CYTOKINES PRODUCED BY CD4+ T CELLS AGAINST A SYNTHETIC GTF-I$_{(1301-1322)}$ PEPTIDE OF *STREPTOCOCCUS MUTANS* IN NATURALLY SENSITIZED HUMANS

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ABSTRACT

Streptococcus mutans (S. mutans) is the main etiological agent in dental caries. Its virulence factors are the proteins PAc and glucosyltransferase (GTF), which are related to its physiopathogenia and have been used in research for a dental caries vaccine. It was reported that using experimental animal models, GTF-I$_{(1301-1322)}$ synthetic peptide from the GLU region of the GTFs has T epitopes, induces production of serum antibodies in saliva and reduces the presence of caries, but little is known about the cellular response in naturally sensitized humans. The aim of this study was to observe whether GTF-I$_{(1301-1322)}$ peptide is capable of activating CD4+ T cells in PBMC from naturally sensitized humans, to classify the response and to establish the relationship with dental caries. The study was conducted on 30 individuals classified into the following 3 groups: active caries (AC), History of Caries (HC), and free of caries (H). A blood sample was drawn from each individual. Specific antigen stimulation and flow cytometry analyses were used to determine cells producing the cytokines IFN-γ (type 1 cytokine) and IL-13 (type 2 cytokine). Cell memory response to GTF-I$_{(1301-1322)}$ peptide was found in naturally sensitized humans. Three different responses were detected: TH0, TH1, and NR. The percentage of CD4+ T cells producing the cytokines IFN-γ (type 1 cytokine) was greater than the percentage producing IL-13 ($p= 0.006$). No statistically significant differences were found among the three groups for the other variables studied ($p\leq 0.05$). In conclusion, specific cellular immune responses against the GTF-I$_{(1301-1322)}$ peptide of S. mutans does not differ between individuals who are naturally sensitized, caries- resistant or with caries.

Key words: Streptococcus mutans, dental caries, GTF-I$_{(1301-1322)}$ peptide, cytokines.

LT CD4+ PRODUCTORES DE CITOQUINAS FRENTE A GTF-I$_{(1301-1322)}$ DE *STREPTOCOCCUS MUTANS* EN HUMANOS NATURALMENTE SENSIBILIZADOS

RESUMEN

Streptococcus mutans (S. mutans) es el principal agente etiológico de la caries dental. Las proteínas PAc y glucosiltransferasas (GTFs) son factores de virulencia de este microorganismo relacionados con su fisiopatogenia y han sido usados en investigación de una vacuna para la caries dental. Modelos animales experimentales han reportado que el péptido sintético GTF-I$_{(1301-1322)}$ de la región GLU de las GTFs, que presenta epítopes T, induce la producción de anticuerpos salivares y séricos disminuyendo la presencia de caries, pero poco se conoce de la respuesta celular en humanos naturalmente sensibilizados. El objetivo de este estudio fue observar si GTF-I$_{(1301-1322)}$ tiene la capacidad de activar las células T CD4+ en CMSP de humanos naturalmente sensibilizados, identificar el tipo de respuesta y establecer su relación con la caries dental. 30 individuos clasificados en los siguientes 3 grupos fueron estudiados: caries activa (AC), historia de caries (HC) y libres de caries (H). Muestras de sangre fueron tomadas de cada individuo. La estimulación antígeno específica y la citometría de flujo fueron usadas para determinar las células productoras de citoquina IFN-γ (citoquinas tipo 1) e IL-13 (citoquinas tipo 2). Se encontró respuesta de memoria celular frente a GTF-I$_{(1301-1322)}$ en humanos naturalmente sensibilizados. Tres tipos de respuesta fueron detectados: TH0, TH1 y NR. Se encontró un mayor porcentaje de LTCD4+ productores de IFN-γ (citoquinas tipo 1) que de IL-13 ($p= 0.006$). No se encontraron diferencias estadísticamente significativas para las otras variables estudiadas para los tres grupos ($p\leq 0.05$). Se concluye que la respuesta inmune celular específica frente al péptido sintético GTF-I$_{(1301-1322)}$ de S. mutans no es diferente entre los individuos sensibilizados naturalmente, resistentes a caries y con caries.

Palabras clave: Streptococcus mutans, caries dental, péptido GTF-I$_{(1301-1322)}$, citoquinas.

INTRODUCTION

Dental caries can be defined as an infectious, localized, post-eruptive, transmissible pathological process ending with the destruction of hard dental tissues by the acids of the microbial deposits adhering to them[1]. Of the many bacteria in the oral cavity, the streptococci belonging to the *mutans* group, particularly *Streptococcus mutans* serotype c (*S. mutans*), have been implicated as the main cause of dental caries[2].

Colonization of tooth surfaces by these microorganisms is considered to be the first step initiating the disease. They adhere by two mechanisms: sucrose-dependent and independent. In sucrose-dependent adhesion, *S. mutans* adheres to the film acquired on the surface of the tooth by means of several cell surface polymers such as the protein PAc[3]. Subsequently, the glucosyltransferase proteins (GTFs) act on the sugar in the diet producing soluble and insoluble glucans[4], which are fixed by glucan-binding proteins (GBP)[4,5], thus facilitating bacterial congregation. Once *S. mutans* is established in the bacterial plaque, the acids synthesized during its metabolism lead to demineralization of the dental tissue[6].

The GTF enzymes have two functional domains: one N-terminal catalytic (CAT) capable of sucrose binding and hydrolysis, and one C-terminal glucan-binding protein (GLU) responsible for binding to glucans[7].

The major role played by the proteins PAc and GTF in the cariogenicity of *S. mutans* makes it a rational target for developing a dental caries vaccine, since the inhibition of these colonization factors may protect the tooth from dental caries[8].

In the search for a vaccine antigen, synthetic GTF peptides, particularly from the CAT and GLU regions have been used. Recent research using an experimental animal model has reported that the peptide GTF-$I_{(1301-1322)}$ from the $GLU_{(1183-1473)}$ region, sequence TGARTINGQHLYFRANGVQVKG, has T and B epitopes, induces the production of antibodies in serum and saliva that inhibits water-insoluble glucan synthesis, and a reduction in caries rate is found in immunized animals[9, 10]. As this peptide has T epitopes, it can stimulate CD4+ T cells, triggering the specific response mechanisms.

In view the aforementioned, the aim of this study was to observe whether the GTF-$I_{(1301-1322)}$ vaccine antigen of the glucan binding region (GLU) of glucosyltransferases, which has been well-studied in animals, has the same capacity for activating the CD4+ T cells in the peripheral blood of naturally sensitized humans, and its corresponding secretion of cytokines such as IFN-γ and IL-13, thus establishing some difference between the immune response and the resistance to disease in a low percentage of the population.

MATERIALS AND METHODS

Population and Sample

Thirty periodontally[11] healthy individuals aged 18 to 30 years were selected, distributed into three groups based on their oral examination and DMTS index (decayed, missing, or filled teeth)[12]: 10 free of caries individuals (H), 10 with history of caries (HC) (with restored lesions, without presence of caries) and 10 patients with active caries (AC) (with multiple carious lesions –at least 2– involving enamel and dentine). Sample size was calculated using the Power and Precision statistical software for Kruskal Wallis, with power=0.8 and α=0.005. The patients included had permanent teeth, without periodontal disease and without fluoride or sealant treatment within the previous 6 months. Except for the presence of caries, all individuals were clinically healthy.

This study was approved by the ethics committee at the School of Dentistry of Pontificia Universdiad Javeriana and conducted according to the Declaration of Helsinki (1975) reviewed in 1983. After the oral examination and classification of DMTS indices, samples of blood anticoagulated with heparin and without anticoagulant were taken. Patients had previously read, approved and signed the informed consent.

Synthetic peptide and antigens

GTF-$I_{(1301-1322)}$ TGARTINGOHLYFRANGVQVKG (25 µg/ml) peptide, was synthesized using the T-Boc method at the Fundación Instito de Inmunología de Colombia (FIDIC) (Bogotá, Colombia). Enterotoxin B from *Staphylococcus aureus* (Sigma) (3.75 µg/ml)[13] was used as a positive control, and non-stimulated cells as a negative control.

Antigen-specific stimulation of peripheral blood mononuclear cells (PBMC)

The PBMCs were purified from samples of heparinized blood by Ficoll-Hypaque density gradient (Amersham Biosciences). A final concentration of $1x10^6$ cells/ml, was cultured in RPMI supplemented with 25mM HEPES, 100 U/ml penicillin, 100 mg/ml streptomycin and 10% fetal bovine serum (FBS) at 37°C with 5% CO_2 for 10 hours. In addition to the antigens for activation, the co-stimulatory antibodies CD28 (1 µg/ml) and CD49d (1 µg/ml) (Becton Dickinson) were used. Brefeldin A (2 µg/ml) (BD) was added. After incubation, two washes were done with PBS, PBS plus EDTA (5nM) and washing buffer (PBS-0.5% fetal bovine serum, 0.02% sodium azide)[14].

Extra- and intracellular marking

The stimulated and non-stimulated cells were resuspended in 200 µl of stain buffer (PBS pH 7.6,

1% FBS, 0.09% sodium azide), the specific antibodies against surface markers CD3 FITC, CD4 PercPe and CD69 APC were added for 15 minutes at 4°C in the dark. The cells were fixed and permeabilized for intracellular marking following the procedure of the Cytofix/Cytoperm kit (Becto Dickinson). The anti-cytokine antibodies IFN-γ PE and IL-13 PE were used with their corresponding controls of isotype mouse IgG2b PE (for IFN-γ) and rat IgG1 PE (for IL-13). Finally, the cells were washed and fixed with 2% paraformaldehyde, acquired in a FACsCalibur flow cytometer (BD Immunocytometry Systems) and analyzed using Cell Quest software. 50.000 events were acquired per tube.

Table 1: Cytokines induced by the peptide GTF-$I_{(1301-1322)}$

Subject	Free of caries (H)		History of Caries (HC)		Active Caries (AC)	
	IFN-γ	IL-13	IFN-γ	IL-13	IFN-γ	IL-13
1	+ ($p=3.98 \times 10^{-8}$)	-	-	-	-	-
2	+ (p=0.003)	-	-	-	-	-
3	-	-	-	-	+ (p=0.04)	-
4	+ ($p=3.95 \times 10^{-5}$)	-	-	-	+ ($p=1.25 \times 10^{-25}$)	-
5	+ (p=0.001)	-	+ ($p=1.31 \times 10^{-8}$)	-	-	-
6	+ ($p=1.55 \times 10^{-5}$)	+ (p=0.03)	-	-	+ ($p=6.57 \times 10^{-5}$)	-
7	-	-	-	-	+ (p=0.007)	-
8	-	-	-	-	-	-
9	-	-	-	-	-	-
10	-	-	-	-	-	-

+ p=<0.05 by Chi-square.

Statistical analysis

The differences between groups were assessed using Kruskal-Wallis and Mann-Whitney U tests to find the association between variables in pairs. Chi-square was used to analyze the differences in cytokine expression between stimulated and non-stimulated CD4+ T cells. All tests were evaluated using a significance level of $p \leq 0.05$.

RESULTS

Cytokines induced by the peptide GTF-$I_{(1301-1322)}$

Of all the patients studied, 5 healthy individuals (free of caries), 1 with history of caries and 4 with active caries produced IFN-γ, and the CD4+ T cells of one healthy individual produced the two cytokines studied. Table 1 shows the number of respondent individuals for each cytokine.

Comparison of respondent individuals per cytokine among study groups showed no statistically significant differences in the production of IFN-γ (p=0,054) or IL-13 (p=0,18), by Chi-square.

Types of immune response

Three types of immune response were found from cytokines produced by CD4+ T cells that responded to the synthetic peptide GTF-$I_{(1301-1322)}$ of the glucan binding region (GLU): type 0 cytokines (TH0), type 1 (TH1) cytokines and non-response (NR) (Table 2).

Table 2: Types of immune response.

	TH0	TH1	TH2	NR
H	1	4	0	5
HC	0	1	0	9
AC	0	4	0	6

H: free of caries; HC: with history of caries; AC: with active caries; NR: no response

Percentage of cytokine-producing CD4+ T cells

Regarding the percentage of IFN-γ-producing CD4+ T cells, no statistically significant differences were found (p=0.86) among groups, but the medians show that there appears to be a tendency towards a greater number of IFN-γ-producing CD4+ T cells in healthy patients and patients with active caries (1.275 and 1.271 respectively) and a lower percentage in the CD4+ T cells of patients with caries history (0.74) (Fig. 1).

Like IFN-γ, the percentage CD4+ T cells producing IL-13 showed no statistical difference (p=0.47) between study groups. The median for the three groups was almost 0 (H=0.44, HC=0.18, AC=0.16).

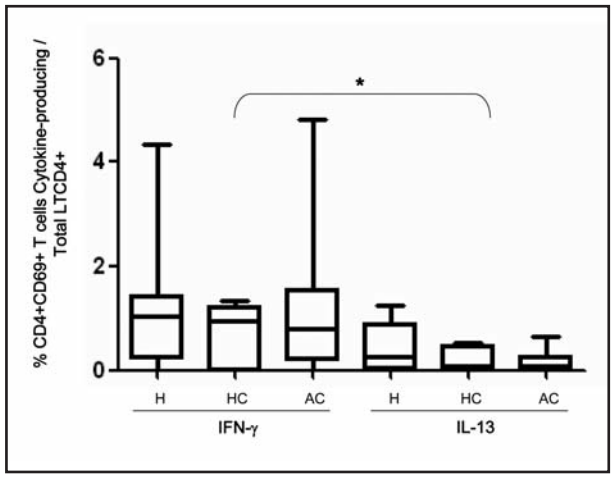

*Fig. 1: Percentage of cytokine-producing LTCD4+, showing inter-quantile distribution or the precentage of IFN-γ or IL-13-producing CD4+ T cells for each study group. * p< 0.05 by Mann Whitney U test. Data shown by medains and ranges.*

There was a greater percentage of $CD4^+$ T cells producing IFN-γ than IL-13 (p=0.006) according to a Mann Whitney U test, correlated to the medians found for the two types of cytokine in the individuals studied (Fig. 1).

DISCUSSION

The study of the specific immune response against cariogenic microorganisms has focused mainly on the knowledge of antibody-mediated immunity against *S. mutans*. *In vitro* studies have shown mechanisms of the action of salivary IgA, including those that prevent *S. mutans* from colonizing the tooth and those that neutralize some of the virulence factors. However, *in vivo*, the results have not allowed a protective role to be established for the specific humoral response in dental caries. Cell-mediated immunity has been less studied and a greater proliferative response of specific T cells against *S. mutans* has been found in individuals with lower caries rates[15].

Studies of synthetic peptides of *S. mutans* virulence factors have been tested on animal models (conventional rats, hamsters and gnotobiotic rats)[16] by means of immunization and co-immunization techniques, to observe the capacity to produce serum and salivary antibodies capable of controlling or reducing the disease[10]; enzymatic function inhibition assays, hydroxyapatite binding, reduction of carious lesions and bacterial colonization.

In this regard, the studies using GLU peptides of different sequences as immunogens, and different immunization pathways[7, 9-10, 17-23], found high levels of IgG and IgA in rodent and human serum. In addition, they found lymphocyte proliferation after 5 days of inoculation[21, 24], suggesting that the GLU peptide contains B and T cell epitopes.

According to the findings of this study, peripheral blood $CD4^+$ T cells were found that were capable of recognizing the synthetic peptide GTF-$I_{(1301-1322)}$ of the glucan-binding region GLU in all the patients studied, under the expression of $CD69^+$ cells (cell memory and activation marker), confirming the presence of T-epitopes in the peptide for the human model with natural *S. mutans* infection.

There are no studies reporting the response of peripheral blood $CD4^+$ T cells against the synthetic peptide GTF-$I_{(1301-1322)}$ of the glucan-binding region GLU in humans and its corresponding cytokine secretion; suggesting that this study might be the first to prove that this peptide, which has frequently been studied in the animal model as a potential vaccine antigen, is recognized by the $CD4^+$ T cells in about half the individuals with natural *S. mutans* infection.

In contrast, there are studies reporting cytokine production and proliferation against whole *S. mutans* as an antigen to stimulate PBMC *in vitro*, which found that there is a response by $CD4^+$ T cells and cytokine production as IFN-γ mainly[25-26], IL-4 and IL-10[25], and of IL-12 and TNF-α. These results show that *S. mutans* is capable of activating the production of cytokines especially associated to inflammation, increase in monocyte function and type of TH1 and TH2 response. These responses may have a correlation with the type of response *in vivo*[26].

The above agrees with the findings of this study, because the peptide induces varied cytokine response (Type 0, Type 1, and non-respondents) in some of the individuals studied[27]. Although most individuals were non-respondents, no statistically significant differences were found between response types; nevertheless, when the individuals were respondent, they were positive for the production of IFN-γ, i.e. there is a tendency towards a type 1 cellular response, as reported for whole *S. mutans*[25-26, 28].

Healthy individuals and those with active caries show similar behavior regarding the response. Four of them were positive for production of IFN-γ and the medians were very similar in percentage of

$CD4^+$ T cells producing it, while individuals with caries history tend to be non-respondent.

This might be explained as follows. The immune response is known to vary according to the amount of antigen present. Thus, the greater the quantity of microorganism, in this case in the oral cavity, the greater the immunologic response as a result of an infectious process, which is what may happen with the response observed in individuals with active caries, who have a greater number of *S. mutans* colonies in saliva samples (data being published) and high immunologic response, as reported in this study. The fact that they have a different number of microorganism colonies was the main reason why the caries-experienced study group was divided into two: active caries and history of caries.

Healthy individuals also show a high response, not because of the presence of antigen (fewer colonies), but because of possible protection against the disease. While the individuals with caries history were once ill and achieve health when the disease and antigen are removed, the immunological system is reduced and appears not to respond, i.e. the immune response does not protect and it is thus deduced that these individuals might be more susceptible to the disease.

Other studies report that in addition to T cells, NK cells can also respond by producing IFN-γ, and to a lesser extent, TNF-β and IL-10 without presence of IL-4 and assign an inhibiting role to *S. mutans* in the production of IL-2[27, 29].

S. mutans antigens such as recombinant proteins GTF-C and GTF-D and raw cell wall extract induce production of a higher proportion of the cytokines IL-6 and TNFα and a lower proportion of IL-2, IFN-γ and IL-10 in PBMC[29]. These experiments all prove that the cell immune response and corresponding cytokine production vary depending on the antigen used, according to whether it is the whole bacteria or recombinants of some of its virulence proteins.

This study showed that human $CD4^+$ T cells in presence of the synthetic peptide GTF-$I_{(1301-1322)}$ of GLU, are capable of being activated and producing more IFN-γ than IL-13 without any relationship to the state of the disease or absence of disease. Theoretically, an immune response mediated by type 1 cytokines implies activation of other cells to induce death of the antigen by lysis, and the activation of B lymphocytes for the production of specific antibodies to protect against colonization by *S. mutans*, in this case IgG, but apparently the response to this peptide does not protect, because it was found equally in healthy and diseased individuals.

These results suggest that further studies are needed on the response of T cells to whole *S. mutans* and other synthetic peptides of the virulence factors, or even of the same peptide providing increased immunogenicity by means of changes in its shape and structure and allowing its use in early stages, before the microorganism colonizes the tooth, so that it can increase the chances of providing a protective response to this vaccine antigen in the population of naturally sensitized humans.

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