Human T-Cell Responses to the Glucosyltransferases of *Streptococcus mutans*

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Received 21 July 2000/Returned for modification 29 September 2000/Accepted 15 November 2000

We previously reported differential humoral responses to glucosyltransferases (GTFs), with significantly higher saliva and serum antibody levels to GtfD than to GtfB or GtfC. To test the hypothesis that cellular immune responses to these molecules also may differ, peripheral blood mononuclear cell (PBMC) and T-cell proliferative responses in young adults and children with distinct genetic backgrounds were determined using purified recombinant GtfC and GtfD. PBMCs from all of the volunteers responded to GtfC and -D, but responses were directed predominantly towards GtfD and were major histocompatibility class II antigen dependent. A predominant T-cell response to GtfD, over GtfC, was detectable at various antigen concentrations ranging from 1 to 20 μg/ml and correlated with the differential serum immunoglobulin G (IgG) and salivary IgA antibody responses to the GTFs. Therefore, in naturally sensitized humans, *Streptococcus mutans* GTFs stimulate differential humoral and cellular immune responses, with the secreted form of GtfD eliciting a stronger response than the cell wall-associated form of GtfC.
spite a readily detectable systemic and mucosal antibody re-
response supports this hypothesis (6). As an initial step to test 
the hypothesis that the various GTFs of *S. mutans* may direct 
differential responses at the cellular level as well, we analyzed 
the T-cell proliferative responses to different GTFs from nat-
urally sensitized humans of different age groups.

The volunteers who participated in the present study were 
30 healthy students, 20 to 22 years of age, from National 
Taiwan University and 6 children, 6 to 8 years of age, from the 
Pediatric Department of National Taiwan University Hospital. 
Umbilical blood was collected routinely from the Gynecology 
Department of National Taiwan University Hospital. The 
statement on the informed consent form for use of human sera 
and umbilical blood samples followed the regulation of the 
university hospital committee. Unstimulated whole saliva was 
collected from students by direct expectoration into sterile 
15-ml containers. The saliva samples were clarified by centrif-
gagation at 6,500 × *g* for 30 min; sediments were discarded, 
and aliquots of clarified saliva were stored frozen at −70°C until 
use for enzyme-linked immunosorbent assay or Western blot 
analysis, as described previously (3). Total IgA concentrations 
in the saliva samples were measured by nephrometry (Behring, 
Marburg, Germany). Genetic typing of HLA-DRB1 of the 
volunteers was carried out by PCR and sequence-specific oli-
gonucleotide probe (SSOP) hybridization (21). In brief, HLA-
 generic and HLA-DRB1-specific PCRs were carried out to 
amply the second exons of generic DRB and HLA-DRB1 
genomes. The PCR product was denatured with NaOH-EDTA 
dotted onto nylon membranes (Pall, Glen Cove, N.Y.). 
Panels of 5'-biotinylated SSOPs were used to characterize the 
polyorphic regions in the exon. The membrane was hybrid-
ized with the 5'-biotinylated SSOPs in the presence of strepta-
vidin-peroxidase and subsequently subjected to stringent wash-
ing. The hybridization signal was viewed by chemilumine-
gence generated by the ECL gene detection system (Amersham). 
Using DRB-generic and DRB1-specific PCR together with 25 
SSOPs, more than 90% of HLA-DRB1 alleles present in the 
Taiwanese population could be determined (15). Subspecifici-
ycts of HLA-DR2 (DRw15 and DRw16), -DR7, and -DR9 
types were not characterized further.

Recombinant GtfC and GtfD expressed in *Escherichia coli* 
were purified by chromatography on an Ni²⁺ affinity resin. The 
gtfC coding sequence in pNH3 (13) was digested with *PshAl* 
and inserted into the *NheI*-HindIII sites of plasmid pRSETA 
(Invitrogen, Carlsbad, Calif.). The resulting plasmid, pRSET-
AgtfC, expressing gtfC with a deletion of its signal sequence 
(amino acids 1 to 43) and an N-terminal 6-His tag, was intro-
duced into *E. coli* BL21(DE3) (Novagen Inc., Madison, Wis.), 
which contains the T7 polymerase gene on the chromosome 
under the control of the lacUV5 promoter. Plasmid pYND72 
(22), expressing gtfD under the control of the lac promoter, was 
altered to introduce a 7-His tag. Cloning of the His tag into 
FYND72 was carried out by insertion of a DNA fragment 
encoding nine amino acid residues into the *salI* site in gtfD. 
The inserted DNA sequence was constructed by annealing two 
oligonucleotides, 5’-TCTGAGCATCAGCATCAGCATCAGCATCAGCAT-3’ 
and 5’-TCGAACTGATGATGATGATGATGATGC-3’, which are complementary and encode seven His residues. The resulting plasmid, which expresses gtfD with a seven-His tag immediately C terminal to the putative signal sequence (amino acids 1 to 29), was termed pYND72-His. *E. coli* har-
boring pRSETAgtfC or pYND72-His was grown to an *Aso* of 
0.4 to 0.5, and the T7 or lac promoter was induced by the 
addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a 
final concentration of 2.0 mM. The cultures were grown for an 
additional 4 h and then harvested. The pellets were resus-
pended in binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM 
Tris-HCl [pH 7.9]), and a cell lysate was prepared by disrupting 
the cells with sonication. The cell debris was removed by cen-
trifugation at 15,000 × *g* for 30 min. Further steps in the 
purification of His-GtfC and -GtfD were performed according 
to the pET instruction manual provided by the manufacturers.

Homogeneity of the purified proteins was confirmed by sodium 
dodecyl sulfate-polyacrylamide gel electrophoresis, followed 
by silver staining or activity staining using periodic acid-Schiff 
reagent (31). The bands were analyzed with an Electrophoresis 
Documentation and Analysis System 120 (Scientific Imaging 
Systems, Eastman Kodak Co., Rochester, N.Y.). Protein concen-
trations were determined using a modification of the 
method of Lowry et al. (18), with bichoninic acid as the 
colorimetric detection reagent (BCA Protein Assay Reagent; 
Pierce). GTF activity was determined by the [¹⁴C]glucose-su-
ccess (New England Nuclear Corp., Boston, Mass.) incorpo-
ration assay as described previously (5). After purification, the 
purity of the GtfD was found to be 98.5%. However, for GtfC, 
a degradation product of lower molecular weight was consist-
etsly found immediately after elution. Only minor bands from 
the *E. coli* host were observed in the final purified GtfC and 
GtfD, following prolonged exposure of sodium dodecyl sulfate-
polyacrylamide gels to silver staining reagent. The authenticity 
of GtfC and -D was confirmed by Western blot analysis using 
PJS-2 and PJS-3 antibodies (2), and their biological activities 
were detectable by activity gel staining.

*S. mutans* MT-8148 was grown in brain heart infusion broth 
(Difco Laboratories, Detroit, Mich.). The extracellular protein 
antigens (EXP-A) and cell wall-associated protein antigens 
(CWP-A) were prepared as described previously (4). Phytohe-
magglutinin (PHA) and staphylococcal enterotoxin B (SEB) 
were purchased from Sigma Chemical Co. (St. Louis, Mo.). 
Glutaraldehyde-inactivated tetanus toxoid (TT) was provided 
by Ming-Yi Liau of the Department of Health, Center for 
Disease Control, Vaccine Center, Taiwan, Republic of China. 
Monoclonal antibody (MAb) MCA477 for HLA-DR DP DQ 
were purchased from Serotec Ltd. (Oxford, United Kingdom). 
Mononuclear cells or enriched T cells were analyzed 
using a FACSscan (Becton Dickinson, San Jose, Calif.), 
and data analysis was performed using the LYSIS II software 
program. All antigens, including purified GtfC and -D and 
reagents used for proliferation assays, exhibited undetectable 
endotoxin levels (<30 pg/ml) as determined by the *Limulus* 
amoebocyte lysate assay (Sigma).

Mononuclear cells were isolated from peripheral blood or 
umbilical blood specimens from healthy children or adult vol-
unteers by Ficoll-Hypaque centrifugation. Irradiated human 
umbilical vein endothelial cells. Suspensions (2 × 10⁵ cells per 50 μl) of PBMC 
in RPMI 1640 medium (Gibco BRL Laboratories, Grand Is-
land, N.Y.), supplemented with 10% fetal calf serum (Gibco 
BRL), (complete RPMI medium) were irradiated at 2,000 rads 
with an X-ray irradiator (Hitachi Medical Co., Tokyo, Japan).
to inhibit proliferation and used as accessory cells in T-cell proliferation assays. For enrichment of T cells, human PBMC or mononuclear suspensions in complete RPMI 1640 medium were passed through a nylon wool column to deplete B cells and macrophages. Alternatively, T cells were enriched directly from whole blood by antibody-mediated separation with RosetteSep (StemCell Technologies Inc., Vancouver, Canada). The enriched T-cell fractions were collected and used in the proliferation assays.

PBMC or mononuclear cells were washed and resuspended in AIM-V (Gibco BRL) supplemented with 2 mM L-glutamine, penicillin (100 μg/ml), streptomycin sulfate (100 μg/ml), and 2% serum replacement TCH (Celsis, St. Paul, Mich.). PBMC (2 × 10⁵ cells per well) were cultured in 96-well round-bottomed plates (Costar, Cambridge, Mass.) in a total volume of 200 μl. Purified T cells (1 × 10⁵ cells per well) were cultured in the presence of irradiated autologous PBMC (2 × 10⁵ cells per well) in RPMI 1640 supplemented with 2% fetal calf serum, 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol, penicillin (100 μg/ml), streptomycin sulfate (100 μg/ml), and 2% TCH (Celox). Three replicates of each culture were incubated with various concentrations (1 to 40 μg/ml) of recombinant GtfC and -D or crude extracts of CWP and EXP or were unsupplemented controls. Incubation was at 37°C in a humidified atmosphere with 5% CO₂ for 4 days. Each culture received 0.2 μCi (7.4 kBq) of [³H]thymidine (Amersham International, Little Chalfont, United Kingdom) 18 h before harvesting. Cultures were harvested onto 96-uniform GF/C plates using a FilterMate cell harvester (Packard, Meriden, Conn.) and dried at 50°C for 30 min, and 30 μl of Microscint (Packard) was added per well. [³H]thymidine incorporation was measured with a Packard microplate scintillation counter. Proliferation was expressed as the stimulation index (SI), which was calculated as the mean counts per minute of antigen-stimulated cultures divided by the counts per minute of antigen-free controls. PHA (10 μg/ml), SEB (0.01 to 20 ng/ml), and TT (5 to 20 μg/ml) were used with every culture as positive controls. The major histocompatibility complex (MHC) dependency of PBMC proliferative responses to GtfC and -D were determined by culturing lymphocytes with antigens, as described above, in the presence of MAb MCA477, an anti-MHC class II MAb. Cultures were incubated with [³H]thymidine and harvested, and [³H]thymidine uptake was determined as described above.

Saliva and plasma samples from 23 volunteers were initially analyzed by Western blotting using purified GtfC and -D. The majority of the samples exhibited significantly higher levels of salivary IgA and serum IgG antibodies to S. mutans GtfD than to GtfC. The differential antibody responses to the S. mutans GTFs were more pronounced in the saliva IgA. These results confirmed our earlier findings that salivary and serum antibody responses to the S. mutans GTFs are different, although the proteins share extensive homology in their primary amino acid sequences.

To test the hypothesis that individual cellular immune responses to GTFs may occur, PBMC from 22 adults and 6 children were examined following stimulation in vitro with purified GtfC and -D. PBMC from either adults or children responded well to the GtfC and -D and TT, with an SI significantly higher than that following stimulation by CWP-A or EXP-A (Table 1). In parallel to the antibody responses, all individuals exhibited significantly higher responses to GtfD than to GtfC at doses of 5 to 20 μg/ml, with peak responses generally at 20 μg/ml (data not shown). Although considerable variation in SI was observed with the different antigens, on the whole, GtfD stimulated PBMC proliferation about two-and-a-half-fold higher than GtfC (P < 0.01) in children and adults. Although the recombinant GtfC and -D preparations included traces of components derived from E. coli, which may themselves induce proliferative responses as indicated by the stimulation of cord blood mononuclear cells (Table 1), no differential stimulatory effects were observed for GtfC and GtfD. Moreover, GtfC and -D stimulated proliferation of these cells to an extent far below that of the mitogen PHA or superantigen SEB, in terms of antigen concentration or SI. These results suggested that recombinant GtfC and -D preparations stimulated proliferation of populations of cells other than T cells nonspecifically. Antigen-dependent stimulation of T cells by GtfC, GtfD, and TT was confirmed by inhibition experiments using MAb blocking of MHC class II molecules (Fig. 1). The percentages of inhibition for GtfC, GtfD, and TT were 57.9, 62.8, and 86.6, respectively. In addition, the dominant cellular response to GtfD, compared to GtfC, could be observed in individuals with distinct HLA-DR antigens. DRB1 genotypes in the tested individuals are randomly distributed.

To further confirm the differential cellular responses stimulated by GtfD versus GtfC, proliferation experiments were carried out using enriched T cells from adults. As a control, T cells were enriched from cord blood samples. Analysis of the homogeneity of the enriched populations using cell sorting indicated that total T cells, enriched by nylon column or antibody depletion methods, are 83 and 93% pure, respectively. T cells enriched from cord blood harvested negligible responses to either GtfD or GtfC (Table 2). These results confirmed our previous observation that the stimulation of cord blood mononuclear cells by GtfC and -D (Table 1) was due to proliferation of cells other than T cells, and, accordingly, such proliferative responses could not be blocked completely by anti-MHC class II antibodies. On the other hand, analogous to the responses of

### Table 1. Mononuclear cell response to bacterial antigens

<table>
<thead>
<tr>
<th>Antigen (conc, μg/ml)</th>
<th>Mononuclear cells from cord blood (n = 6)</th>
<th>PBMC from:</th>
<th>Children (n = 6)</th>
<th>Adults (n = 22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GtfC (20)</td>
<td>13.07 ± 7.38</td>
<td>8.08 ± 2.26</td>
<td>8.61 ± 10.73</td>
<td></td>
</tr>
<tr>
<td>GtfD (20)</td>
<td>11.78 ± 7.13</td>
<td>18.64 ± 6.18b</td>
<td>18.55 ± 15.78b</td>
<td></td>
</tr>
<tr>
<td>CWP-A (20)</td>
<td>7.12 ± 5.48</td>
<td>4.12 ± 3.4</td>
<td>3.73 ± 3.01</td>
<td></td>
</tr>
<tr>
<td>EXP-A (20)</td>
<td>2.45 ± 1.03a</td>
<td>2.71 ± 2.49a</td>
<td>2.68 ± 1.71c</td>
<td></td>
</tr>
<tr>
<td>TT (10)</td>
<td>0.98 ± 0.72</td>
<td>6.64 ± 3.82</td>
<td>8.79 ± 5.88</td>
<td></td>
</tr>
<tr>
<td>SEB (0.01)</td>
<td>35.5 ± 5.20</td>
<td>ND⁻</td>
<td>35.5 ± 8.92</td>
<td></td>
</tr>
</tbody>
</table>

* Positive responses of different groups to the recombinant GtfC and -D and other antigens were those with SIs of greater than the mean plus two standard deviations of the response to the control protein TT in the cord blood group. With medium only, the response was 678 ± 85 cpm. PHA (10 μg/ml) stimulated PBMC efficiently, with a mean SI ranging from 170 to 180 in the three different groups. Control eluents from E. coli harboring a nonrecombinant plasmid exhibited undetectable stimulation in all tested groups.

<sup>a</sup> P < 0.01 compared to the value for GtfC by student's t test.

<sup>b</sup> no response.

<sup>ND</sup>, not determined.
PBMC, enriched T cells responded better to GtfD than to GtfC. The intensity of T-cell proliferative responses to GtfD was comparable to that with TT but was far less than that with SEB (Table 2). The fold increase in T-cell proliferation induced by GtfD versus GtfC exhibited a positive correlation with the IgA or IgG levels quantitated on the Western blots (r = 0.85). These results confirmed our hypothesis that cellular responses to GTF molecules may differ in a manner similar to that of levels of saliva and serum antibody GtfD or -C.

The proliferative responses of either PBMC or enriched T-cell populations were primary responses without prior antigen feeding or stimulation during culture. In addition, all tested individuals, regardless of age group, responded well to GtfD, with an SI comparable to that of the potent immunogen TT. These results confirmed that, in naturally sensitized humans, differential cellular immune responses to S. mutans GTFs exist in an antigen-specific manner at the T-cell level, with a stronger response to the secreted form of GtfD than to the cell wall-associated form of GtfC. Recombinant, His-tagged GtfC and -D both were biologically active. Thirty-four of the 42 amino acids deleted from the N terminus of GtfC are predicted to comprise a signal sequence, and it is unlikely, therefore, that differences in cellular immune responses to GtfC and -D resulted from truncation of the N terminus of GtfC. Genetic and biochemical analyses revealed that GtfB and GtfC share an overall 79% identity in amino acid residues and about 58% homology with GtfD. Considering the linear nature of T-cell epitopes, we are currently investigated the possibility of identifying major T-cell epitopes, which are recognized by most of the population, by constructing truncated GtfD fragments.

Although cell wall polysaccharides from S. mutans showed mitogenic activity on B cells (11), protein antigens of cell wall-associated or secreted forms does not contain mitogenic or superantigenic integredients, as indicated by the proliferative responses of either PBMC or T cells to CWP-A and EXP-A. By analogy, GtfB and -C do not possess superantigenic effecs, as confirmed by the lack of response in the T cells from cord blood samples (Table 2). Therefore, the nonspecific stimulation by either GtfC or GtfD observed on the PBMC was probably due to minor contaminating fractions from the E. coli host, although the final preparation revealed that lipopolysaccharide integredients were undetectable. This nonspecific stimulation was class II MHC independent and might result from cell populations other than T cells, as suggested by the anti-MHC antibody blocking experiments and the nonresponsive-ness of the T cells from cord blood samples (Table 2). Therefore, the primary responses measured with the enriched T-cell samples were antigen specific.

An interesting question is why GTFs should elicit differential antibody and cellular immune responses. It has been demonstrated previously that GtfB and -C are more important, structurally and functionally, than is GtfD in establishing bacterial adherence to smooth surfaces (14). Additional studies from two independent groups also reported that GtfC is the molecule essential for adherence and colonization (8, 30). We have shown previously that levels of salivary antibody to GtfC were significantly higher in caries-free than in caries-active young adults. This suggests that GtfC may serve as a protective antigen, although the responses in saliva and serum induced by this protein are lower than those induced by GtfD. Results from the present study confirmed that GtfC and -D stimulate different T-cell responses. Taking the results together, one possible speculation might be that S. mutans directs antibody and cellular immune responses away from molecules essential for adherence and colonization to evade immune surveillance. In other words, extracelluar secretion of GtfD may provide a decoy antigen to neutralize circulating IgA or IgG, preventing reaction with cell wall-associated GtfC and evading antibody clearance. However, it should be pointed out that despite the higher response to GtfD, there is a robust response to GtfC as well.

We thank H. K. Kuramitsu for providing plasmids pNH3 and

![Image](http://cvi.asm.org/)

**FIG. 1.** Inhibition of proliferation by anti-MHC class II antibody. Mononuclear cells from cord blood (□) or adult PBMC (●) were preincubated with MAb MCA477, and assays of proliferation to different antigens were carried out. Inhibition was expressed as the SI for treated cells compared to that for untreated cells, which was normalized to 100%. Each bar represents the mean and standard deviation from three assays.

**TABLE 2. T-cell response to bacterial antigens**

<table>
<thead>
<tr>
<th>Antigen (concn. µg/ml)</th>
<th>T-cell response (mean SI ± SD)</th>
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<tbody>
<tr>
<td></td>
<td>Cord blood (n = 4)</td>
</tr>
<tr>
<td>GtfC (10)</td>
<td>ND*</td>
</tr>
<tr>
<td>GtfC (20)</td>
<td>1.7 ± 0.38</td>
</tr>
<tr>
<td>GtfD (1)</td>
<td>ND</td>
</tr>
<tr>
<td>GtfD (10)</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>CWP-A (20)</td>
<td>1.24 ± 0.09</td>
</tr>
<tr>
<td>TT (10)</td>
<td>1.02 ± 0.12</td>
</tr>
<tr>
<td>SEB (0.01)</td>
<td>36.8 ± 4.12</td>
</tr>
</tbody>
</table>

* Positive responses in T cells from the adult group to different antigens were those with. SIs of greater than mean plus two standard deviations of the response to the same protein in the cord blood group. With irradiated PBMC only, the response was 108 ± 25 cpm.
* ND, not determined
* P < 0.01 compared to the value for GtfC at 10 µg/ml by Student's t test.
* No response.
REFERENCES