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Immuno-enhancement effects of Huang Qi Liu Yi Tang in a murine model of cyclophosphamide-induced leucopenia

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Abstract

Tonic Chinese prescriptions are widely used in daily foods to improve health in Taiwan. Huang Qi Liu Yi Tang (HQLYT) is one such tonic Chinese prescription. In this study, the immuno-enhancement effects of HQLYT tonic were explored using an in vitro splenocyte proliferation assay and by evaluating improvements in cyclophosphamide-induced leucopenia in a BaLb/c mice model. HQLYT is composed of Radix Astragali, licorice, and jujube, and extracted in boiling water. The contents of glycyrrhizin and formononetin were used as bioactive markers to control the quality of the HQLYT water extract, at 7.26 ± 0.04 mg/g and 112.07 ± 0.08 μg/g, respectively. In the in vitro assay, HQLYT (12.5 μg/ml) showed better immuno-enhancing effects than Radix Astragali, licorice, or jujube alone, and enhanced the survival rate of splenocytes by 88%. Moreover, HQLYT significantly enhanced the cytotoxic effects of natural killer cells of splenocytes against YAC-1 cells.

The BaLb/c mice were fed HQLYT once a day for 14 days. After oral treatment with HQLYT 400 mg/kg body weight (BW), the IL-2 release in BaLb/c mice and the natural killer cell activity of the splenocytes were both enhanced, and the WBC level of cyclophosphamide-induced leucopenic mice was improved by treatment with 400 mg HQLYT/kg BW. HQLYT significantly delayed the decline in the WBC level and affected a faster recovery of the WBC level back to a normal status. In summary, HQLYT enhanced immunologic functions in both in vitro and in vivo studies. These results offer scientific evidence to prove the efficacy of this tonic Chinese prescription.

Keywords: Tonic Chinese prescription; Immuno-enhancement; Huang Qi Liu Yi Tang (HQLYT); Natural killer cells activity; Splenocyte; Cyclophosphamide-induced leucopenic mice model

1. Introduction

Tonic Chinese prescriptions are used to supplement normal diets, and to treat deficiency syndromes. In traditional Chinese medicine theory, deficiency syndromes can generally be divided into four types: the qi, blood, yin, and yang (Hsu and Hsu, 1980). As compared with Western medicine, a deficiency of qi means a weakness in immune functions; of blood, in blood circulatory functions; of yin, in the endocrine functions; and of yang, in the growth and functions of bone and muscle (Hsu and Hsu, 1980). Huang Qi Liu Yi Tang (HQLYT) is a Chinese medicinal prescription widely used in daily foods to improve health in Taiwan. HQLYT is composed of Radix Astragali, licorice, and jujube (Yen, 1980). Many species of *Astragalus* are used for Radix Astragali (Ma et al., 2002). However, in Taiwan markets, a red substitute *Hedysarum polybotrys* is commonly used as Radix Astragali (Yen, 1980). The major components of *H. polybotrys* are isoflavonoids, which differ from the astragalosides in *Astragalus membranaceus* (Ma et al., 2002). The name HQLYT originates from the ratio of Radix Astragali and licorice of 6:1. The traditional functions of both Radix Astragali and licorice are in enhancing the immune system. Jujube is used to improve blood circulatory functions. (Yen, 1980). Recently, the pharmacological functions of Radix Astragali, licorice, and jujube were individually reported as exhibiting immune-enhancing effects (Yamaoka et al., 1996; Kurashige et al., 1999; Dai et al., 2001). However, the three elements used in a single formula, as HQLYT, have not been explored. Therefore,

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we hypothesized that the HQLYT formula could improve the immune functions of immune system-weakened people.

In order to ensure the quality control of the water extracts of HQLYT used in this study, HPLC analysis was performed, and glycyrrhizin and formononetin were used as substance markers. Formononetin and glycyrrhizin are principle compounds of Radix Astragali and licorice, respectively (Dai et al., 2001; Ma et al., 2002; Xiao et al., 2004). The immuno-modulation effects of the integrated quality extract of HQLYT was evaluated using in vitro and in vivo models.

HQLYT stimulation of the proliferation of splenocyte and natural killer (NK) cell activity was measured by MTT and lactate dehydrogenase (LDH) leakage in vitro (Sepp et al., 1996; Tsai and Won, 2001). NK cell activity and the amount of interleukin 2 (IL-2) in BaLb/c mice fed HQLYT were also measured in vivo. Leucopenia was induced in BaLb/c mice with cyclophosphamide, and then they were fed HQLYT. This model was used to evaluate the immune-enhancing effects of HQLYT in vivo. Cyclophosphamide is an anticancer and immunosuppressive drug, and was used as an inducer of leucopenia (Davis and Kuttan, 1998; Shalit et al., 2001). HQLYT can prevent cyclophosphamide injury to mice, and has the potential to be developed as a chemopreventive drug.

2. Materials and methods

2.1. Preparation of HQLYT

The Huang Qi Liu Yi Tang (HQLYT) used in this study was purchased from a traditional Chinese medicine store in Taipei, Taiwan. The medicinal plants and materials used in the experiment included the root of *Hedysarum polybotrys* Handel-Mazzetti (Fabaceae, Radix Astragali), the root of *Glycyrrhiza uralensis* Fisch. (Fabaceae, licorice), and *Zizyphus jujube* Miller (Rhamnaceae, jujube). The medicinal materials were authenticated by Associate Prof. H.C. Chang, National Laboratories of Food and Drugs, Department of Health, Executive Yuan, Taipei, Taiwan. Voucher specimens (Nos. HP-0001, GU-0001, and ZJ-0001) were deposited at the Herbarium of the College of Pharmacy, Taipei Medical University.

Huang Qi Liu Yi Tang (HQLYT) is composed of Radix Astragali (67.5 g), licorice (11.25 g), and jujube (11.25 g) (Yen, 1980). The herbal medicines of the prescription were purchased from traditional medicine stores in Taipei, Taiwan. HQLYT was boiled in 1.8 L distilled water for 1 h and then filtered. The aqueous solution was lyophilized to obtain a powder, and the powder was diluted with sterile water before using. The extract powders of Radix Astragali (67.5 g), licorice (11.25 g), and jujube (11.25 g) were prepared by the same method. Glycyrrhizin and formononetin were purchased from Nacalai Tesque (Japan) and Extrasynthese (France), respectively, and dissolved to a concentration of 10 mM in DMSO.

2.2. Quality control of HQLYT

The HPLC system consisted of a Shimadzu (Kyoto, Japan) LC-10ATvp liquid chromatograph equipped with a DGU-14A degasser, an FCV-10ALvp low-pressure gradient flow control valve, an SIL-10ADvp auto injector, an SPD-M10Avp diode array detector, and an SCL-10Avp system controller. Peak areas were calculated with Shimadzu Class-VP software (version 6.12 sp5).

The mobile phase was composed of 0.05% trifluoroacetic acid–acetonitrile (65:35, v/v). A Tosoh ODS-80TM column (250 mm × 4 mm i.d., 5 μm, Japan) was used. The flow-rate was 1.0 ml/min with UV absorbance detection at 254 nm. The operation was carried out at 40 °C. The retention times of the biomarker substances were 26.35 and 19.76 min for glycyrrhizin and formononetin, respectively. The linearity of the peak area (y) versus concentration (x, μg/ml) curve for glycyrrhizin and formononetin was used to calculate the contents of the biomarker substances in HQLYT.

2.3. Animals

Five-week-old BaLb/c female mice weighing between 20 and 25 g were purchased from the National Laboratory Animal and Research Center (Taipei, Taiwan). These animals were especially pathogen-free and kept in environmentally controlled quarters (temperature maintained at 24 °C with a 12-h light–dark cycle) for at least 1 week before use. Water and pelleted feed were freely available. All laboratory food pellets were obtained from the National Laboratory Animal and Research Center (Taipei, Taiwan).

2.4. Splenocyte proliferation assay

Splenocytes were obtained by gentle disruption of the spleen of BaLb/c female mice and filtration *via* a 40-μm Nylon cell strainer (Falcon, NJ, USA). The erythrocytes were lysed with 0.38% NH_4Cl–Tris buffer (pH 7.4), while the remaining cells were resuspended in RPMI-1640 with 10 mM Hepes, 10% fetal bovine serum (FBS), 100 mg/l streptomycin, and 100 IU/ml penicillin.

Splenocytes (2×10^6 cells/ml) were, respectively, treated with three kinds of mitogens (lipopolysaccharide, LPS; concanavalin A, ConA; phytohemagglutinin, PHA) at 10 μg/ml, and co-cultured with test samples in 24-well plates for 48 h at 37 °C in a humidified atmosphere of 5% CO_2. The proliferation of splenocytes was measured by an MTT assay. Data are presented as the mean ± standard deviation (S.D.). Student's t-test was used for comparison of the absorbance values between the test and blank groups.

2.5. Natural killer cell activity of splenocytes in vitro

YAC-1 cells (BCRC60147) were purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan) and maintained in RPMI-1640 supplemented with 10% FBS, 100 mg/l streptomycin, and 100 IU/ml penicillin. YAC-1 cells were used as the target cells, while splenocytes were the effector cells. The ratios of effector to target cells were 12.5:1, 25:1 and 50:1, respectively, and these were co-treated with 12.5 μg/ml HQLYT for 16 h. The NK cell activity of

the splenocytes was determined by cytotoxicity against YAC-1 cells using a lactate dehydrogenase leakage assay (Sepp et al., 1996; Tsai and Won, 2001). The NK activity of effector cells was calculated by the following formula: cytotoxicity (%) = $100 \times \{(A-B)-(C-D)\}/(E-F)$; where A is the experimental release, B the spontaneous release of effector cells, C the spontaneous release of target cells, D the blank for the spontaneous release of effector cells, E the maximum release of target cells (after 2% Triton X-100 treatment for 1 h), and F is the blank for the maximum release. Data are presented as the mean ± standard deviation (S.D.). The Student's t-test was used for comparison of cytotoxicity (%) values between the test and blank groups.

2.6. Cytotoxicity assay

2.6.1. MTT assay

The yellow MTT solution was converted to a purple formazan product by the mitochondrial dehydrogenase of the cells. The cytotoxic effects of cells were dependent on mitochondrial function. After treatment of the splenocytes, the cells were washed with PBS, and then 5 mg/ml of the MTT solution was added for 4 h at 37 °C. The formazan products were dissolved by 0.1N HCl in DMSO, and determined by the absorbance at 600 nm (Bruggisser et al., 2002).

2.6.2. Lactate dehydrogenase leakage assay

When the cell membrane was damaged, lactate dehydrogenase (LDH) was released into the culture medium. The amount of LDH in the cultured medium was an indicator of the cytotoxicity. After the end point of the experiment, the LDH activity of the cells in the culture medium was determined, following the instructions of the LDH cytotoxicity detection kit (Roche, Germany).

2.7. The IL-2 assay in BaLb/c mice

BaLb/c mice were divided into four groups, a control (sterile water) and three test groups (400, 800, and 1600 mg HQLYT/kg body weight, BW); each group contained three mice. The test samples were orally administered to the mice once a day for 14 days. The whole blood of the hearts and spleens was obtained from BaLb/c mice killed by cervical dislocation, under sterile conditions. The whole blood was centrifuged at $500 \times g$ and 4 °C for 30 min; the upper layer contained the serum. The amount of IL-2 in the serum was analyzed by commercial mouse interleukin-2, using the Biotrak ELISA system assay kit (Amersham Biosciences, USA). Data are presented as the mean ± standard deviation (S.D.). Student's t-test was used for comparison of the IL-2 levels between the test and blank groups.

2.8. Natural killer cell activity of splenocytes in vivo

Splenocytes were taken from the above experimental procedure and used as effector cells. The effector cells (splenocytes) were reacted with target cells (YAC-1) in ratios of 15:1, 20:1, and 25:1 (effector:target) for 16 h. The NK cell activity of the splenocytes was measured using the same method as that described above.

2.9. Cyclophosphamide-induced leucopenia in mice

BaLb/c mice were divided into blank, induced, and test groups, with each group containing five mice. BaLb/c mice in the blank group were injected intraperitoneally (i.p.) with normal saline once on day 0, and then sterile water was orally administered once a day for 6 days; in the induced group, 100 mg cyclophosphamide/kg BW was administered once on day 0, and then sterile water was orally administered once a day for 6 days; in the treated group, 100 mg cyclophosphamide/kg BW was administered once on day 0, and then 400 mg of HQLYT/kg BW was orally administered once a day for 6 days. Whole blood was obtained from an eyehole vein in the BaLb/c mice each day before treatment with HQLYT. The white blood cell (WBC) levels of the whole blood were analyzed by an automatic multi-parameter blood cell counter (Sysmex KX-21N). Data are presented as the mean ± standard deviation (S.D.). The multiple analysis of variance (MANOVA) was used for comparing the WBC levels among the blank, induced, and test groups. MANOVA is used to see the main and interaction effects of categorical variables on multiple dependent interval variables.

3. Results

3.1. Effects of HQLYT on splenocyte proliferation

The water extract of HQLYT amounted to about 27.31% of the input herbs. The quality was monitored by the amount of glycyrrhizin and formononetin in the water extract. According to the results of the HPLC analysis, HQLYT contained 7.26 ± 0.04 mg/g and 112.07 ± 0.081 μg/g of the two chemicals, respectively.

Splenocytes were co-treated with HQLYT and three kinds of mitogens (LPS, ConA, and PHA), respectively. The proliferation of splenocytes was more significantly enhanced by HQLYT co-treated with LPS than with ConA or PHA (Table 1). The enhanced percentage of splenocytes showed dose dependence after co-treatment of HQLYT at 3.13–12.5 with 10 μg/ml LPS; the concentration which produced 50% enhanced proliferation

Table 1
Enhanced proliferation percentage of Huang Qi Liu Yi Tang treated-splenocytes co-cultured in lipopolysaccharide (LPS), concanavalin A (ConA) or phytohemagglutinin (PHA) for 48 h

	Huang Qi Liu Yi Tang		
	3.125 μg/ml	6.25 μg/ml	12.5 μg/ml
LPS	11.86%	27.34%	87.82%
ConA	10.40%	14.85%	17.47%
PHA	–	–	–

The concentration of these mitogens was 10 μg/ml; –: no enhanced effects when co-treated with PHA.

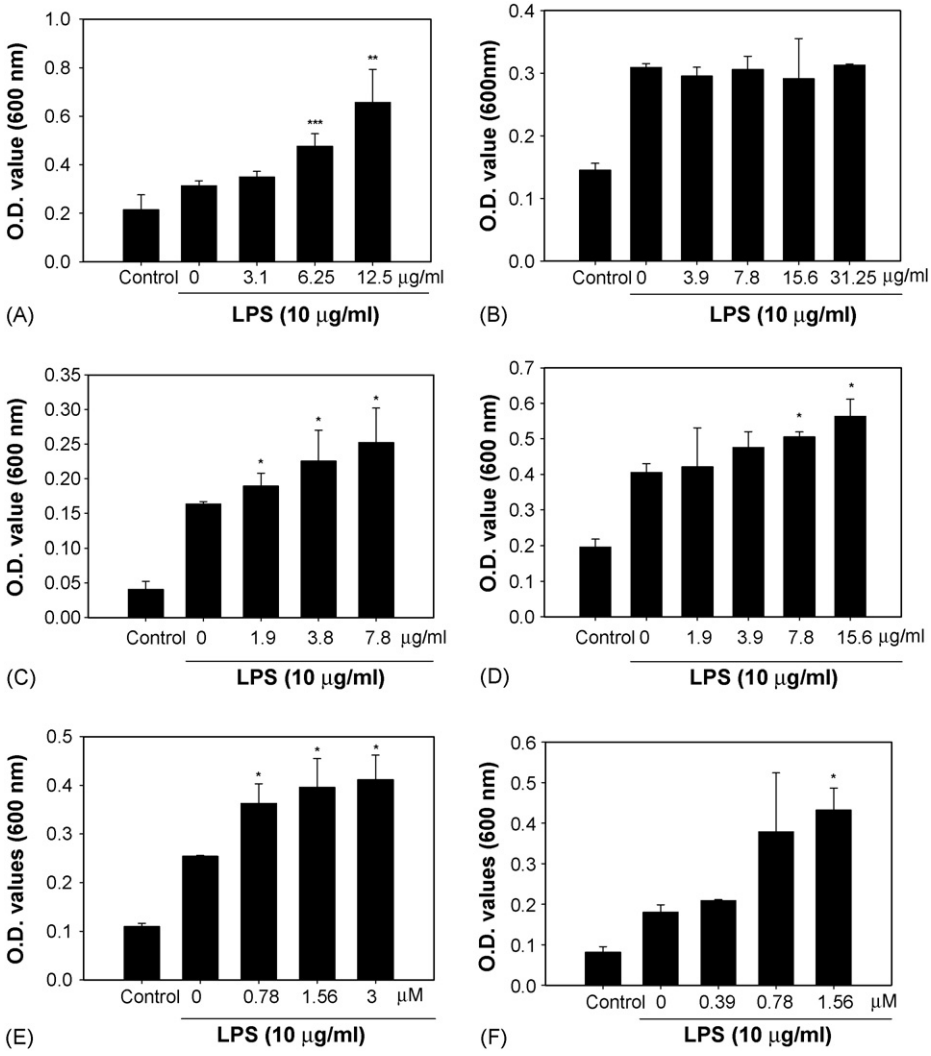

Fig. 1. Effects of Huang Qi Liu Yi Tang (HQLYT) and its constitutive elements on splenocyte proliferation in response to lipopolysacchride (LPS). Splenocytes were cultured in the presence and absence of LPS (10 μg/ml) with various concentrations of HQLYT (A), Radix Astragali (B), licorice (C), jujube (D), formononetin (E) and glycyrrhizin (F) for 48 h. Cell proliferation was measured with the MTT assay. The viability of the splenocytes was detected by measuring the absorbance (o.d. value) at a wavelength of 600 nm. Data are represented as the mean ± S.D. of their separate observations performed in duplicate. Student's *t*-test: $^{*}p < 0.05$; $^{**}p < 0.005$.

was 8.2 μg/ml (Table 1). However, HQLYT only slightly enhanced the proliferation of splenocytes co-treated with ConA, and the maximum enhancement rate was about 15%. PHA in HQLYT-treated splenocytes showed no significant effects.

The prescription of HQLYT includes Radix Astragali, licorice, and jujube. The enhanced proliferation effects of the splenocytes, using the three herbal medicines, were measured after co-treatment with LPS. The water extracts of licorice and jujube both significantly enhanced the proliferation of splenocytes with dose dependence, but Radix Astragali did not. Moreover, HQLYT was more effective than the three herbal medicines individually (Fig. 1). As shown in Table 2, if the enhanced percentages of the three elements at the same concentration (3.9 or 7.8 μg/ml) were added together, the sum total was still lower than HQLYT at 12.5 μg/ml. The substance markers of HQLYT were glycyrrhizin and formononetin, whose enhanced proliferation effects in splenocytes were also measured. The results

Table 2
Enhanced proliferation percentage of splenocytes treated with Huang Qi Liu Yi Tang (HQLYT) and its elements co-cultured with lipopolysaccharide (LPS) for 48 h

	Enhanced proliferation percentage of splenocytes (%)		
	12.5 μg/ml	3.9 μg/ml	7.8 μg/ml
HQLYT	87.8		
AR		0	0
GR		38.0	54.6
ZF		17.3	24.7
Total		55.3	79.3

Total: the enhance proliferation percentage of splenocytes of the three elements at the same concentration were added to the sum; AR, Radix Astragali; GR, licorice; ZF, jujube.

Table 3
Serum IL-2 concentration in HQLYT treated BaLB/c mice

	IL-2 (pg/ml)
Control group	
Sterile water	20.57 ± 8.73
Treated group	
400 mg/kg	69.39 ± 29.98
800 mg/kg	58.45 ± 54.23
1600 mg/kg	84.52 ± 46.93

Each group contained three mice.

showed that both compounds significantly enhanced the proliferation effects of splenocytes and glycyrrhizin to a greater extent than did formononetin (Fig. 1).

3.2. Effects of HQLYT on natural killer cell activity of splenocytes in vivo

NK cell activity of splenocytes was determined by examining cytotoxicity against YAC-1 cells using an LDH assay. As shown by the above data, HQLYT at 12.5 μg/ml significantly enhanced the proliferation effects of splenocytes. Therefore, splenocytes were pretreated with 12.5 μg/ml HQLYT and added to target cells (YAC-1) in ratios of 12:1, 25:1, and 50:1, with an effector (pretreated splenocytes) to target cell ratio of 50:1, and the NK cell activity of the splenocytes was enhanced (Fig. 2A).

3.3. Effects of HQLYT on the IL-2 assay in BaLb/c mice

HQLYT was orally administered to BaLb/c mice once a day for 14 days. Serum of whole blood was obtained from BaLb/c mice after 14 days of treatment. The amount of IL-2 in HQLYT-treated BaLb/c mice was higher than in the control group at 400, 800, and 1600 mg/kg, but there was no significant dose dependence (Table 3). Therefore, spleens were obtained from 400 mg/kg HQLYT-treated BaLb/c mice, and splenocytes were prepared and used as effector cells in the NK cell activity assay.

3.4. Effects of HQLYT on natural killer cell activity of splenocytes in vivo

The effector cells (splenocytes from 400 mg/kg HQLYT-treated BaLb/c mice) were reacted with target cells (YAC-1). The cytotoxic activity of NK cells was enhanced by more than 50% at effector and target cell ratios of 15:1, 20:1, and 25:1 (Fig. 2B).

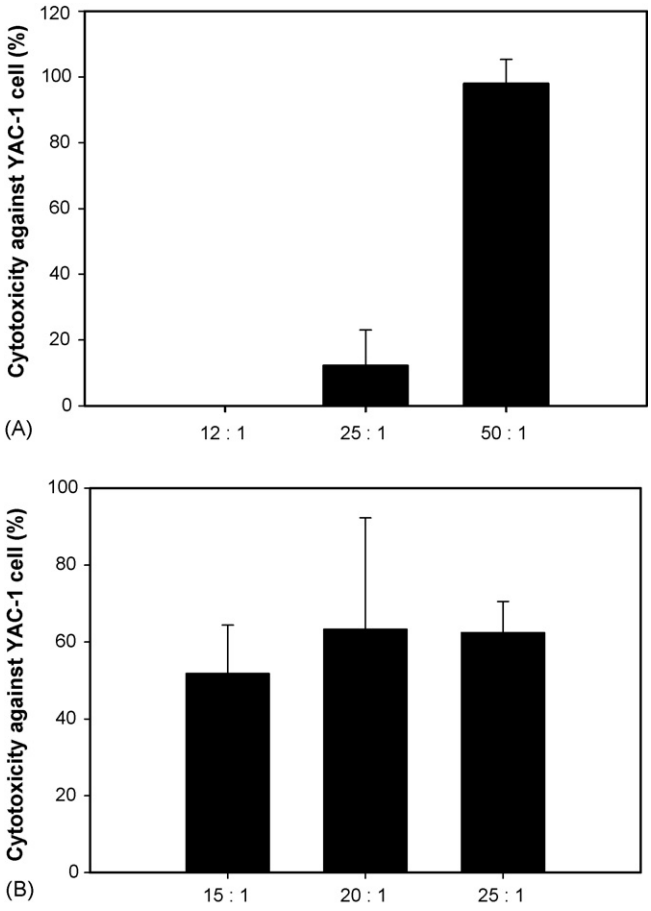

Fig. 2. Effects of 12.5 μg/ml Huang Qi Liu Yi Tang (HQLYT) on natural killer cell activity of splenocytes treated in vitro (A); from mice after administering p.o. 400 mg HQLYT/kg for 14 days (B). Cytotoxic activity against YAC-1 cells was measured by the lactate dehydrogenase (LDH) release assay. Data are presented as the mean ± S.D. of their separate observations performed in duplicate.

3.5. Effects of HQLYT on cyclophosphamide-induced leucopenia in mice

Cyclophosphamide is an anticancer and immunosuppressant drug that was used to induce leucopenia in mice in this study. When the leucopenic mice were treated with HQLYT at 400 mg/kg, the decline in the WBC levels of the mice was

Table 4
Rise in the white blood cell (WBC) level of Huang Qi Liu Yi Tang (HQLYT)-treated cyclophosphamide-induced leucopenic mice

WBC count ($\times 10^3$ μl^{-1})	Blank group	Induced group	Treated group	p-Value
0 day	3.89 ± 1.33			
1st day	2.54 ± 0.65	2.02 ± 0.30	3.74 ± 1.22	**
2nd day	4.48 ± 0.68	2.04 ± 0.15	3.22 ± 1.39	**
3rd day	3.06 ± 0.53	2.08 ± 0.36	2.82 ± 1.03	
4th day	4.00 ± 1.02	2.32 ± 0.25	3.76 ± 1.69	**
5th day	4.38 ± 1.09	2.64 ± 0.19	3.93 ± 0.60	**

Blank group: normal saline i.p. once on day 0, and sterile water orally administered to the mice once a day for 6 days; induced group: cyclophosphamide 100 mg/kg, i.p. once on day 0, and then sterile water orally administered to the mice once a day for 6 days; treated group: cyclophosphamide 100 mg/kg, i.p. once on day 0, and the HQLYT at 400 mg/kg orally administered to the mice once a day for 6 days; each group contained five mice. MANOVA, $^{**}p < 0.01$.

significantly delayed, and there was a faster recovery to a normal status (Table 4). However, the WBC level of mice with cyclophosphamide-induced leucopenia was minimal on the third day, and was not raised by the 400 mg/kg HQLYT treatment.

4. Discussion

HQLYT is a tonic Chinese prescription and is usually compared with other Chinese formulas for treating deficiency syndromes used by Chinese medicinal doctors. In daily use, Taiwanese make HQLYT tea to enhance the immune system and improve blood circulation in the fall and winter. HQLYT tea is sweet due to the Radix Astragali, licorice, and jujube content in the recipe. These three herbal medicines are all rich in saponin glycosides and polysaccharides, and their extracts all have immune-enhancing effects (Matsuda et al., 1999; Erdal et al., 2000; Verotta et al., 2001; Wang and Nixon, 2001). This is the first report of the immune-enhancing effects of these three herbs used in one prescription, HQLYT.

LPS, ConA, and PHA are well-known mitogens, and stimulate different lymphocytes, B cells, and T cells (Th. CD5/8 and T cell Th. CD4). As shown in Table 1, HQLYT co-treated with LPS more significantly enhanced the proliferation of splenocytes than did either ConA or PHA. Therefore, HQLYT is suggested to have greater sensitivity for the B cells of splenocytes. On the other hand, HQLYT more strongly stimulated the proliferation effects of splenocytes than did Radix Astragali, licorice, and jujube used individually (Table 2). The results support the theory of traditional Chinese medicine, in that combined Chinese medicine prescriptions have greater effects than the single herbs used individually.

T cells play an important role in the immune system and are known to be of different types: cytotoxic (Tc) cells, suppressor (Ts) cells, and helper (Th) cells. Moreover, the cells produce interleukin (IL)-2, IL-4, IL-6, and IL-12, and various cytokines, to directly or indirectly regulate immune reactions (Mosmann and Coffman, 1989). For example, IL-2 activates NK cells to decrease tumor masses and inhibit tumor cell metastases (Vujanovic et al., 1995; Yasumura et al., 1999). In this study, BaLb/c mice were orally administered HQLYT once a day for 14 days. The amount of IL-2 in BaLb/c mice was increased, and activated the NK cells of the splenocytes after treatment with HQLYT (Table 1, Fig. 2B). Moreover, the NK cell cytotoxicity of the splenocytes was also activated by treatment with HQLYT in splenocyte culture (Fig. 2A). These results show the probability that HQLYT stimulates IL-2 to activate NK cells.

Immunostimulatory effects of a drug or nutritional supplement are difficult to evaluate in healthy people and animals. Therefore, we used cyclophosphamide, an immunosuppressant, as a leucopenia inducer in mice. In the murine model of cyclophosphamide-induced leucopenia, the WBC level of mice will decrease when 100 mg/kg cyclophosphamide is injected intraperitoneally (i.p.), and then allowed to spontaneously recover for 4 days (Davis and Kuttan, 1998; Shalit et al., 2001). In leucopenic mice orally administered HQLYT, it was found that the decline in the WBC level was significantly delayed, and that the WBC level recovered to its normal status more quickly (Table 3). In summary, it is probable that HQLYT has immunostimulatory effects both in vitro and in vivo, and can be used in daily food intake to promote health and improve immune functions during chemotherapy.

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