens is considered to be a key function in the elimination of most pathogens (35). Further, such immune responses are not only critical for eliminating intracellular pathogens, but also dictate the amounts and diversity of the antibodies produced (27, 49). Studies that have examined the complex series of events following exposure of human peripheral blood mononuclear cells (hu-PBMC) to intact microorganisms have generally been limited to examining the responses to individual bacterial components. However, because the response to

an intact organism may not necessarily be equal to the sum of the responses to its individual molecules, additional studies that examine responses to whole organisms are still required. Due to recent technological advances (57) and increased understanding of T cell subsets (35), effective tools for examining those responses using human mixed lymphocyte populations, such as hu-PBMC, are now available. The purpose of the present study was to examine the complex cellular and humoral immune responses of hu-PBMC to biofilm bacteria including oral streptococci, as the results could provide valuable

information regarding the control of infectious biofilm organisms. We chose hu-PBMC because they are often the first cells to encounter foreign pathogens in humans (35). In addition, nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice reconstituted with hu-PBMC (hu-PBMC-NOD/SCID) have been used as *in vivo* models for studying human-specific infectious pathogens, such as human immunodeficiency virus (HIV) (9, 15, 46, 47) and oral streptococci (4, 36), as that strain can support levels of hu-cell grafting that are 5- to 10-fold greater than those obtained in SCID mice (35).

Dental caries is one of the most common infectious diseases affecting humans (23) and several different types of bacteria, especially species of viridans streptococci found in dental plaque biofilm, have been shown to be associated with oral infections (2, 19) as well as dental caries (23, 25, 29). Oral streptococci are also important agents of infective endocarditis that are suspected to contribute to its etiology in over 50% of the cases encountered (42, 54). In addition, the viridans group of streptococci has been shown to be responsible for alpha-streptococcal shock syndrome in neutropenic patients (48). However, the mechanisms by which viridans streptococci cause pathogenic lesions and bacteremia associated with severe clinical manifestations have not been elucidated.

In the present study, we examined *Streptococcus mutans*, *Streptococcus sobrinus*, *Streptococcus sanguinis*, and *Streptococcus anginosus* as representative viridans oral biofilm streptococci. *S. mutans* are thought to be present in large numbers in dental plaque biofilm, and there is a strong correlation between the presence of cariogenic organisms in plaque and the occurrence of dental caries in humans (14, 29). *S. sobrinus* is also one of the most common species encountered in human dental plaque, and is considered to be a principal agent of dental caries and oral infections in humans (6, 16) as well as animals (59). In addition, *S. sanguinis* is among the first bacteria to adhere selectively to and colonize saliva-coated teeth (11, 22), while it also interacts with salivary film, which is known to contain  $\alpha$ -amylase, secretory IgA (10, 26), mucin (50), and agglutinin (5). *S. anginosus*, another member of the oral viridans group of streptococci, is a common commensal organism found in the human oral cavity and on other mucosal surfaces, such as in the upper respiratory and gastrointestinal tracts (43). This organism is considered to have a low level of pathogenicity; however, it has been reported to cause serious purulent abscesses in various body sites (11, 40, 55) and subacute bacterial endocarditis (40, 56).

A critical advance in cellular immunology was the discovery of two types of functionally distinct T cell subsets, T-helper type 1 (Th1) and T cytotoxic type 1 (Tc1) cells, and type 2 (Th2 and Tc2) cells, which are classified on the basis of their cytokine expression. Th1 and Tc1 cells produce cytokines that are associated with inflammatory reactions, as well as cell-mediated immune response factors such as interferon- $\gamma$  (IFN- $\gamma$ ) (41). In contrast, Th2 and Tc2 cells release cytokines, including

interleukin (IL)-4, IL-5, IL-6, and IL-10, that promote antibody-mediated immune responses (1, 33, 41). Therefore, IFN- $\gamma$  and IL-4 have important and cross-regulatory roles in infections (38, 52).

In the present study, we examined the effects of the oral viridans streptococci *S. mutans*, *S. sobrinus*, *S. sanguinis*, and *S. anginosus* on leukocyte recruitment, as well as their stimulation of cytokine and antibody production by mononuclear cells *in vivo* in hu-PBMC grafted NOD/SCID mice, an animal model recently shown to be effective for evaluating host responses to oral pathogens (13, 60). Our findings have important implications regarding the regulation of immunity to oral streptococci and the capacity of those pathogens to stimulate the host during oral infection.

## Material and methods

### Mice

Specific pathogen-free female NOD/SCID mice from 6 to 8 weeks old were used in the experiments and housed in polypropylene mouse cages with pine sawdust on the floor. Each experiment utilized six mice, including two controls, and each mouse was transplanted with hu-PBMC from a donor. Animal care was performed in accordance with the guidelines of the National Institute of Infectious Diseases.

### Microorganisms

*S. mutans* MT8148, *S. anginosus* ATCC 33397, *S. sobrinus* 6715, and *S. sanguinis* ATCC 10556 bacterial strains were taken from our laboratory stocks and used as representative strains. The microorganisms were cultured in brain-heart infusion media in anaerobic gas jars (Type AZ-Hard, Hirasawa, Tokyo, Japan) with an atmosphere of 10% CO<sub>2</sub> and 10% H<sub>2</sub> in nitrogen, then harvested by centrifugation and washed twice with 40 mM of sterile phosphate buffered saline (PBS, pH 7.4).

### Antibodies and chemicals

The following monoclonal antibodies were purchased from BD Pharmingen (San Diego, CA): R-phycoerythrin-conjugated anti-hu-CD8 and anti-hu-IFN- $\gamma$ ; fluorescent isothionate (FITC)-conjugated anti-hu-CD4 (clone RPA-T4), anti-hu-CD8 (RPA-T8), anti-hu-CD45 (30-F11), and antimouse CD45 (30-FH); and antigen presenting cell-conjugated anti-hu-IL-4. Alkaline phosphatase-conjugated goat antihuman IgG ( $\gamma$ ), IgG<sub>1</sub>, IgG<sub>2</sub>, and anti-hu-IgM ( $\mu$ )

were also obtained (Organo, West Chester, PA), as were mouse antihuman IgG<sub>3</sub> (5G12) and IgG<sub>4</sub> (HP6025) (Biogenesis, England, UK, and Chemicon International Inc., Temecula, CA). Alkaline phosphatase-conjugated goat antimouse IgG (H+L) antibody (Zymed Laboratory, South San Francisco, CA) was used as a third antibody to detect human IgG<sub>3</sub> and IgG<sub>4</sub>. We also purchased cell separating medium Lympholyte-M (Cedarlane, Hornby, Canada), Eagle's minimal essential medium (Nissui, Tokyo, Japan), RPMI-1640 (Sigma, St. Louis, MO), FACS lysing solution and FACS permeabilizing solution (BD Pharmingen), fetal bovine serum (ICN, Aurora, OH), Saponin and DNase I (Sigma, St. Louis, MO), disodium *P*-nitrophenyl-phosphate hexahydrate (Wako, Osaka, Japan), and trypan blue (Chroma, Stuttgart, Germany).

### Transplantation of hu-PBMC

hu-PBMC were isolated from 400 ml of peripheral blood taken from an individual healthy adult volunteer by separation using Ficoll-Hypaque gradients, with a gravity of  $1.077 \pm 0.001$  (Immuno-Biological Laboratory, Gunma, Japan). The cells were washed three times in Hanks' balanced salt solution (Gibco Laboratories, Life Technologies, Paisley, UK) and adjusted to a concentration of  $2.5 \times 10^7$  in 500  $\mu$ l of Hanks' balanced salt solution. Suspensions of hu-PBMC were then administered intraperitoneally at 500  $\mu$ l per mouse using the transplantation technique reported by Mosier et al. and Senpuku et al. (31, 44). The NOD/SCID model grafted intraperitoneally with hu-PBMC has been employed for long-term *in vivo* analysis of human lymphocyte activation and pathogens (44). For each experimental group, six female mice from a single litter were grafted with PBMC from a donor and used.

### Injection of humanized NOD/SCID mice with oral streptococci

One day after the experimental groups, each consisting six NOD/SCID mice, were transplanted with hu-PBMC from a single donor, the mice were injected intraperitoneally with 100  $\mu$ g of each bacterial strain (dry weight/mouse,  $1 \times 10^7$  colony forming units) suspended in 300  $\mu$ l of 40 mM PBS, pH 7.0. Intraperitoneal injections of *S. mutans*, *S. anginosus*, *S. sobrinus*, *S. sanguinis*, or PBS (control) were given to five mice in separate groups, with the remaining

noninjected mouse in each group used as a negative control. Intraperitoneal injection has been reported to be a useful method to induce specific immunoresponses by hu-lymphocytes to immunogens in the hu-PBMC-NOD/SCID system (34, 44). The injections were given once a week for 3 weeks to both the experimental and the control mice. Three weeks after the final injection, blood serum, spleen cells, and peritoneal exudate cells were sampled for the following experiments. Each experiment was performed independently three or four times.

#### Flow cytometric analysis

Peritoneal exudate cells were collected by washing the peritoneal cavities twice with RPMI-1640 containing 10% heat-inactivated fetal bovine serum. The spleens were removed, then teased and filtered through a stainless steel mesh (Ikemoto, Tokyo, Japan) in ice-cold Hanks' balanced salt solution, after which mononuclear cells were isolated by density gradient centrifugation and contaminating erythrocytes were lysed with 0.83% ammonium phosphate buffer solution (pH 7.6). After washing, the cells were counted using a hemacytometer. All cell suspensions were washed once in ice-cold Hanks' balanced salt solution as described below. The spleen cells and peritoneal exudate cells were stained with FITC-, phycoerythrin-, and antigen presenting cell-conjugated antihuman-marker mAbs in PBS/1% bovine serum albumin, then washed with Hanks' balanced salt solution medium. At least  $10^4$ – $10^5$  live spleen cells, including mouse and hu-lymphoid cells, were acquired in each run. For each mouse analyzed, cells were also stained with mouse IgG conjugated to FITC, phycoerythrin, and antigen presenting cell as an isotype control. Spleen cells and peritoneal exudate cells from a nontransplanted NOD/SCID mouse were stained in parallel as an additional negative control. Fluorescence levels that excluded greater than 98% of the cells in the negative controls were considered to be positive and specific for hu-staining. The cells were fixed in a 3% formalin/Hanks' balanced salt solution and stored at 4°C until flow cytometric analysis. Samples gated on the forward light scatter and side light scatter were used to identify viable lymphocytes. Proportions of the major subsets were determined by single and quadrant analyses. Single cell suspensions were stained with the following antibodies: fluorescein isothiocyanate (FITC)-

conjugated antimouse CD45, antihuman-CD45, antihuman-CD4, and phycoerythrin-conjugated antihuman-CD8. The percentages of FITC- and phycoerythrin-positive cells were measured using a FACS with the CELLQUEST program (Beckton Dickinson, San Jose, CA). Negative control samples were incubated with irrelevant, isotype-matched antibodies in parallel with all of the experimental samples, and produced no more than 0.2% fluorescent CD4<sup>+</sup> or CD8<sup>+</sup> T cells.

In a detection assay of intracellular cytokine production, spleen cells and peritoneal exudate cells were incubated for 4 h at 37°C in brefeldin A (10 µg/ml), then 100 µl of each were incubated for 15 min at room temperature with either FITC-conjugated antihuman-CD4 or antihuman-CD8 monoclonal antibodies (mAbs). Next, the cells were lysed with FACS lysing solution and permeabilized using FACS permeabilizing solution according to the manufacturer's instructions. The cells were then washed with PBS containing 2% fetal bovine serum and incubated for 30 min at room temperature in the dark with either phycoerythrin-conjugated anti-IFN-γ or antigen presenting cell-conjugated anti-IL-4. The cells were washed and resuspended in 500 µl of 1% paraformaldehyde, and cell fluorescence was measured using a Becton Dickinson FACScan. Cell events were acquired using gates set by forward and side scatter plots to determine the proportion of each T cell subset, as well as by a side scatter plot and FL1 (FITC), FL2 (phycoerythrin), or FL3 (antigen presenting cell) to determine the proportion of cytokine-producing T cells for each subset (CD4<sup>+</sup> or CD8<sup>+</sup>). To ensure that only intracellular proteins were quantified, the cells were fixed but not permeabilized, resulting in less than 0.2% fluorescence per subset of T cells (CD4<sup>+</sup> or CD8<sup>+</sup>). The analysis gate was set on CD4<sup>+</sup> and CD8<sup>+</sup> T cells and invariant quadrant histograms were constructed for IL-4<sup>+</sup>/CD4<sup>+</sup>, IFN-γ<sup>+</sup>/CD4<sup>+</sup>, IL-4<sup>+</sup>/CD8<sup>+</sup>, and IFN-γ<sup>+</sup>/CD8<sup>+</sup> T cells. Data are expressed as the percentage of positive cells.

#### Enzyme-linked immunosorbent assay (ELISA)

Three weeks after the final injection of streptococci, the mice were killed under anesthesia with diethyl ether and serum was collected from a retinal vein puncture. A conventional sandwich ELISA method was used to detect serum IgG and IgM antibodies against all of the tested streptococci. Briefly, 96-well microtiter H-plates (Sumitomo Bakelite, Tokyo, Japan) were

coated at 4°C with 20 µg of each of the streptococci per well in 50 mM of carbonate buffer (pH 9.6) after sonication. The plates were washed with PBS containing 0.1% (v/v) Tween 20 (PBST) and blocked with 1% (wt/vol) skim milk in PBST at 37°C for 1 h. Excess skim milk in PBST was removed by three washes with PBST, and 100 µl of a 1/200 dilution of the serum sample including serum/0.5% skim milk in PBST was added to the wells and the plates incubated at 37°C for 1 h. The wells were then washed five times with PBST and further incubated at 37°C for 1 h with 100 µl of a 1/1000 dilution of alkaline phosphatase-conjugated goat antihuman-IgG, IgM, IgG<sub>1</sub>, or IgG<sub>2</sub> antiserum, and mouse antihuman-IgG<sub>3</sub> and -IgG<sub>4</sub> monoclonal antibodies. To detect IgG<sub>3</sub> and IgG<sub>4</sub> after washing five times with PBST, 100 µl of a 1/1000 dilution of alkaline phosphatase-conjugated goat antimouse IgG (H +L) antibody was added to the wells and incubated at 37°C for 1 h. After five washes with PBST, bound antibodies were detected by the addition of 100 µl of 3 mg/ml of *p*-nitro-phenyl phosphate hexahydrate (Wako) as a substrate and incubated at 37°C for 1 h. The absorbance at 405 nm ( $A_{405}$ ) was measured with a Multiskan Bichromatic microplate reader (Labsystems, Helsinki, Finland).

#### Statistical analysis

Statistical analyses were performed using ANOVA. *P*-values of 0.05 or less were considered to indicate statistical significance.

#### Results

##### CD45<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> cell responses to oral streptococci

Three weeks after the final injections of the oral streptococci, the mice were killed, and the hu-leukocyte contents and subset distributions in suspensions of spleen cells and peritoneal exudate cells were analyzed by flow cytometry. hu-CD45<sup>+</sup> cells represented up to 72.6 ± 9.3% (range 19.0 ± 4.3% to 72.6 ± 9.3%) and 75.6 ± 12.3% (range 40.0 ± 5.4% to 75.6 ± 12.3%) of the cells ( $2.5 \times 10^7$ ) recovered from the spleen and among the peritoneal exudate cells, respectively, in both streptococci-injected and PBS-injected hu-PBMC-NOD/SCID mice (Figs. 1A and 2). The percentages and total numbers of hu-CD45<sup>+</sup> cells were significantly increased among the spleen cells following immunization with *S. mutans* (72.6 ± 9.3% and  $20.2 \pm 4.3 \times 10^6$ ,

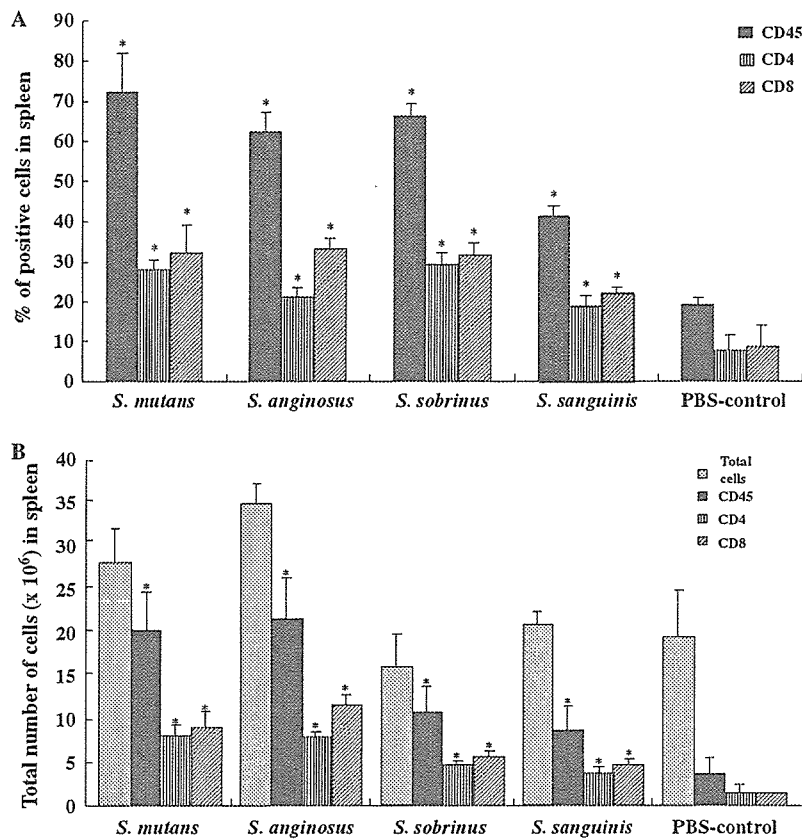


Fig. 1. Flow cytometric analyses of proportions of hu-CD45<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> cells present in the spleens from grafted NOD/SCID mice injected with *Streptococcus mutans*, *Streptococcus anginosus*, *Streptococcus sobrinus*, *Streptococcus sanguinis*, and phosphate-buffered saline (PBS; control). The analyses were performed on gated lymphocytes with forward light scatter/side light scatter characteristics. Lymphocytes were distinguished in the quadrant analysis. Data are shown as proportions (A) and populations (B) of hu-CD45<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> cells, and are expressed as the mean ± SD of four independent assays. Asterisks denote significant differences (\**P* < 0.05, vs. PBS).

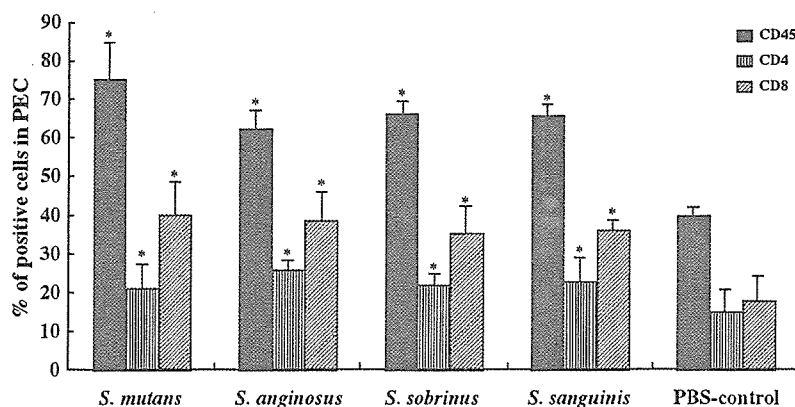


Fig. 2. Flow cytometric analyses of proportions of hu-CD45<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> cells present in peritoneal exudate cells from grafted NOD/SCID mice injected with *Streptococcus mutans*, *Streptococcus anginosus*, *Streptococcus sobrinus*, *Streptococcus sanguinis*, and phosphate-buffered saline (PBS; control). The analyses were performed on gated lymphocytes with forward light scatter/side light scatter characteristics. Lymphocytes were distinguished in the quadrant analysis. Proportions of hu-CD45<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> cells are shown as the mean ± SD of four independent assays. Asterisks denote significant differences (\**P* < 0.05, vs. PBS).

respectively), *S. anginosus* ( $62.5 \pm 4.3\%$  and  $21.5 \pm 4.7 \times 10^6$ , respectively), *S. sobrinus* ( $66.4 \pm 3.3\%$  and  $10.7 \pm 4.3 \times 10^6$ , respectively), and *S. sanguinis* ( $41.1 \pm 5.8\%$  and  $8.6 \pm 2.8 \times 10^6$ , respectively) in comparison with the controls, which were injected with PBS ( $19.0 \pm 3.6\%$  and  $3.6 \pm 1.9 \times 10^6$ , respectively) (Fig. 1A, B). The percentages of hu-CD4<sup>+</sup> and CD8<sup>+</sup> T cells were also significantly increased among the spleen cells following injection with *S. mutans* ( $28.1 \pm 2.3\%$  and  $32.4 \pm 6.6\%$ , respectively), *S. anginosus* ( $21.2 \pm 2.3\%$  and  $33.2 \pm 2.6\%$ , respectively), *S. sobrinus* ( $29.3 \pm 3.3\%$  and  $31.7 \pm 4.3\%$ , respectively), and *S. sanguinis* ( $18.6 \pm 2.8\%$  and  $21.9 \pm 1.8\%$ , respectively) in comparison with the control ( $7.5 \pm 3.9\%$  and  $8.5 \pm 5.4\%$ , respectively) (Fig. 1A). In addition, the total numbers of CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets among the spleen cells from hu-PBMC-NOD/SCID mice were significantly increased following immunization, in comparison with the PBS-injected control mice (Fig. 1B). The relative percentages of CD45<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> cells among peritoneal exudate cells were also significantly increased following immunization with *S. mutans* ( $75.6 \pm 9.3\%$ ,  $21.0 \pm 6.6\%$ , and  $40.1 \pm 8.6\%$ , respectively), *S. anginosus* ( $62.5 \pm 4.8\%$ ,  $26.1 \pm 2.2\%$ , and  $38.8 \pm 7.3\%$ , respectively), *S. sobrinus* ( $66.4 \pm 4.3\%$ ,  $21.9 \pm 3.3\%$ , and  $35.31 \pm 6.9\%$ , respectively), and *S. sanguinis* ( $65.9 \pm 6.8\%$ ,  $22.9 \pm 6.2\%$ , and  $36.3 \pm 3.2\%$ , respectively) in comparison with the PBS controls ( $40.0 \pm 3.9\%$ ,  $15.2 \pm 5.4\%$ , and  $17.9 \pm 6.4\%$ , respectively) (Fig. 2). In contrast, the ratios of CD4<sup>+</sup>/CD8<sup>+</sup> T cells from mice infected with *S. mutans*, *S. anginosus*, *S. sobrinus*, and *S. sanguinis* were slightly reduced among spleen cells ( $0.76 \pm 0.25\%$ ,  $0.68 \pm 0.12\%$ ,  $0.66 \pm 0.31\%$ , and  $0.76 \pm 0.25\%$ , respectively), as well as among peritoneal exudate cells ( $0.59 \pm 0.18\%$ ,  $0.57 \pm 0.12\%$ ,  $0.62 \pm 0.25\%$ , and  $0.68 \pm 0.19\%$ , respectively) in comparison with PBS-injected mice ( $0.85 \pm 0.32\%$  and  $0.91 \pm 0.32\%$ , respectively). These results demonstrate that the tested oral streptococci influenced human T cells engrafted in NOD/SCID mice to penetrate, activate, and proliferate.

#### Inductions of IFN- $\gamma$ and IL-4 in CD4<sup>+</sup> and CD8<sup>+</sup> T cells by oral streptococcal stimulation

To explore the mechanisms involved with the differences in inflammatory

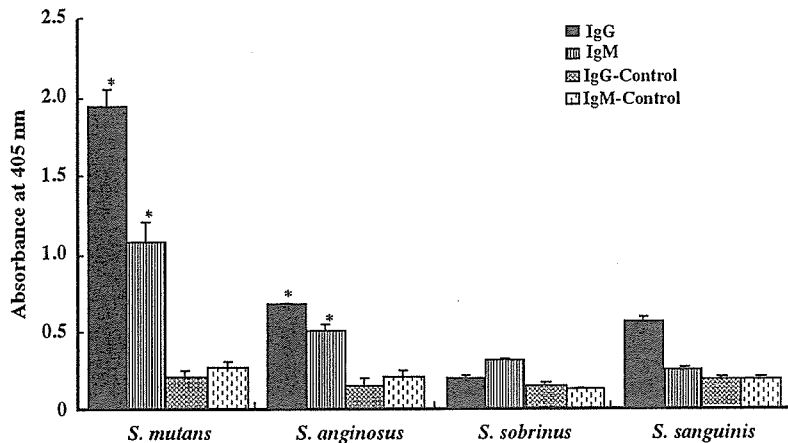


Fig. 5. Production of anti-hu-IgG and IgM antibodies in serum samples from grafted NOD/SCID mice injected with *Streptococcus mutans*, *Streptococcus anginosus*, *Streptococcus sobrinus*, *Streptococcus sanguinis*, and phosphate-buffered saline (PBS; control). Sera were collected 3 weeks after the last injection of bacteria. Anti-human IgG and IgM antibodies in the samples were analyzed by enzyme-linked immunosorbent assay. Results are expressed as the mean  $\pm$  SD of absorbance at 405 nm in triplicate assays. The experiments were performed on three lots of serum antibodies from the injected hu-PBMC-NOD/SCID mice, with similar results obtained in each experiment. Asterisks denote significant differences ( $*P < 0.01$ , other streptococci).

with *S. mutans* showed increases in levels of IgG<sub>1</sub> and IgG<sub>2</sub> anti-*S. mutans* antibody responses following the third immunization, and the specific antibody levels in those mice were significantly different from the levels in the other groups injected with oral streptococci (Fig. 6,  $P = 0.001$ ), as well as the *S. sobrinus* and *S. sanguinis* groups (data not shown). However, significant increases in serum IgG<sub>3</sub> and IgG<sub>4</sub> anti-*S. mutans* antibody levels were not

observed in mice injected with *S. mutans* or in the other groups (data not shown).

#### Discussion

The hu-PBMC-NOD/SCID mouse model has been shown to be useful for *in vivo* studies of hu-immune functions under normal and pathologic conditions (3, 37), including oral streptococci and HIV infections (30, 34, 45). Further, optimization of

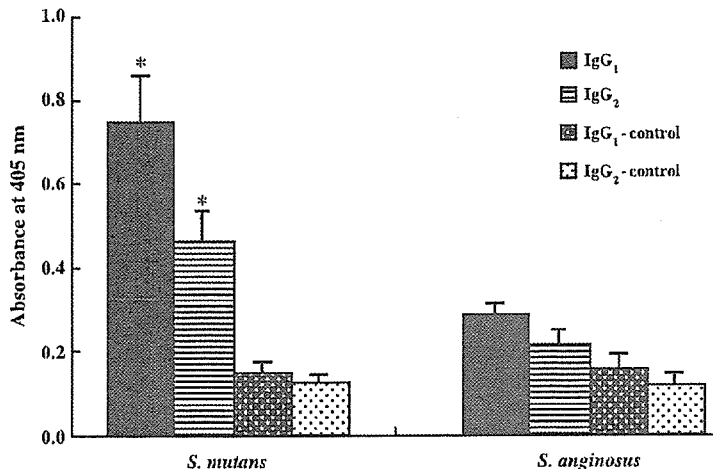


Fig. 6. Production of anti-hu-IgG<sub>1</sub> and IgG<sub>2</sub> in serum samples from grafted NOD/SCID mice injected with *Streptococcus mutans* and *Streptococcus anginosus*. Sera were collected 3 weeks after the last injection of bacteria. Anti-hu-IgG<sub>1</sub> and anti-IgG<sub>2</sub> antibodies were analyzed by enzyme-linked immunosorbent assay. Results are expressed as the mean  $\pm$  SD of absorbance at 405 nm in triplicate assays. The experiments were performed on three lots of serum antibodies from the injected hu-PBMC-NOD/SCID mice, with similar results obtained in each experiment. Asterisks denote significant differences vs. *S. anginosus* ( $*P < 0.01$ , *S. mutans* vs. *S. anginosus*).

hu-T cell engrafting in a suitable small animal model like the NOD/SCID mouse is of substantial importance for studies of oral pathogens, as well as AIDS and HIV infection (24, 30, 34), as T cells have been shown to play important roles in both cellular and humoral responses to intra- and extracellular pathogens (27, 35). Grafted hu-T cells are likely to have secondary immune responses to *S. mutans* in the NOD/SCID mouse system, because oral streptococci are common commensal bacteria and initialize lymphocytes at an early age in humans (45). In the present experiments, we found increased levels of engrafted hu-CD45<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> cells among the spleen cells and peritoneal exudate cells from hu-PBMC-NOD/SCID mice 3 weeks after the final immunization with the tested oral streptococci (Fig. 1 and 2). *S. anginosus* induced higher levels than the other tested oral streptococci of IFN- $\gamma$ -producing CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the spleens of hu-PBMC-NOD/SCID mice, as well as among the peritoneal exudate cells from those mice. However, there were no significant differences in the numbers of CD4<sup>+</sup> and CD8<sup>+</sup> T cells that produced IL-4 in the spleens and among peritoneal exudate cells from hu-PBMC-NOD/SCID mice after infection with the tested streptococci, whereas IFN- $\gamma$  production in mice injected with *S. anginosus* was higher than that of IL-4. These results show that among the oral streptococci tested in the present study, *S. anginosus* induced Th1 and Tc1 cell responses to a greater degree than Th2 and Tc2 cell responses in comparison with the other streptococci. *S. anginosus*, *Streptococcus constellatus*, and *Streptococcus intermedius* are known for purulent infections that occur following local disruption of the mucosal barrier, such as in cases of ulceration, perforation, inflammation, and surgery (17, 40). Our results indicate that stimulation and recruitment of CD45<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> lymphocytes, as well as IFN- $\gamma$ -producing CD4<sup>+</sup> and CD8<sup>+</sup> T cells, are involved in the pathogenesis of purulent infections caused by *S. anginosus*.

We also found that anti-*S. mutans* hu-IgG and IgM antibody responses in sera from mice immunized with *S. mutans* were significantly higher than those of specific antibodies in mice infected with the other streptococci. We observed higher levels of IgG<sub>1</sub> and IgG<sub>2</sub> antibody responses to the antigens in hu-PBMC-NOD/SCID mice immunized with *S. mutans* as compared to mice immunized with the other streptococci, though the level of IgG<sub>1</sub> was significantly higher than

that of IgG<sub>2</sub>. In mice, Th1 and Th2 cells mediate the production of IgG<sub>2a</sub> and IgG<sub>1</sub> antibodies, respectively (20, 32, 49). In humans, Th2 cytokine enhances the switching to IgG<sub>2</sub> and IgG<sub>4</sub> in B cells (18). Whole cells of *S. mutans* induced specific IgG<sub>1</sub> and IgG<sub>2</sub>, but not IgG<sub>3</sub> or IgG<sub>4</sub>, production, as well as an increase in the average levels of Th1 and Tc1 type reactions in comparison with the other streptococci, except for *S. anginosus*. In addition, *S. mutans* did not induce significant IL-4 production in these mice (Fig. 4). It was previously reported that immune responses and susceptibility to IL-4 production are low in the NOD/SCID mouse system, whereas the responses and susceptibility to IFN- $\gamma$  production are high (45). In addition, the production of CD8 and IFN- $\gamma$  has been found to be dominant in long-term experiments with those mice (34, 44). Further, immunization with the peptide or protein antigen from *S. mutans* may induce antigen specific hu-antibodies in NOD/SCID mice (45, 53), and the noncorrelation between IL-4 production or stimulation and antibody production may indicate a unique *in vivo* immune response by hu-lymphocytes to *S. mutans* in these mice. The induction of antigen specific hu-IgG and IgM antibodies following immunization with *S. mutans* in the mice in the present study supports the results of various vaccine studies that used *S. mutans* antigens (28, 51).

*S. sanguinis* did not strongly induce CD45<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> cells, intracellular cytokine-producing cells or antigen specific antibodies in comparison with the other streptococci tested in the present study. Generally, *S. sanguinis* together with *Streptococcus gordonii*, *Streptococcus mitis*, *Streptococcus oralis*, and *Streptococcus parasanguis* (21) are prominent components of human oral microbiota and play a significant role as biofilm pioneer colonizers in the development of dental biofilm (8, 39). *S. sanguinis* is frequently found in the oral cavities of young children and is known to induce microbial oral flora. Therefore, it is possible that an immunologic tolerance to *S. sanguinis* may develop in human immune systems. Humoral antibody responses to *S. sanguinis*, which is consistently found in the subgingival plaque of periodontal patients, were reported to be depressed in periodontitis patients compared with plaque of healthy controls (7). In contrast, serum samples from patients with Behcet's disease, a chronic multisystemic inflammatory disorder, showed significantly higher anti-

body titers to *S. sanguinis* clinical strains than samples from healthy controls (58). It has been hypothesized that *S. sanguinis* is harmonized as a nonstimulator in normal immune systems, but not with lymphocytes in irregular immune systems. Further, the saliva IgA antibody to the surface protein antigen from *S. mutans* has been speculated to be a negative contributor as compared to the number of *S. mutans* organisms in the oral cavity (26), as the saliva antibody may regulate the attachment, colonization, and biofilm formation of *S. mutans* on the tooth surface. *S. sobrinus* produces insoluble and soluble glucans, and an antigenic surface protein, which is similar to *S. mutans*. However, neither *S. sobrinus* nor *S. sanguinis* induced significant stimulation of Th and Tc cells, or hu-IgG and IgM production in the present study. Taken together, *S. mutans* may act as a stimulator of specific antibody production in humans, which leads to immune reactivity that is different from other streptococci.

In conclusion, we found that *S. anginosus* infection induced significant levels of the proinflammatory cytokine IFN- $\gamma$  in CD4<sup>+</sup> and CD8<sup>+</sup> cells, as well as T cells in hu-PBMC-NOD/SCID mice in comparison with other streptococci, and infection with *S. mutans* caused increased levels of hu-IgG, IgG<sub>1</sub>, IgG<sub>2</sub>, and IgM antibodies in serum samples. In contrast, *S. sanguinis* and *S. sobrinus* showed poor responses to stimulation by hu-PBMC. Our results suggest that biofilm bacteria have various properties and roles in the human immune system, such as the stimulation of Th1 and Tc1 by *S. anginosus* and antibody production by *S. mutans*. These findings may contribute to a better understanding of lymphocyte interactions among cytokines and immunoglobulins to biofilm bacteria, the control of oral flora by immune functions, and the impact on dental caries accompanied by pulpal infections, as well as inflammatory diseases in mucosal tissues and endocarditis.

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