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Immunomodulating Effects of "Tien-Hsien Liquid" on Peripheral Blood Mononuclear Cells and T-Lymphocytes 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. In our institution, an immunomodulating agent, levamisole, is used to treat RAU with satisfactory therapeutic effect. Tien-Hsien liquid (THL) is an extract of Chinese medicinal herbs with immunomodulating effects. To test whether THL has immunomodulating effects on antigen-stimulated proliferation response (PR) of peripheral blood mononuclear cells (PBMC) and T-cells isolated from RAU patients and to test whether THL is a potential drug for treating RAU, PBMC or T-cells isolated from RAU patients were incubated with lipopolysaccharides (LPS) from *Escherica coli*, phytohemagglutinin (PHA), staphylococcal enterotoxin B (SEB), glutaraldehyde-inactivated tetanus toxoid (TT), glucosyltransferase D (GtfD), or antigens of *Streptococcus mutans* in the presence or absence of THL. We found that THL significantly increased the LPS-stimulated PR of PBMC from active RAU patients, the GtfD-stimulated PR of PBMC and of T-cells from inactive RAU patients, and the *S. mutans*-stimulated PR of PBMC from inactive RAU patients. However, THL could also significantly reduce the SEB-stimulated PR of PBMC and of T-cells from active RAU patients and *S. mutans*-stimulated PR of T-cells from active RAU patients. These results suggest that THL can modulate the antigen-stimulated PR of PBMC and T-cells from RAU patients. Therefore, it may be a potential immunoceutical agent for treatment of RAU.

Keywords: Chinese Medicinal Herbs; Tien-Hsien Liquid; *Cordyceps sinensis*; *Oldenlandia diffusae;* Peripheral Blood Mononuclear Cells; T-Lymphocyte; Recurrent Aphthous Ulcerations.

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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; however, alterations of humoral and cellular immunities in RAU patients have been reported (Sun *et al*., 1983; Sun *et al*., 1987; Savage *et al*., 1988, Landesberg *et al*., 1990; Sun *et al*., 1991; Sun *et al*., 2000). An early study found significantly higher serum levels of IgG, IgM and IgA in RAU patients in the active stage (active RAU patients) than in normal control subjects. The high levels of IgM and IgA return to normal in RAU patients in the inactive stage (inactive RAU patients), but the IgG level remains the same in inactive RAU patients (Sun *et al*., 1983). Depressed natural killer (NK) cell activity has been observed in active RAU patients (Sun *et al*., 1991). In addition, RAU patients have depressed $CD4^+$ cell numbers and elevated $CD8^+$ cell numbers. The $CD4^+/CD8^+$ ratio is also depressed (Sun *et al*., 1987; Savage *et al*., 1988). The markedly decreased $CD4^+/CD8^+$ ratio returns to normal in the remission stage of RAU (Sun *et al*., 1987). In a more detailed recent study, we further showed a significant increase in the percentages of $CD3^+$, $CD4^+$, $CD4^+$ interleukin-2 receptor $(IL\text{-}2R)^+$, $CD8^+$ $IL\text{-}2R^+$ cells, in $CD4^+/CD8^+$ ratio, and in the serum level of IL-2 in the exacerbation stage of RAU (from the onset of oral mucosal ulceration to the peak of oral discomfort). Moreover, a significant decrease in the percentage of $CD4^+$ cells and in the $CD4^+/CD8^+$ ratio, as well as a significant increase in $CD8^+$ cells, are found in the post-exacerbation stage of RAU (from the peak of oral discomfort to the healing of oral ulceration) (Sun *et al*., 2000). The increase in the number of activated T-cells ($CD4^+$ $IL\text{-}2R^+$ and $CD8^+$ $IL\text{-}2R^+$ cells) in RAU patients in the exacerbation stage supports the role of cell-mediated cytotoxicity in the immunopathogenesis of RAU. Treatment with levamisole for RAU patients for a few months can result in a significant improvement in clinical symptoms, normalization of the decreased $CD4^+/CD8^+$ ratio, and normalization of increased serum immunoglobulin levels (Sun *et al*., 1994). In addition, our recent study also shows that levamisole and levamisole plus Chinese medicinal herbs (a water extract of *Radix astragale, Fructus lycii* and *Fructus ziziphi jujubae*) can significantly reduce the serum IL-6 level in RAU patients. Furthermore, the mean reduction of serum IL-6 level in RAU patients after treatment with levamisole plus Chinese medicinal herbs is significantly higher than that in RAU patients after treatment with levamisole only (Sun *et al*., 2003). These results suggest that RAU can be treated by immunomodulation and correction of the patient's deranged cellular and humoral immunities.

A viral or bacterial etiology has long been suggested in RAU. Herpes simplex virus (HSV), varicella zoster virus (VZV), human cytomegalovirus (HCMV) and Epstein-Barr virus (EBV) genomes have been found by PCR in biopsies of ulcerative and pre-ulcerative oral aphthae from RAU patients (Studd *et al*., 1991; Pedersen *et al*., 1993; Sun *et al*., 1996; Sun *et al*., 1998). Our previous study also demonstrated the presence of EBV-DNA and EBV-associated antigens in pre-ulcerative oral aphthous tissues by *in situ* hybridization (ISH) and immunohistochemistry (IHC), respectively (Sun *et al*., 1998). The presence of herpes virus DNAs and their associated antigens in oral aphthous tissues indicates that herpes viruses are the possible etiologic agents in RAU. *Sreptococcus mutans* is a common pathogen

found in the dental plaque on the surfaces of 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 that exacerbate the oral ulceration. Our recent study found 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 patients with erosive oral lichen planus (EOLP) or healthy control subjects (Sun *et al*., 2002). Therefore, we suggest that in addition to herpes viruses, *S. mutans* antigens and GtfD may also be involved in the disease process of RAU, especially in the exacerbation stage.

Tien-Hsien liquid (THL, Feida Union Pharmaceutical Manufactory, El Monte, CA, USA) is an extract of Chinese medicinal herbs and has been used as an anticancer agent for more than ten years. It contains major ingredients, such as *Cordyceps sinensis* (CS), *Oldenlandia diffusae* (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). Compositions as well as pharmacological and immunological effects of major ingredients of THL are summarized in Table 1 (Hu, 1996; Luoh, 1999). All of these constituents have an anticancer effect. CS, RA, PG and GR can increase the cytotoxicity activity of murine NK cells. OD can increase the cytotoxicity activity of murine cytotoxic T-lymphocytes. CS, OD, IPL, PU, RA, PG, AMR, TR, LLA and GR can enhance the phagocytic activity of murine macrophages and/or polymorphonuclear leukocytes. CS and GR can increase the secretion of interleukin-1 (IL-1) by murine macrophages. RA, PG and GR can induce the secretion of interferon-γ (IFN-γ) by mouse spleen cells. CS, OD, PU, RA, PG, AMR, LLA and GR can induce the secretion of interleukin-2 (IL-2) by mouse spleen cells, but CS also selectively inhibits the phytohemagglutinin (PHA)-induced IL-2 secretion and LLA also depresses the antigen-stimulated high IL-2 secretion by mouse spleen cells. CS, PU, RA and AMR can increase the expression of IL-2R by murine lymphocytes. CS, OD, AMR, LLA and GR have anti-inflammatory effects, whereas TR enhances the inflammatory reaction. CS, OD, IPL, PU, RA, PG, AMR and LLA stimulate a PR of murine lymphocytes, but TR inhibits a blast transformation of murine lymphocytes and in some conditions (e.g. high concentration) CS and RA also inhibit the PR of murine lymphocytes. CS, OD, IPL, PU, RA, PG, AMR, TR, LLA and GR have immunopotentiation effect. However, CS, PU and TR also have immunosuppression effect in some circumstances and are shown to possess immunomodulating effects on murine immune system (Hu, 1996; Luoh, 1999). Because THL has both immunopotentiation and immunosuppression effects and can either stimulate or inhibit the lymphoproliferation response, it may be used as an immunomodulating agent to restore the altered cellular or humoral immunity. RAU is an oral mucosal disease with deranged cellular and humoral immunities. It was worth testing whether THL has immunomodulating effects on the antigen-stimulated PR of PBMC and T-cells isolated from RAU patients and whether THL is a potential drug for treating RAU.

Table 1. Composition and Pharmacological and Immunological Effects of Major Ingredients of Tien-Hsien Liquid

	Composition	Anti-cancer	NK Activity	CTL Activity	Phagocytosis	IL-1	IFN-γ	IL-2	IL-2R Expression	Anti-inflammatory	Lympho-proliferation	Immuno-potentiation	Immuno-suppression	Immuno-modulation
***Cordyceps sinensis* (CS)**	1150 mg	↑	↑		↑	↑		↑↓	↑	↑	↑↓	↑	↑	↑
***Oldenlandia diffusae* (OD)**	425 mg	↑		↑	↑			↑		↑	↑	↑		
***Indigo pulverata levis* (IPL)**	425 mg	↑			↑						↑	↑		
***Polyporus umbellatus* (PU)**	270 mg	↑			↑			↑	↑		↑	↑	↑	↑
***Radix astragale* (RA)**	255 mg	↑	↑		↑		↑	↑	↑		↑↓	↑		
***Panax ginseng* (PG)**	255 mg	↑	↑		↑		↑	↑			↑	↑		
***Solanum nigrum* L. (SNL)**	140 mg	↑												
***Pogostemon cablin* (PC)**	140 mg	↑												
***Atractylodie macrocephalae rhizoma* AMR)**	125 mg	↑			↑			↑	↑	↑	↑	↑		
***Trichosanthis radix* (TR)**	125 mg	↑			↑					↓	↓	↑	↑	↑
***Clematidis radix* (CR)**	125 mg	↑												
***Margarita* (M)**	75 mg	↑												
***Ligustrum lucidum Ait* (LLA)**	65 mg	↑			↑			↑↓		↑	↑	↑		
***Glycyrrhizae radix* (GR)**	65 mg	↑	↑		↑	↑	↑	↑		↑		↑		

NK activity = natural killer activity, CTL activity = cytotoxic T-lymphocyte activity, IFN-γ = interferon-γ, IL-1 = interleukin-1, IL-2 = interleukin-2, IL-2R = interleukin-2 receptor.

Active hexose correlated compound (AHCC) is a proprietary extract prepared from co-cultured mycelia of several species of Basidiomycete mushrooms, including shitake (*Lentinus edodes*). The extract is made using hot water following 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). Kamiyama (1999) conducted a clinical trial in Japan to evaluate the preventive effect of AHCC against recurrence of hepatocellular carcinoma (HCC) following surgical resection. He found that after one year the patients taking AHCC show a significantly higher survival rate than the control patients without taking AHCC (Kamiyama, 1999). Further follow-up study by Matsui *et al*. (1999) demonstrated that liver tumor recurrence is not lower in the AHCC group than in the control group, although the survival rate of HCC patients in the AHCC group is higher than that in the control group. In addition, the AHCC-treated patients have significantly decreased levels of liver damage makers SGOT and SGPT. Among these patients, significant improvements are noted in lymphocyte and red cell counts (Kidd, 2000). These findings suggest that AHCC may have liver-protection and immunopotentiation effect and can prolong the survival of HCC patients after surgical resection.

In this study, PBMC and T-cells were isolated from RAU patients. The immunomodulating effects of THL or AHCC on antigen-stimulated PR of PBMC or T-cells from active or inactive RAU patients were examined by culturing PBMC or T-cells with mitogens (lipopolysaccharides, LPS; PHA), superantigen (staphylococcal enterotoxin B, SEB), or specific antigens (glutaraldehyde-inactivated tetanus toxoid, TT; glucosyltransferase D, GtfD; antigens of *S. mutans*) in the presence or absence of THL or AHCC. We found that THL could depress the SEB-stimulated higher PR of PBMC and T-cells from active RAU patients to a lower level, and could augment the GtfD- or *S. mutans*-stimulated lower PR of PBMC or T-cells from inactive RAU patients to a higher level. Because THL also has anti-inflammatory and immunomodulating effects that may correct the altered cellular and humoral immunities in RAU patients, we suggest that THL may be a potential immunoceutical agent for treatment of RAU.

Materials and Methods

Subjects

Thirty-five RAU patients (17 men and 18 women, mean age 36 ± 10 years, range 15–79 years) and five normal control subjects (two men and three women, mean age 32 ± 14 years, range 18–56 years) were included in this study. All the RAU patients had at least one episode of oral ulcerations per month during the preceding years. The development of RAU was divided into two stages: (1) active stage: from the onset of oral mucosal ulceration to the complete healing of oral ulceration, and (2) inactive stage: 1 or 2 weeks after remission of oral mucosal ulceration. The normal control group included five healthy dental patients without any oral mucosal diseases. All the patients were diagnosed and treated in the Department of Oral Pathology and Oral Diagnosis of National Taiwan University Hospital, Taipei, Taiwan. All of them did not take any prescribed drug before entering the study.

Stimulation Antigens

S. mutans GS-5 was grown in brain heart infusion broth (Difco Laboratories, Detroit, MI, USA). 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). LPS from *E. coli*, PHA and SEB were purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA). TT was provided by Ming-Yi Liau of the Department of Health, Center for Disease Control, Vaccine Center, Taiwan. All stimulation antigens used for proliferation assays, including GtfD and reagents, exhibited undetectable endotoxin levels (< 30 pg/ml) as determined by the Limulus amebocyte lysate assay (Sigma).

Cell Preparation and Proliferation Assay

Peripheral blood samples were collected from the 35 RAU patients and five normal control subjects. PBMC were isolated from blood samples by Ficoll-Hypaque centrifugation. Suspensions (2×10^5 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 T-cell proliferation 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 proliferation assays.

PBMC 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 (Celox, St. Paul, MI, USA). PBMC (2×10^5 cells per well) were cultured in 96-well round-bottomed plates (Costar, Cambridge, MA, USA) in a total volume of 200 µl. Purified T-cells (1×10^5 cells per well) were cultured in the presence of irradiated autologous PBMC (2×10^5 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% TCH (Celox). To test if THL and AHCC have mitogenic effects on T-lymphocytes, three replicates of five T-cell cultures from five healthy control subjects were incubated with 1/100, 1/1000 or 1/10,000 dilution of THL or 500 µg/ml of AHCC, or 5, 1 or 0.5 µg/ml of PHA (positive controls). To test if THL and AHCC have immunomodulating effects on the PR of PBMC or T-cells from active or inactive RAU patients, three replicates of PBMC or T-cell culture from 35 active or inactive RAU patients were incubated with LPS (5 µg/ml), PHA (1 µg/ml), SEB (0.01 ng/ml), TT (10 µg/ml), recombinant GtfD (10 µg/ml), or antigens of *S. mutans* (2×10^5 CFU) in the presence or absence of THL (1/1000 dilution) or AHCC (5 µg/ml). Because previous studies have shown that AHCC has immunopotentiating 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 immunopotentiating effects for antigen-stimulated PR of PBMC and of T-cells from either active or inactive RAU patients. In addition, our previous studies have shown that healthy young adults are naturally sensitized with *S. mutans* in their lives

and exhibit readily detectable antibodies and cellular responses to Gtfs (Chia *et al*., 1997; Chia *et al*., 2000; Chia *et al*., 2001). Among the three Gtfs (GtfB, GtfC and GtfD), GtfD preferentially stimulates a higher PR of T-cells than GtfB or GtfC. The differential response to GtfD versus GtfC at cellular level is correlated with the response of salivary or serum antibody response to these Gtf molecules (Chia *et al*., 2001). Therefore, only GtfD, but not GtfB or GtfC, was used as a stimulation antigen for proliferation assays in this study. Incubation was performed at 37°C in a humidified atmosphere with 5% CO_2 for 5 days. Each culture was treated with 0.2 μCi (7.4 kBq) of [^{3}H]-thymidine (Amersham International, Little Chalfont, Bucks, UK) 18 hours before harvesting. Cultures were harvested into 96-unifilter GF/C plates using a FilterMate cell harvester (Packard, Meriden, CN, USA), dried at 50°C for 30 minutes, and 30 μl of Microscint (Packard) was added per well. [^{3}H]-thymidine incorporation was measured with a Packard microplate scintillation counter. PR was expressed as the stimulation index (SI, mean ± standard error of the mean), calculated as the mean counts per minute in antigen-stimulated cultures divided by counts per minute in antigen-free cultures.

Statistical Analysis

The PR of PBMC or T-cells to each of six different mitogens or antigens in the presence or absence of THL or AHCC were compared between groups by Wilcoxon signed rank test. The result was considered to be significant if the p value was less than 0.05.

Results

In this study, we first examined if THL and AHCC have mitogenic effects on the PR of human PBMC and T-cells. PHA alone at various concentrations could elicit a mild PR of T-cells from healthy control subjects. However, THL or AHCC alone at various concentrations did not stimulate the PR of T-cells from healthy control subjects after 5 days of incubation (Table 2). Analogous results were obtained when PBMC from healthy control subjects were tested (data not shown). In addition, THL and AHCC exhibited no cytotoxic effects on PBMC and T-cells cultured *in vitro*, as revealed by the estimation of aldehyde dehydrogenase activity (data not shown).

The immunomodulating effects of THL or AHCC on PBMC or T-cells from RAU patients were next examined by culturing PBMC or T-cells with mitogen (LPS or PHA), superantigen (SEB), or specific antigen (TT, GtfD, or antigens of *S. mutans*) in the presence or absence of THL or AHCC. When stimulated with sub-optimal dose of LPS (5 μg/ml), PHA (1 μg/ml) or SEB (0.01 ng/ml), PBMC or T-cells from active or inactive RAU patients responded efficiently. When LPS-stimulated PRs of PBMC from active RAU patients were compared between groups, the SI of LPS plus THL group (7.9 ± 1.9, $p = 0.011$) or of LPS plus AHCC group (10.8 ± 3.6, $p = 0.041$) was significantly higher than that of LPS alone group (2.7 ± 0.4) (Table 3). However, no significant difference in the LPS-stimulated PR of PBMC from inactive RAU patients was found when THL or AHCC was added to LPS. In addition,

Table 2. Proliferative Response of T-cells from Healthy Control Subjects in the Presence of Various Concentrations of PHA, THL or AHCC

	Antigens	Proliferative Response of T-cells (n = 5) Stimulation Index (Mean ± SD)
PHA		
	5 μg/ml	7.5 ± 3.4
	1 μg/ml	5.6 ± 2.3
	0.5 μg/ml	2.6 ± 2.0
THL		
	1/100 dilution	0.8 ± 0.2
	1/1000 dilution	1.0 ± 0.1
	1/10,000 dilution	0.8 ± 0.2
AHCC		
	5 μg/ml	0.9 ± 0.1
	50 μg/ml	0.9 ± 0.1
	500 μg/ml	1.1 ± 0.2

No mitogenic effects of THL and AHCC on T-cells were found.
SD = Standard deviation.

Table 3. LPS-stimulated Proliferative Response of PBMC from either Active or Inactive RAU Patients in the Presence or Absence of THL and AHCC

Antigens	Proliferative Response of PBMC Stimulation Index (Mean ± SEM)	
	Active Stage (n = 26)	Inactive Stage (n = 9)
LPS (5 μg/ml)	2.7 ± 0.4	2.3 ± 0.6
LPS (5 μg/ml) + THL (1/1000)	7.9 ± 1.9*	6.6 ± 2.3
LPS (5 μg/ml) + AHCC (5 μg/ml)	10.8 ± 3.6†	7.2 ± 4.7

Comparison between the LPS group and LPS + THL or LPS +AHCC groups by Wilcoxon signed rank test with *p = 0.011 and †p = 0.041.
SEM = Standard error of the mean.

no significant difference in the PHA-stimulated PR of T-cells from either active or inactive RAU patients was observed when T-cells were incubated with PHA in the presence or absence of THL or AHCC (Table 4). As shown in Table 5, the addition of THL significantly reduced the SEB-stimulated PR of PBMC (SI dropped from 107.4 ± 18.9 to 75.6 ± 12.3, $p = 0.043$) or of T-cells (SI dropped from 117.3 ± 33.8 to 72.4 ± 29.4, $p = 0.049$) from active RAU patients. Although the addition of THL also reduced the SEB-stimulated PR of PBMC (SI dropped from 126.9 ± 42.4 to 99.5 ± 44.3) or of T-cells (SI dropped from 42.8 ± 12.2 to 30.4 ± 10.5) from inactive RAU patients, no significant difference in SI was found ($p > 0.05$). Furthermore, no significant difference in SI of PBMC or T-cells from either active or inactive RAU patients was found when AHCC was added to SEB (Table 5).

Table 4. PHA-stimulated Proliferative Response of T-cells from either Active or Inactive RAU Patients in the Presence or Absence of THL and AHCC

Antigens	Proliferative Response of T-cells Stimulation Index (Mean ± SEM)	
	Active Stage (n = 25)	Inactive Stage (n=7)
PHA (1 μg/ml)	10.8 ± 3.8	3.7 ± 0.6
PHA (1 μg/ml) + THL (1/1000)	11.6 ± 3.4	10.7 ± 3.3
PHA (1 μg/ml) + AHCC (5 μg/ml)	21.1 ± 5.4	12.7 ± 6.3

No significant difference in stimulation index was found between the PHA group and PHA + THL or PHA +AHCC groups by Wilcoxon signed rank test.
SEM = Standard error of the mean.

Table 5. SEB-stimulated Proliferative Response of PBMC and T-cells from either Active or Inactive RAU Patients in the Presence or Absence of THL and AHCC

Antigens	Stimulation Index (Mean ± SEM)			
	Proliferative Response of PBMC		Proliferative Response of T-cells	
	Active Stage (n = 18)	Inactive Stage (n = 4)	Active Stage (n = 13)	Inactive Stage (n = 4)
SEB (0.01 ng/ml)	107.4 ± 18.9	126.9 ± 42.4	117.3 ± 33.8	42.8 ± 12.2
SEB (0.01 ng/ml) + THL (1/1000)	75.6 ± 12.3*	99.5 ± 44.3	72.4 ± 29.4†	30.4 ± 10.5
SEB (0.01 ng/ml) + AHCC (5 μg/ml)	120.5 ± 29.6	101.4 ± 30.8	103.8 ± 31.8	37.5 ± 19.9

Comparison between the SEB group and SEB + THL or SEB +AHCC groups by Wilcoxon signed rank test with *p = 0.043 and †p = 0.049.
SEM = Standard error of the mean.

When AHCC was added to the cell cultures in the presence of TT (10 μg/ml), it significantly increased the PR of PBMC (SI raised from 5.6 ± 1.4 to 14.4 ± 4.2, p = 0.046) or of T-cells (SI raised from 3.5 ± 0.6 to 21.0 ± 7.2, p = 0.025) from active RAU patients, and also significantly increased the PR of T-cells (SI raised from 1.6 ± 0.2 to 13.7 ± 5.0, p = 0.049) from inactive RAU patients. Although THL also enhanced the TT-stimulated PR of PBMC or of T-cells from either active or inactive RAU patients, no significant difference in SI of PBMC or T-cells was found when THL was added to TT (Table 6).

When THL was added to the cell cultures in the presence of GtfD (10 μg/ml), it significantly raised the PR of PBMC (SI increased from 4.8 ± 1.2 to 12.0 ± 2.1, p = 0.013) or of T-cells (SI increased from 3.3 ± 0.6 to 13.9 ± 4.5, p = 0.044) from inactive RAU patients. No significant difference in SI of PBMC or T-cells from active RAU patients was found when THL was added to GtfD. In addition, AHCC did not significantly increase the GtfD-stimulated PR of PBMC or T-cells from either active or inactive RAU patients (p > 0.05) (Table 7).

Table 6. TT-stimulated Proliferative Response of PBMC and T-cells from either Active or Inactive RAU Patients in the Presence or Absence of THL and AHCC

Antigens	Stimulation Index (Mean ± SEM)			
	Proliferative Response of PBMC		Proliferative Response of T-cells	
	Active Stage (n = 20)	Inactive Stage (n = 7)	Active Stage (n = 20)	Inactive Stage (n = 7)
TT (10 μg/ml)	5.6 ± 1.4	3.9 ± 1.2	3.5 ± 0.6	1.6 ± 0.2
TT (10 μg/ml) + THL (1/1000)	8.7 ± 1.9	8.7 ± 2.0	7.3 ± 3.3	7.5 ± 2.5
TT (10 μg/ml) + AHCC (5 μg/ml)	14.4 ± 4.2*	11.6 ± 5.1	21.0 ± 7.2†	13.7 ± 5.0‡

Comparison between the TT group and TT + THL or TT + AHCC groups by Wilcoxon signed rank test with *p = 0.046, †p = 0.025 and ‡p = 0.049.
SEM = Standard error of the mean.

Table 7. GtfD-stimulated Proliferative Response of PBMC and T-cells from either Active or Inactive RAU Patients in the Presence or Absence of THL and AHCC

Antigens	Stimulation Index (Mean ± SEM)			
	Proliferative Response of PBMC		Proliferative Response of T-cells	
	Active Stage (n = 11)	Inactive Stage (n = 6)	Active Stage (n = 10)	Inactive Stage (n = 6)
GtfD (10 μg/ml)	24.4 ± 8.0	4.8 ± 1.2	24.4 ± 7.0	3.3 ± 0.6
GtfD (10 μg/ml) + THL (1/1000)	26.6 ± 14.3	12.0 ± 2.1*	31.9 ± 9.3	13.9 ± 4.5†
GtfD (10 μg/ml) + AHCC (5 μg/ml)	25.9 ± 7.8	7.9 ± 1.5	33.4 ± 8.2	5.1 ± 0.4

Comparison between the GtfD group and GtfD + THL or GtfD +AHCC groups by Wilcoxon signed rank test with *p = 0.013 and †p = 0.044.
SEM = Standard error of the mean.

When THL was added to the cell cultures in the presence of *S. mutans* (2×10^5 CFU), it significantly raised the PR of PBMC (SI increased from 4.6 ± 0.9 to 6.4 ± 1.6, p = 0.047) from inactive RAU patients, and significantly reduced the PR of T-cells (SI decreased from 8.6 ± 3.3 to 4.0 ± 1.2, p = 0.049) from active RAU patients. Although THL also enhanced the *S. mutans*-stimulated PR of PBMC from active RAU patients (SI increased from 3.6 ± 0.7 to 13.2 ± 8.2, p > 0.05, Table 8), no significant increase in the SI was found because of the great variation of the data (SEM = 8.2). AHCC also significantly raised the *S. mutans*-stimulated PR of T-cells (SI increased from 6.2 ± 1.9 to 9.4 ± 2.7, p = 0.046) from inactive RAU patients. However, no significant difference in the SI was observed when AHCC was added to PBMC from either active or inactive RAU patients or to T-cells from active RAU patients in the presence of *S. mutans* (Table 8).

Table 8. ***S. mutans*-stimulated Proliferative Response of PBMC and T-cells from either Active or Inactive RAU Patients in the Presence or Absence of THL and AHCC**

Antigens	Stimulation Index (Mean ± SEM)			
	Proliferative Response of PBMC		Proliferative Response of T-cells	
	Active Stage (n = 13)	Inactive Stage (n = 8)	Active Stage (n = 14)	Inactive Stage (n = 8)
S. mutans ($2 \cdot 10^5$ CFU)	3.6 ± 0.7	4.6 ± 0.9	8.6 ± 3.3	6.2 ± 1.9
S. mutans ($2 \cdot 10^5$ CFU) + THL (1/1000)	13.2 ± 8.2	6.4 ± 1.6*	4.0 ± 1.2†	6.7 ± 2.6
S. mutans ($2 \cdot 10^5$ CFU) + AHCC (5 μg/ml)	3.4 ± 0.8	5.7 ± 0.8	6.0 ± 1.5	9.4 ± 2.7‡

Comparison between the *S. mutans* group and *S. mutans* + THL or *S. mutans* +AHCC groups by Wilcoxon signed rank test with *p = 0.047, †p = 0.049 and ‡p = 0.046.
SEM = Standard error of the mean.

Discussion

This study found that both THL and AHCC had no mitogenic and cytotoxic effects on PBMC and T-cells from healthy control subjects. THL significantly increased the LPS-stimulated PR of PBMC from active RAU patients, the GtfD-stimulated PR of PBMC and of T-cells from inactive RAU patients, and the *S. mutans*-stimulated PR of PBMC from inactive RAU patients. In addition, the THL could also significantly reduce the SEB-stimulated PR of PBMC and of T-cells from active RAU patients and the *S. mutans*-stimulated PR of T-cells from active RAU patients. These findings suggest that THL can either depress the antigen-stimulated higher PR of PBMC and T-cells from active RAU patients to a lower level or elevate the antigen-stimulated lower PR of PBMC and T-cells from inactive RAU patients to a higher level. Therefore, THL may have both immunopotentiation and immunosuppression effects. In contrast, AHCC only significantly enhanced the LPS-stimulated PR of PBMC from active RAU patients, the TT-stimulated PR of PBMC and of T-cells from active RAU patients, the TT-stimulated PR of T-cells from inactive RAU patients, and the *S. mutans*-stimulated PR of T-cells from inactive RAU patients, suggesting that AHCC may have only immunopotentiation effect.

The reasons why THL has immunomodulating effects on PBMC and T-cells from RAU patients are still not clear. Previous studies on mice have shown that ingredients of THL, CS and GR, can increase the secretion of IL-1 by murine macrophages; 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 (Table 1). IL-1 can induce the expression of IL-2 and IL-2R by lymphocytes. IL-2 is a T-cell growth factor and can stimulate a second round of IL-2 synthesis by the activated T-cells. Moreover, IL-2 itself can further increase the expression of IL-2R (Abbas *et al*., 1991). The binding of IL-2 to IL-2R initiates a series of events that culminate in T-cell proliferation. Because the ingredients of THL can induce the production of IL-2 and the expression of IL-2R by lymphocytes, it was easy to explain why THL can augment the PR of PBMC or T-cells to

LPS, GtfD or antigens of *S. mutans*. 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 (Table 1). 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 (Table 1). Because the ingredients of THL can also 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 PR of PBMC or T-cells to SEB or antigens of *S. mutans*. Previous murine studies also showed that CS, OD, IPL, PU, RA, PG, AMR, TR, LLA and GR have immunopotentiation effect, whereas CS, PU and TR also have immunosuppression effect in some circumstances (Hu, 1996; Luoh, 1999). Because THL can either stimulate or inhibit the PR of murine lymphocytes and of human PBMC and T-cells and possesses both immunopotentiation and immunosuppression effects, it is easy to understand why THL has immunomodulating effects. Moreover, the THL-mediated two-direction effects of immunopotentiation and immunosuppression on the immune system of RAU patients could be through the actions of different THL ingredients, respectively.

LPS is a B-cell mitogen. In this study, LPS was used to stimulate the PR of PBMC from either active or inactive RAU patients. THL significantly increased the lower LPS-stimulated PR of PBMC from active RAU patients to a higher level (SI raised from 2.7 ± 0.4 to 7.9 ± 1.9, $p < 0.05$, Table 3), but it did not significantly elevate the LPS-stimulated PR of PBMC from inactive RAU patients (SI increased from 2.3 ± 0.6 to 6.6 ± 2.3, $p > 0.05$, Table 3). PHA is a polyclonal activator. Although PHA transforms a major proportion of the T-cells, helper T-cells are preferentially stimulated by PHA. In this study, PHA was used to stimulate the PR of T-cells from either active or inactive RAU patients. We found that THL did not significantly augment the PHA-stimulated PR of T-cells from either active or inactive RAU patients ($p > 0.05$, Table 4). Because LPS and PHA specifically stimulate the B-cells and T-cells, respectively, the differences in the results of THL-mediated LPS- or PHA-stimulated PR of PBMC or of T-cells could be due to modulation of different immunological pathways in various assay systems.

AHCC has anticancer efficacy. Clinical studies in Japan have shown that HCC patients taking AHCC have a significantly higher survival rate than control patients not taking AHCC (Kamiyama, 1999; Matsui *et al*., 1999). In addition, AHCC significantly increase the lymphocyte and red cell counts in HCC patients (Kidd, 2000). These findings suggest that AHCC may restore the suppressed immune system in HCC patients, and thus, has immunopotentiation effect. In the present study, we found that AHCC could only augment the LPS-, TT- or *S. mutans*-stimulated PR of PBMC or T-cells from active or inactive RAU patients. No inhibitory effect of AHCC on the antigen-stimulated PR of PBMC or T-cells from RAU patients was found. Therefore, this study also showed that AHCC had only immunopotentiation effect but not immunosuppression effect.

HSV, VZV, HCMV and EBV genomes and their virus-associated antigens have been demonstrated in biopsies of ulcerative and pre-ulcerative oral aphthae from RAU patients by PCR, ISH or IHC (Studd *et al*., 1881; Pedersen *et al*., 1993; Sun *et al*., 1996; Sun *et al*.,

1998), suggesting that herpes viruses are the possible etiologic agents in RAU. Virus-induced early oral ulceration may promote the invasion of bacteria such as *S. mutans* into the oral aphthous lesions. Our recent study found that *S. mutans* antigens and GtfD could stimulate significantly higher PRs in PBMC and T-cells from RAU patients in the exacerbation stage than in those from normal control subjects (Sun *et al*., 2002). Therefore, we suggest that *S. mutans* antigens and GtfD may be involved in the disease process of RAU, especially in the middle and late exacerbation stage of RAU. In this study, we found that THL could raise the GtfD-stimulated lower PR of PBMC and T-cells from inactive RAU patients to a higher level, could raise the *S. mutans*-stimulated lower PR of PBMC from inactive RAU patients to a higher level, and could depress the *S. mutans*-stimulated higher PR of T-cells from active RAU patients to a lower level. These findings suggest that THL can modulate the GtfD- or *S. mutans*-stimulated PR of PBMC or T-cells from active or inactive RAU patients. Previous studies also found that some of the THL ingredients have anti-inflammatory and immunomodulating effects and can increase the cytotoxicity activity of murine NK cells (Hu, 1996; Luoh, 1999). Correction of the altered cellular and humoral immunities in RAU patients may result in a marked improvement of symptoms of RAU. Therefore, we suggest that THL may be a potential immunoceutical agent for treatment of RAU.

In summary, this study found that THL could either depress the SEB-stimulated higher PR of PBMC and T-cells from active RAU patients to a lower level or augment the GtfD- or *S. mutans*-stimulated lower PR of PBMC or T-cells from inactive RAU patients to a higher level. In contrast, AHCC only augmented the LPS-, TT- and *S. mutans*-stimulated PR of PBMC or T-cells from either active or inactive RAU patients. We conclude that THL has an immunomodulating effect on PBMC and T-cells from either active or inactive RAU patients and may be a potential immunoceutical agent for treatment of RAU or other diseases with altered cellular and humoral immunities.

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