

## “Tien-Hsien Liquid” Can Modulate Antigen-Stimulated Cytokine Production by T-Cells Isolated from Patients with Recurrent Aphthous Ulcerations

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**Abstract:** Recurrent aphthous ulcerations (RAU) represent a common oral mucosal disease with altered humoral and cellular immunities. Tien-Hsien liquid (THL) is an extract of Chinese medicinal herbs with immunomodulating effects. Our previous study found that THL can modulate the antigen-stimulated proliferative response of peripheral blood mononuclear cells and T-cells isolated from RAU patients. In this study, we further tested whether THL can modulate the antigen-stimulated cytokine production by T-cells isolated from RAU patients. To achieve this goal, T-cells isolated from 19 RAU patients were incubated with phytohemagglutinin (PHA), glutaraldehyde-inactivated tetanus toxoid (TT), glucosyltransferase D (GtfD), or antigens of *Streptococcus mutans* in the presence or absence of THL. The levels of interleukin (IL)-2, interferon- $\gamma$  (IFN- $\gamma$ ), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), IL-6, or IL-10 in the supernatants of T-cell cultures were measured by cytokine enzyme-linked immunosorbent assay (ELISA) kits. We found that THL significantly increased the PHA- or TT-stimulated TNF- $\alpha$ , IL-6, and IL-10 production by T-cells isolated from RAU patients. However, THL could also significantly decrease the TT-stimulated IL-2 production, the GtfD-stimulated IL-2, TNF- $\alpha$ , IL-6 and IL-10 production, and the *S. mutans*-stimulated IFN- $\gamma$ , TNF- $\alpha$ , and IL-10 production by T-cells isolated from RAU patients. These results indicate that THL can modulate the antigen-stimulated cytokine production by T-cells isolated from RAU patients. Because RAU is probably a Th1-mediated disease with elevated levels of IL-2, IFN- $\gamma$ , TNF- $\alpha$  and IL-6 in either the patient's sera or oral lesions and these increased levels of

cytokines can be reduced by THL, we suggest that THL may be a potential immunocutaneous agent for treatment of RAU.

*Keywords:* Chinese Medicinal Herbs; Tien-Hsien Liquid; *Cordyceps sinensis* (CS); *Oldenlandia diffusae* (OD); *Indigo pulverata levis*; T-Lymphocyte; Recurrent Aphthous Ulcerations.

## Introduction

Recurrent aphthous ulcerations (RAU) represent a common oral mucosal disease characterized by recurrent and painful ulcerations of the oral non-keratinized mucosa. The etiology of RAU is still obscure. Previous studies have found significantly higher than normal serum levels of immunoglobulin (Ig)G, IgM and IgA (Sun *et al.*, 1983) and a depressed natural killer (NK) cell activity in RAU patients in the active stage (active RAU patients) (Sun *et al.*, 1991). In addition, RAU patients have a decreased CD4<sup>+</sup>/CD8<sup>+</sup> ratio (Sun *et al.*, 1987; Savage *et al.*, 1988). Recently, we further showed a significant increase in the percentages of CD3<sup>+</sup>, CD4<sup>+</sup>, CD4<sup>+</sup> interleukin-2 receptor (IL-2R)<sup>+</sup>, CD8<sup>+</sup> IL-2R<sup>+</sup> cells, a significant increase in CD4<sup>+</sup>/CD8<sup>+</sup> ratio in the exacerbation stage (from the onset of oral mucosal ulceration to the peak of oral discomfort) of RAU patients, and a significant decrease in CD4<sup>+</sup>/CD8<sup>+</sup> ratio in the post-exacerbation stage (from the peak of oral discomfort to the healing of oral ulceration) of RAU patients (Sun *et al.*, 2000). The increase in the number of activated T-cells (CD4<sup>+</sup> IL-2R<sup>+</sup> and CD8<sup>+</sup> IL-2R<sup>+</sup> cells) in RAU patients in the exacerbation stage supports the role of cell-mediated cytotoxicity in the immunopathogenesis of RAU. Treatment of RAU patients with levamisole for a few months can result in a significant improvement of clinical symptoms, normalization of the decreased CD4<sup>+</sup>/CD8<sup>+</sup> ratio, and normalization of increased serum immunoglobulin levels (Sun *et al.*, 1994). These results suggest that RAU can be treated by immunomodulation and correction of the patient's deranged cellular and humoral immunities.

A bacterial etiology has long been suggested in RAU. *Streptococcus mutans* is a common pathogen found in the dental plaque on the surfaces of the teeth (Loesche, 1986). In the early ulcerative stage of RAU, *S. mutans* may secondarily infect the oral aphthous lesions through the mucosal break. Therefore, *S. mutans* antigens and its secreted proteins, such as glucosyltransferase D (GtfD), may penetrate into the ulcerative oral mucosa and elicit specific immune reactions which exacerbate the oral ulceration. Our previous study demonstrated that peripheral blood mononuclear cells (PBMC) and T-cells isolated from RAU patients in the exacerbation stage showed a significantly higher proliferative response (PR) to *S. mutans* antigens and GtfD than those isolated from healthy control subjects (Sun *et al.*, 2002). Therefore, we suggest that *S. mutans* antigens and GtfD may also be involved in the disease process of RAU, especially in the exacerbation stage. Our recent study found that Tien-Hsien liquid (THL), an extract of Chinese medicinal herbs, significantly increased the GtfD-stimulated PR of PBMC and of T-cells isolated from inactive RAU patients, and the *S. mutans*-stimulated PR of PBMC isolated from inactive RAU patients.

However, THL could also significantly reduce *S. mutans*-stimulated PR of T-cells isolated from active RAU patients. These results suggest that THL can modulate the GtFD- or *S. mutans*-stimulated PR of PBMC and T-cells isolated from RAU patients. Therefore, it may be a potential immunocutaneous agent for treatment of RAU.

IL-2 is secreted by activated T-cells. It induces T-cell proliferation, potentiates B-cell growth, and enhances NK-cell and monocyte activation (Roitt *et al.*, 1998). Buno *et al.* (1998) detected an elevated level of IL-2 mRNA in RAU lesions compared with that in normal oral mucosa. Our previous study found a significantly higher serum level of IL-2 in RAU patients in the exacerbation stage than in normal control subjects (Sun *et al.*, 2000).

Interferon- $\gamma$  (IFN- $\gamma$ ) is a pleiotropic cytokine that plays an essential role in both the innate and adaptive phases of an immune response. NK, CD8, and CD4 Th1 cells are the most potent sources of IFN- $\gamma$  (Szabo *et al.*, 2003). Clearly one essential role of IFN- $\gamma$  is to activate macrophages to kill intracellular pathogens (Boehm *et al.*, 1997). Elevated levels of IFN- $\gamma$  mRNA have been detected in RAU lesions (Buno *et al.*, 1998).

Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) is a pro-inflammatory cytokine which is secreted by activated monocytes, macrophages, and many other cells including B-cells, T-cells, mast cells and fibroblasts (Vilcek and Lee, 1991; Vasalli, 1992). TNF- $\alpha$  exerts stimulatory activities on activated T-cells. It also induces the secretion of IL-1, IFN- $\gamma$  and IL-6 (Ebersole, 1992). Natah *et al.* (2000) found the expression of TNF- $\alpha$  in macrophages and lymphocytes in RAU lesions. Buno *et al.* (1998) reported an elevated level of TNF- $\alpha$  mRNAs in lesional and non-lesional mucosa of RAU. Increased TNF- $\alpha$  has been demonstrated in the unstimulated peripheral blood leukocyte culture supernatant from active RAU patients compared with controls (Taylor *et al.*, 1992).

IL-6 is a multifunctional cytokine that participates in the inflammatory and immune responses. IL-6 is produced by activated monocytes, macrophages, endothelial cells, fibroblasts, keratinocytes and activated T- and B-cells in response to induction by a variety of stimuli, which include other cytokines (Toruniowa *et al.*, 1995). Its immunological activities include B-cell differentiation and stimulation of IgG secretion, T-cell growth and differentiation, and cytotoxic T-lymphocyte (CTL) differentiation (Hibi *et al.*, 1996). Yamamoto *et al.* (1994) demonstrated that an elevated serum IL-6 concentration in RAU patients is decreased after treatment. Our recent study also found that a higher than normal serum level of IL-6 in RAU patients can be reduced by treatment with levamisole or levamisole plus Chinese medicinal herbs (Sun *et al.*, 2003).

IL-10 is an important immunosuppressive and anti-inflammatory cytokine released by both T-cells and antigen-presenting cells (Moore *et al.*, 2001). IL-10 can inhibit the activation and effector function of several cell types including T-cells, monocytes, and macrophages. IL-10 directly affects the function of Th1 cells by inhibiting the production of a number of cytokines, including IL-2, IFN- $\gamma$ , and TNF- $\alpha$  (Roncarolo *et al.*, 2003).

Previous studies showed an increased production of IL-2, IFN- $\gamma$ , and TNF- $\alpha$  mRNAs in RAU lesions (Buno *et al.*, 1998), elevated levels of IL-2 (Sun *et al.*, 2000), TNF- $\alpha$  (Taylor *et al.*, 1992), and IL-6 (Yamamoto *et al.*, 1994; Sun *et al.*, 2003) in sera of RAU patients, and a decreased level of IL-10 mRNA in RAU lesions (Buno *et al.*, 1998). These

findings suggest that the altered balance between the pro-inflammatory cytokines (IL-2, IFN- $\gamma$ , TNF- $\alpha$ , and IL-6) and the anti-inflammatory cytokine (IL-10) in the oral mucosa of RAU patients may be implicated in the pathogenesis of RAU (Buno *et al.*, 1998). Our recent study found that THL has immunomodulating effects on PBMC and T-lymphocytes isolated from RAU patients (Sun *et al.*, 2004). Because THL has both immunopotential and immunosuppression effects and can either stimulate or inhibit the cytokine production (Hu, 1996; Luoh, 1999; Sun *et al.*, 2004), it may be used as an immunomodulating agent to restore the altered cellular or humoral immunity in RAU patients.

In this study, we tested whether THL can modulate the phytohemagglutinin (PHA)-, glutaraldehyde-inactivated tetanus toxoid (TT)-, glucosyltransferase D (GtfD)-, or *S. mutans*-stimulated cytokine production by T-cells isolated from RAU patients. We found that THL significantly increased the PHA- or TT-stimulated TNF- $\alpha$ , IL-6, and IL-10 production by T-cells isolated from RAU patients. However, THL could also significantly decrease the TT-stimulated IL-2 production, the GtfD-stimulated IL-2, TNF- $\alpha$ , IL-6 and IL-10 production, and the *S. mutans*-stimulated IFN- $\gamma$ , TNF- $\alpha$ , and IL-10 production by T-cells isolated from RAU patients. Therefore, we suggest that THL can modulate the cytokine production by T-cells isolated from RAU patients and may be a potential immunocutaneous agent for treatment of RAU.

## Materials and Methods

### *Subjects*

The study group consisted of 19 RAU patients (nine men and ten women, mean age  $33 \pm 10$  years, range 24–56 years). All the RAU patients had at least one episode of oral ulcerations per month during the preceding years and were in the active stage of the disease, i.e. from the onset of oral mucosal ulceration to the complete healing of oral ulceration. All of them were diagnosed and treated in the Department of Oral Pathology and Oral Diagnosis of National Taiwan University Hospital, Taipei, Taiwan, ROC. None of them had taken any prescription medication for at least 3 months before entering the study.

### *Stimulation Antigens*

PHA was purchased from Sigma Chemical Co. (St. Louis, MO, USA). TT was provided by Ming-Yi Liao of the Department of Health, Center for Disease Control, Vaccine Center, Taiwan. Recombinant GtfD was made in our laboratory and the detailed procedures for production and purification of recombinant GtfD have been described in recent reports (Chia *et al.*, 2001; Sun *et al.*, 2002). *S. mutans* GS-5 was grown in brain heart infusion broth (Difco Laboratories, Detroit, MI, USA). All antigens used for stimulating cytokine production by T-cells isolated from RAU patients, including GtfD and reagents, exhibited undetectable endotoxin levels ( $< 30$  pg/ml) as determined by the Limulus amoebocyte lysate assay (Sigma).

### *Modulating Drugs*

THL (Feida Union Pharmaceutical Manufactory, El Monte, CA, USA) (1:1000 dilution) and active hexose correlated compound (AHCC, 5 µg/ml) were used to modulate the antigen-stimulated cytokine production by T-cells isolated from RAU patients. The major ingredients of THL contain ingredients such as *Cordyceps sinensis* (CS), *Oldenlandia diffusa* (OD), *Indigo pulverata levis* (IPL), *Polyporus umbellatus* (PU), *Radix astragale* (RA), *Panax ginseng* (PG), *Solanum nigrum L.* (SNL), *Pogostemon cablin* (PC), *Atractylodie macrocephalae rhizoma* (AMR), *Trichosanthis radix* (TR), *Clematidis radix* (CR), *Margarita* (M), *Ligustrum lucidum Ait* (LLA), and *Glycyrrhizae radix* (GR). Composition, pharmacological and immunological effects of these major ingredients of THL have been described in previous studies and our recent report (Hu, 1996; Luoh, 1999; Sun *et al.*, 2004). AHCC is a proprietary extract prepared from co-cultured mycelia of several species of Basidiomycete mushrooms, including shiitake (*Lentinus edodes*). The extract is made by boiling in the hot water followed by an enzyme pre-treatment; it contains polysaccharides, amino acids, and minerals, and is orally bioavailable (Kidd, 2000). Animal research and preliminary human studies indicate AHCC has anticancer efficacy (Kidd, 2000). In addition, AHCC have liver protection and immunopotential effect, and can prolong the survival of patients with hepatocellular carcinoma (HCC) after surgical resection (Kamiyama, 1999; Matsui *et al.*, 1999; Kidd, 2000).

### *Cell Preparation and Antigen-Stimulated Cytokine Production Assay*

Peripheral blood samples were collected from the 19 RAU patients in the active stage. PBMC were isolated from blood samples by Ficoll-Hypaque centrifugation. Suspensions ( $2 \times 10^5$  cells/50 µl) of PBMC in RPMI 1640 medium (Gibco BRL Laboratories, Grand Island, NY, USA), supplemented with 10% fetal calf serum (Gibco BRL), (complete RPMI medium) were irradiated at 4500 rads with an X-ray irradiator (Hitachi Medical Co., Tokyo, Japan) to inhibit proliferation, and used as accessory cells in antigen-stimulated cytokine production assays. T-cells were enriched directly from whole blood by antibody-mediated separation with RosetteSep (StemCell Technologies Inc., Vancouver, BC, Canada). The enriched T-cell fractions were collected and used in the antigen-stimulated cytokine production assays.

Enriched T cells ( $1 \times 10^5$  cells/well) were cultured in the presence of irradiated autologous PBMC ( $2 \times 10^5$  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% thiophene-2-carboxylic acid hydrazide (Celox). To test whether THL and AHCC have modulating effects on the cytokine production by T-cells, three replicates of T-cell culture from 19 RAU patients were incubated with PHA (1 µg/ml), TT (10 µg/ml), recombinant GtfD (10 µg/ml), or antigens of *S. mutans* ( $2 \times 10^5$  CFU) in the presence or absence of THL (1:1000 dilution) or AHCC (5 µg/ml). Because previous studies have shown that AHCC has immunopotential effects on the immune system of HCC patients (Kamiyama, 1999; Matsui *et al.*, 1999; Kidd, 2000), in

this study, AHCC was used as a positive control agent with potentiating effects on the antigen-stimulated cytokine production by T-cells isolated from RAU patients. Incubation was performed at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> for 5 days. Cultured supernatant was collected on day 5 and frozen at -20°C for future analysis.

### *Detection of Cytokines*

Cytokines were quantitated by enzyme-linked immunosorbent assay (ELISA) kits (Quantikine; R & D systems Inc., Minneapolis, Minnesota, USA) used according to the manufacturer's instruction as described previously (Chia *et al.*, 2002). The minimum detectable cytokine concentrations were estimated to be 1 pg/ml for IL-2, IFN- $\gamma$ , TNF- $\alpha$ , and IL-10, and 1.6 pg/ml for IL-6. Cytokine level was expressed as mean  $\pm$  standard error of the mean (mean  $\pm$  SEM).

### *Statistical Analysis*

The mean cytokine levels were compared between no antigen and antigen only groups as well as between antigen only and antigen plus THL or antigen plus AHCC groups by Wilcoxon signed rank test. The result was considered to be significant if the  $p < 0.05$ .

## **Results**

In this study, we tested whether THL (1:1000 dilution) or AHCC (5  $\mu$ g/ml) had modulating effects on PHA (1  $\mu$ g/ml)-, TT (10  $\mu$ g/ml)-, GtfD (10  $\mu$ g/ml)-, and *S. mutans* ( $2 \times 10^5$  CFU)-stimulated secretion of IL-2, IFN- $\gamma$ , TNF- $\alpha$ , IL-6, and IL-10 by T-cells isolated from RAU patients. Compared to the spontaneous release of IL-2 (5  $\pm$  1 pg/ml) by T-cells from RAU patients, PHA and TT could stimulate T-cells to secrete significantly higher levels of IL-2 (27  $\pm$  4 pg/ml,  $p < 0.001$  and 31  $\pm$  8 pg/ml,  $p < 0.005$ , respectively). GtfD and *S. mutans* had no significant modulating effect on the IL-2 production by T-cells (Table 1). THL significantly decreased the TT-stimulated IL-2 production by T-cells from 31  $\pm$  8 pg/ml to 11  $\pm$  4 pg/ml ( $p < 0.05$ ) and the GtfD-stimulated IL-2 production by T-cells from 4  $\pm$  1 pg/ml to 1.3  $\pm$  0.2 pg/ml ( $p < 0.05$ ). However, AHCC had no significant modulating effects on the antigen-stimulated IL-2 production by T-cells from RAU patients (Table 1).

IFN- $\gamma$  secretion by T-cells could be elicited to significantly higher levels with the stimulation of PHA (47  $\pm$  13 pg/ml,  $p < 0.001$ ), TT (23  $\pm$  10 pg/ml,  $p < 0.05$ ), GtfD (215  $\pm$  65 pg/ml,  $p < 0.005$ ), or *S. mutans* (90  $\pm$  23 pg/ml,  $p < 0.001$ ) compared to the spontaneous release of IFN- $\gamma$  (2  $\pm$  1 pg/ml) by T-cells without antigen stimulation. It was obvious that GtfD and *S. mutans* were stronger antigens than PHA and TT to stimulate the IFN- $\gamma$  secretion by T-cells. THL significantly reduced the *S. mutans*-stimulated IFN- $\gamma$  production by T-cells from 90  $\pm$  23 pg/ml to 13  $\pm$  6 pg/ml ( $p < 0.005$ ). Furthermore, AHCC could significantly enhance the PHA-stimulated IFN- $\gamma$  production by T-cells from

**Table 1. Modulation of Antigen-Stimulated IL-2 Production by THL or AHCC in T-Cells Isolated from 19 Patients with RAU**

Antigens	IL-2 Level (pg/ml, mean $\pm$ standard error of the mean)			
	No Antigen	Antigen Only	Antigen + THL (1:1000 dilution)	Antigen + AHCC (5 $\mu$ g/ml)
PHA (1 $\mu$ g/ml)	5 $\pm$ 1	27 $\pm$ 4*	30 $\pm$ 6	32 $\pm$ 8
TT (10 $\mu$ g/ml)	5 $\pm$ 1	31 $\pm$ 8 <sup>†</sup>	11 $\pm$ 4 <sup>‡</sup>	30 $\pm$ 7
GtfD (10 $\mu$ g/ml)	5 $\pm$ 1	4 $\pm$ 1	1.3 $\pm$ 0.2 <sup>‡</sup>	1.8 $\pm$ 0.8
<i>S. mutans</i> (2 $\times$ 10 <sup>5</sup> CFU)	5 $\pm$ 1	5 $\pm$ 1	4 $\pm$ 2	2 $\pm$ 1

Comparison by Wilcoxon signed rank test between no antigen and antigen only groups with \*p < 0.001 and <sup>†</sup>p < 0.005 as well as between antigen only and antigen + THL groups with <sup>‡</sup>p < 0.05.

**Table 2. Modulation of Antigen-Stimulated IFN- $\gamma$  Production by THL or AHCC in T-Cells Isolated from 19 Patients with RAU**

Antigens	IFN- $\gamma$ Level (pg/ml, mean $\pm$ standard error of the mean)			
	No Antigen	Antigen Only	Antigen + THL (1:1000 dilution)	Antigen + AHCC (5 $\mu$ g/ml)
PHA (1 $\mu$ g/ml)	2 $\pm$ 1	27 $\pm$ 13*	101 $\pm$ 37	168 $\pm$ 52 <sup>¶</sup>
TT (10 $\mu$ g/ml)	2 $\pm$ 1	23 $\pm$ 10 <sup>‡</sup>	41 $\pm$ 21	60 $\pm$ 15 <sup>¶</sup>
GtfD (10 $\mu$ g/ml)	2 $\pm$ 1	215 $\pm$ 65 <sup>†</sup>	136 $\pm$ 37	256 $\pm$ 61
<i>S. mutans</i> (2 $\times$ 10 <sup>5</sup> CFU)	2 $\pm$ 1	90 $\pm$ 23*	13 $\pm$ 6 <sup>§</sup>	123 $\pm$ 30

Comparison by Wilcoxon signed rank test between no antigen and antigen only groups with \*p < 0.001, <sup>†</sup>p < 0.005, and <sup>‡</sup>p < 0.05 as well as between antigen only and antigen + THL or antigen + AHCC groups with <sup>§</sup>p < 0.005 and <sup>¶</sup>p < 0.05.

47  $\pm$  13 pg/ml to 168  $\pm$  52 pg/ml (p < 0.05) and the TT-stimulated IFN- $\gamma$  production from 23  $\pm$  10 pg/ml to 60  $\pm$  15 pg/ml (p < 0.05) (Table 2).

A significantly higher level of TNF- $\alpha$  production by T-cells from 22  $\pm$  8 pg/ml to 342  $\pm$  43 pg/ml (p < 0.001) and to 388  $\pm$  50 pg/ml (p < 0.001) could be elicited with the stimulation of GtfD and *S. mutans*, respectively. PHA and TT had no significant modulating effect on the TNF- $\alpha$  production by T-cells (Table 3). THL significantly augmented the PHA-stimulated TNF- $\alpha$  production by T-cells from 28  $\pm$  12 pg/ml to 147  $\pm$  36 pg/ml (p < 0.05) and the TT-stimulated TNF- $\alpha$  production from 25  $\pm$  17 pg/ml to 168  $\pm$  37 pg/ml (p < 0.001). THL could also significantly lower the GtfD-stimulated TNF- $\alpha$  production by T-cells from 342  $\pm$  43 pg/ml to 208  $\pm$  50 pg/ml (p < 0.05) and the *S. mutans*-stimulated TNF- $\alpha$  production from 388  $\pm$  50 pg/ml to 259  $\pm$  42 pg/ml (p < 0.05). AHCC had no significant modulating effect on the antigen-stimulated TNF- $\alpha$  production by T-cells (Table 3).

Stimulation with GtfD or *S. mutans* could significantly increase the IL-6 secretion by T-cells from 517  $\pm$  334 pg/ml to 11787  $\pm$  866 pg/ml (p < 0.001) and to 10089  $\pm$  1241 pg/ml (p < 0.001), respectively. PHA and TT had no significant modulating effect on the IL-6 production by T-cells (Table 4). THL significantly enhanced the PHA-stimulated

**Table 3. Modulation of Antigen-Stimulated TNF- $\alpha$  Production by THL or AHCC in T-Cells Isolated from 19 Patients with RAU**

Antigens	TNF- $\alpha$ Level (pg/ml, mean $\pm$ standard error of the mean)			
	No Antigen	Antigen Only	Antigen + THL (1:1000 dilution)	Antigen + AHCC (5 $\mu$ g/ml)
PHA (1 $\mu$ g/ml)	22 $\pm$ 8	28 $\pm$ 12	147 $\pm$ 36 <sup>‡</sup>	46 $\pm$ 19
TT (10 $\mu$ g/ml)	22 $\pm$ 8	25 $\pm$ 17	168 $\pm$ 37 <sup>†</sup>	63 $\pm$ 26
GtfD (10 $\mu$ g/ml)	22 $\pm$ 8	342 $\pm$ 43 <sup>*</sup>	208 $\pm$ 50 <sup>‡</sup>	345 $\pm$ 48
<i>S. mutans</i> (2 $\times$ 10 <sup>5</sup> CFU)	22 $\pm$ 8	388 $\pm$ 50 <sup>*</sup>	259 $\pm$ 42 <sup>‡</sup>	368 $\pm$ 49

Comparison by Wilcoxon signed rank test between no antigen and antigen only groups with <sup>\*</sup>p < 0.001 as well as between antigen only and antigen + THL or antigen + AHCC groups with <sup>†</sup>p < 0.001 and <sup>‡</sup>p < 0.05.

**Table 4. Modulation of Antigen-Stimulated IL-6 Production by THL or AHCC in T-Cells Isolated from 19 Patients with RAU**

Antigens	IL-6 Level (pg/ml, mean $\pm$ standard error of the mean)			
	No Antigen	Antigen Only	Antigen + THL (1:1000 dilution)	Antigen + AHCC (5 $\mu$ g/ml)
PHA (1 $\mu$ g/ml)	517 $\pm$ 334	788 $\pm$ 253	9426 $\pm$ 1226 <sup>†</sup>	8016 $\pm$ 952 <sup>†</sup>
TT (10 $\mu$ g/ml)	517 $\pm$ 334	692 $\pm$ 366	10781 $\pm$ 980 <sup>†</sup>	9925 $\pm$ 816 <sup>†</sup>
GtfD (10 $\mu$ g/ml)	517 $\pm$ 334	11787 $\pm$ 866 <sup>*</sup>	9053 $\pm$ 1067 <sup>‡</sup>	11357 $\pm$ 1033
<i>S. mutans</i> (2 $\times$ 10 <sup>5</sup> CFU)	517 $\pm$ 334	10089 $\pm$ 1241 <sup>*</sup>	9853 $\pm$ 1033	9503 $\pm$ 1341

Comparison by Wilcoxon signed rank test between no antigen and antigen only groups with <sup>\*</sup>p < 0.001 as well as between antigen only and antigen + THL or antigen + AHCC groups with <sup>†</sup>p < 0.001 and <sup>‡</sup>p < 0.05.

IL-6 production by T-cells from 788  $\pm$  253 pg/ml to 9426  $\pm$  1226 pg/ml (p < 0.001) and the TT-stimulated IL-6 production from 692  $\pm$  366 pg/ml to 10781  $\pm$  980 pg/ml (p < 0.001). THL could also significantly reduced the GtfD-stimulated IL-6 production by T-cells from 11787  $\pm$  866 pg/ml to 9053  $\pm$  1067 pg/ml (p < 0.05). AHCC could also significantly increase the PHA-stimulated IL-6 production by T-cells from 788  $\pm$  253 pg/ml to 8016  $\pm$  952 pg/ml (p < 0.001) and the TT-stimulated IL-6 production from 692  $\pm$  366 pg/ml to 9925  $\pm$  816 pg/ml (p < 0.001) (Table 4).

The spontaneously released IL-10 level by T-cells without antigen stimulation was too low to be detected (< 1 pg/ml). Significantly higher levels of IL-10 could be produced by T-cells with the stimulation of GtfD (83  $\pm$  21 pg/ml, p < 0.001) or *S. mutans* (73  $\pm$  20 pg/ml, p < 0.001) compared with the spontaneously released IL-10 production by T-cells without antigen stimulation (< 1 pg/ml) (Table 5). THL significantly elevated the PHA-stimulated IL-10 production by T-cells from 4  $\pm$  2 pg/ml to 13  $\pm$  4 pg/ml (p < 0.05) and the TT-stimulated IL-10 production from 5  $\pm$  3 pg/ml to 15  $\pm$  4 pg/ml (p < 0.05). THL could also significantly depress the GtfD-stimulated IL-10 production by T-cells from 83  $\pm$  21 pg/ml to 22  $\pm$  8 pg/ml (p < 0.05) and the *S. mutans*-stimulated IL-10 production from 73  $\pm$  20 pg/ml to 27  $\pm$  9 pg/ml (p < 0.05). However, AHCC had no significant modulating effects on the antigen-stimulated IL-10 production by T-cells from RAU patients (Table 5).



**Table 5. Modulation of Antigen-Stimulated IL-10 Production by THL or AHCC in T-Cells Isolated from 19 Patients with RAU**

Antigens	IL-10 Level (pg/ml, mean $\pm$ standard error of the mean)			
	No Antigen	Antigen Only	Antigen + THL (1:1000 dilution)	Antigen + AHCC (5 $\mu$ g/ml)
PHA (1 $\mu$ g/ml)	< 1	4 $\pm$ 2	13 $\pm$ 4 <sup>†</sup>	7 $\pm$ 3
TT (10 $\mu$ g/ml)	< 1	5 $\pm$ 3	15 $\pm$ 4 <sup>†</sup>	8 $\pm$ 3
GtfD (10 $\mu$ g/ml)	< 1	83 $\pm$ 21*	22 $\pm$ 8 <sup>†</sup>	68 $\pm$ 20
<i>S. mutans</i> (2 $\times$ 10 <sup>5</sup> CFU)	< 1	73 $\pm$ 20*	27 $\pm$ 9 <sup>†</sup>	75 $\pm$ 21

Comparison by Wilcoxon signed rank test between no antigen and antigen only groups with \* $p$  < 0.001 as well as between antigen only and antigen + THL groups with <sup>†</sup> $p$  < 0.05.

## Discussion

This study found that GtfD and *S. mutans* were more potent antigens than PHA and TT for stimulating the cytokine production by T-cells isolated from RAU patients, except for the stimulation of IL-2 secretion. THL not only significantly increased the PHA- or TT-stimulated TNF- $\alpha$ , IL-6, and IL-10 production by T-cells isolated from RAU patients, but also significantly decreased the TT-stimulated IL-2 production, the GtfD-stimulated IL-2, TNF- $\alpha$ , IL-6, and IL-10 production, and the *S. mutans*-stimulated IFN- $\gamma$ , TNF- $\alpha$ , and IL-10 production by T-cells isolated from RAU patients. These findings suggest that THL is an immunomodulator that can either potentiate or suppress the cytokine secretion by T-cells isolated from RAU patients.

In this study, GtfD and *S. mutans* significantly increase the IFN- $\gamma$ , TNF- $\alpha$ , IL-6 and IL-10 secretion by T-cells isolated from RAU patients. Our previous study showed that GtfD and *S. mutans* can significantly augment the PR of T-cells isolated from RAU patients compared to those from healthy control subjects (Sun *et al.*, 2002). Therefore, the significant GtfD- and *S. mutans*-induced elevation of IFN- $\gamma$ , TNF- $\alpha$ , IL-6 and IL-10 secretion could be due to an increase in the number of T-cells that are capable of secreting these four cytokines with the stimulation of GtfD and *S. mutans*. In addition, IFN- $\gamma$  and TNF- $\alpha$  itself can promote the TNF- $\alpha$  synthesis and/or release from the activated macrophages (Ebersole, 1992). IFN- $\gamma$  may induce the production of IL-6 mRNA (Faggioli *et al.*, 1997). TNF- $\alpha$  can induce the secretion of IFN- $\gamma$  by T-lymphocytes (Sugermann *et al.*, 1996) and can stimulate the secretion of IL-6 by activated macrophages (Sugermann *et al.*, 1996). IL-6 can also induce the production of TNF- $\alpha$  by activated monocytes (Ebersole, 1992). Thus, the significant GtfD- and *S. mutans*-induced elevation of IFN- $\gamma$ , TNF- $\alpha$ , and IL-6 secretion by T-cells may also be attributed to the reciprocal stimulation by these three cytokines on one another.

IL-2, TNF- $\alpha$  and IL-6 play important roles for proliferation and activation of T-cells. IL-2 is a T-cell growth factor. Its major function is to enhance proliferation of activated T-cells (Ebersole, 1992). TNF- $\alpha$  exerts multiple stimulatory activities on activated T-cells, including increasing the proliferation in response to antigens, increasing IL-2 receptor

expression, and increasing the response to IL-2 stimulus. IL-6 acts together with IL-2 to induce T-cell proliferation and CTL generation (Ebersole, 1992). Increased serum levels of IL-2 (Sun *et al.*, 2000) and of IL-6 (Yamamoto *et al.*, 1994; Sun *et al.*, 2003) are found in RAU patients. Significantly higher than normal amounts of TNF- $\alpha$  are released from unstimulated monocyte-enriched and monocyte-depleted leukocyte fractions from active RAU patients (Taylor *et al.*, 1992). Elevated levels of IL-2, IFN- $\gamma$ , and TNF- $\alpha$  mRNAs are detected in RAU lesions (Buno *et al.*, 1998). TNF- $\alpha$  immunoreactivity has been detected in macrophages, lymphocytes, mast cells, and vascular endothelial cells in oral aphthous lesion (Natah *et al.*, 2000). Our previous study showed that *S. mutans* and GtfD may be involved in the disease process of RAU. *S. mutans*- and GtfD-induced secretion of cytokine profile by T-cells isolated from RAU patients, the serum cytokine profile in RAU patients, and the expression patterns of cytokine in RAU lesions suggest that RAU is probably a disease of enhanced Th1-mediated immune response directed toward the focal area of the oral mucosa.

This study showed that THL could significantly decrease the GtfD-stimulated IL-2, TNF- $\alpha$ , IL-6, and IL-10 production, and significantly lower the *S. mutans*-stimulated IFN- $\gamma$ , TNF- $\alpha$ , and IL-10 production by T-cells isolated from RAU patients. These results indicate that THL can reduce the antigen-stimulated Th1-associated cytokine production by T-cells isolated from RAU patients. Because RAU is probably a Th1-mediated disease with elevated levels of IL-2, IFN- $\gamma$ , TNF- $\alpha$ , and IL-6 in either the patient's sera or oral lesions and these increased levels of cytokines can be reduced by THL, we suggest that THL may be a potential immunocutaneous agent for treatment of RAU.

IL-10 is an important immunosuppressive and anti-inflammatory cytokine that can counteract the immunopotential effects of IL-2, IFN- $\gamma$ , and TNF- $\alpha$ . Buno *et al.* (1998) reported elevated levels of IL-2, IFN- $\gamma$ , and TNF- $\alpha$  mRNAs and a reduced level of IL-10 mRNA in lesional and non-lesional mucosa of RAU patients compared with controls. They suggested that cytokine imbalance resulting in failure to suppress the inflammatory reaction initiated by trauma or other external stimuli might be important in the pathogenesis of RAU. In the present study, THL not only significantly increased the PHA- or TT-stimulated IL-10 secretion by T-cells, but also significantly decreased GtfD- or *S. mutans*-stimulated IL-10 secretion by T-cells isolated from RAU patients. These findings suggest that THL can modulate the IL-10 secretion by T-cells isolated from RAU patients. Therefore, THL may benefit RAU patients through modulating the T-cell secretion of both Th1-associated cytokines (IL-2, IFN- $\gamma$  and TNF- $\alpha$ ) and Th2-associated cytokine (IL-10).

The reasons why THL has modulating effects on cytokine production by T-cells isolated from RAU patients are still not very clear. Previous studies on mice have shown that ingredients of THL, RA, PG and GR, can induce the secretion of IFN- $\gamma$  by mouse spleen cells; CS, OD, PU, RA, PG, AMR, LLA and GR can induce the secretion of IL-2 by mouse spleen cells; and CS, PU, RA and AMR can increase the expression of IL-2R by murine lymphocytes (Hu, 1996; Luoh, 1999; Sun *et al.*, 2004). IL-2 is a T-cell growth factor that can stimulate the T-cell proliferation. In fact, previous murine studies have also demonstrated that CS, OD, IPL, PU, RA, PG, AMR and LLA can induce the proliferation of murine lymphocytes (Hu, 1996; Luoh, 1999; Sun *et al.*, 2004). Furthermore, as stated

before, IFN- $\gamma$ , TNF- $\alpha$ , and IL-6 are closely related inflammatory cytokines and one can induce the production of another (Ebersole, 1992). Because the ingredients of THL can induce the production of IL-2 and IFN- $\gamma$  and the expression of IL-2R by lymphocytes, it was not difficult to explain why THL can augment the PHA- or TT-stimulated IFN- $\gamma$ , TNF- $\alpha$ , and IL-6 production by T-cells isolated from RAU patients. On the contrary, CS selectively inhibits the PHA-induced IL-2 secretion by murine spleen cells, and LLA can also depress the antigen-stimulated high IL-2 secretion by murine spleen cells. Furthermore, TR can inhibit a blast transformation of murine lymphocytes, and in high concentration CS and RA can also inhibit the proliferation of murine lymphocytes (Hu, 1996; Luoh, 1999; Sun *et al.*, 2004). Because the ingredients of THL can inhibit the production of IL-2 by lymphocytes and can inhibit the lymphoproliferation directly, it was not difficult to understand why THL can decrease the GtfD- or *S. mutans*-stimulated IL-2, IFN- $\gamma$ , TNF- $\alpha$ , and IL-6 production by T-cells from RAU patients.

In summary, this study found that THL could not only significantly augment the lower PHA- or TT-stimulated TNF- $\alpha$ , IL-6, and IL-10 production by T-cells isolated from RAU patients to a higher level, but also significantly depress the higher levels of TT-stimulated IL-2 production, the GtfD-stimulated IL-2, TNF- $\alpha$ , IL-6, and IL-10 production, and the *S. mutans*-stimulated IFN- $\gamma$ , TNF- $\alpha$ , and IL-10 production by T-cells isolated from RAU patients to lower levels. These results indicate that THL can modulate the antigen-stimulated cytokine production by T-cells isolated from RAU patients. Because RAU is probably a Th1-mediated disease with elevated levels of IL-2, IFN- $\gamma$ , TNF- $\alpha$ , and IL-6 in either the patient's sera or oral lesions, and these increased levels of cytokines can be reduced by THL, we suggest that THL may be a potential immunocutaneous agent for treatment of RAU.

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