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Cytokine production by human lymphocytes stimulated by a herbal compound containing *Bupleurum* (KY88 LIVER LIVO)

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KEY WORDS *Bupleurum*; KY88 LIVER LIVO; Chinese traditional medicine; lymphocyte proliferation; interleukins; tumor necrosis factor

ABSTRACT

AIM: Compounds containing *Bupleurum* possess immunomodulating effects. KY88 LIVER LIVO (KY88) is a blend of such compound. The aim of this study is to investigate the effects of KY88 on the production of cytokines by lymphocytes *in vitro*. **METHODS**: Seventy Sprague Dawley rats were used of which 40 were orally fed with 4 mg purified KY88 for 35 d. Normal human lymphocytes were isolated and cultured in standard conditions. The culture medium was collected at zero and 72 h after the KY88 treatment. The cytokines, including interleukin-1β (IL-1β), IL-2, IL-4, IL-6, tumor necrosis factor-α (TNF-α), and interferon-γ, were measured by ELISA kits. **RESULTS**: TNF-α levels in the supernatant of cultured human lymphocytes significantly increased after the treatment of PHA and KY88. The mean levels were (855±251), (399±145), and (176±49) ng/L after the treatment with KY88 at the concentrations of 10, 1 and 0.1 g/mL respectively. However, the level in the control group without specific treatment was only (68±4) ng/L. The difference between KY88 10 g/mL and control groups was significant ($P<0.05$). All other cytokines did not show significant variations between KY88 and the control groups. KY88 may regulate the immune function through the induction of TNF-α expression.

INTRODUCTION

It is well accepted that the immune system can eliminate cancer cells[1,2]. T-lymphocytes can be used as adoptive immunotherapy[3]. Recently, cytotoxic T lymphocytes specifically against tumor-associated antigens have been shown to be present in cancer patients and healthy individuals[2]. Therefore, more and more researches are focused on the finding of reagents, which can up-regulate the immune function.

Traditional herbal medicine has been used regularly in ‘modulating immunity’ in China. Experiments on *Imperata cylindrica* (Beauv) and other purified polysaccharides have demonstrated that these substances are excellent immunomodulating agents[4-13]. Other herbs such as *Tripterygium wilfordii*, Aconitum and Artemisiae species have immunosuppressive effects. Some of these are now used clinically for the treatment of rheumatoid arthritis, chronic nephritis, systemic lupus erythematosis, and various skin disorders[7]. KY88 LIVER LIVO (KY88) is a blend of the compound containing bupleurum, which possesses anti-inflammatory effects. It is used in the treatment of acute and chronic hepatitis B virus infection. Our previous work has demonstrated that KY88 could stimulate human lympho-

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cytes to form colonies and to produce granulocyte-monocyte colony stimulating factor (GM-CSF)[14]. It may be, thus, involved in the regulation of humoral and cellular immune responses.

Cytokines interact with their receptors and control a wide range of innate and adaptive immune response functions[15,16]. A key feature of these cytokines is their pleiotropy and redundancy. Lymphocyte culture *in vitro* has been used to evaluate the effects of mitogens and proliferation inhibitors on cell proliferation[1,17]. Secretion of cytokines and GM-CSF by the mononuclear cells was markedly increased *in vitro* in the treatment with a low concentration of *Zingiberis rhizoma* extract[18]. Therefore, this study aimed to observe the changes of cytokine production by *in vitro* cultured human lymphocytes under the stimulation of KY88.

MATERIALS AND METHODS

KY88 preparation KY88 is obtained from Hon Ding (HK) International Limited. The ingredients of KY88 are: *Schizandrae fructus, Bupleuri* radix, *Artemisiae capillaris, Desmodii herba, Poria sclerotium, Lithospermi* radix, *Paeoniae* radix, *Phellodendri cortex,* Scutellariae radix, and Trichosanthis radix[14]. Ten grams of each of the above ingredients were primarily washed. Through the process of extraction, concentration, and purification, the essence of the herbal extracts– KY88 was formed. This capsule had been tested by SGS Hong Kong Limited (Société Générale de Surveillance) for the presence of heavy metals and micro-organisms, which was confirmed to be above the international safety level and free from micro-organisms, respectively.

Prior to conduction of experiments, KY88 (50 g) was extracted with methanol (500 mL×3). The solid residue of the crude extract was then dissolved in dimethyl sulphoxide to a concentration of 92 g/L and stored at 4 °C until use. For use, the KY88 was diluted (10 g/L) in Hanks' Balanced Salt Solution (HBSS) (Invitrogen, Carlsbad, California, US) and filtered using 0.22 MicroCellulose Acetate (Corning, New York, US) for sterilization.

Subject selection Fifty volunteers with age ranging from 20 to 45 a, were recruited for this study. Written consent was obtained from each participant and 30 mL of peripheral venous blood was collected in sterilized ethylenediamine tetraacetic acid (EDTA) tubes.

Lymphocyte isolation and culture The blood samples were centrifuged at 1200 revolutions per minute at room temperature for 10 min. Cells were collected and mixed thoroughly with 30 mL ice cold HBSS (Hanks' Balanced Salt Solution, Invitrogen, Carlsbad, California, US) containing HEPES 10 mmol/L (Invitrogen, Carlsbad, California, US). The samples were carefully layered over Ficoll Hypaque (Pharmacia, Uppsala, Sweden) followed by centrifugation at 2000 rpm at 4 °C for 20 min . Peripheral blood mononucelar cells were isolated and the interface was harvested and washed thrice with cold RPMI-1640 medium (serum free).

For initial culture, the isolated cells were distributed into a 6-well plate (Corning Glass Works, Corning, NY, USA) to be cultured in RPMI-1640 medium mixed with 10 % deactivated fetal bovine serum, 10 mmol/L HEPES, and 1 % antibiotics (100 kU/L penicillin and 100 mg/L streptomycin). The plate was incubated at 37 °C in 95 % air and 5 % CO_2 for 1 h. After overnight incubation, a pilot test on the separated lymphocytes was carried out using various concentrations of KY 88 initially. These were eventually stimulated with 10 g/ mL phytohaemagglutinin (PHA) (Roche, Sandhofer, Strasse, Germany), and 0.1 g/mL, 1 g/mL and 10 g/mL KY88 for 72 h. PHA is commonly used for the stimulation of proliferation of lymphocytes. The optimal concentrations of KY88 were calculated according to the recommended dosage from the supplier. RPMI-1640 medium only was added as control.

Proliferation assay for KY88 Proliferation of lymphocytes was assessed with the cell proliferation reagent WST-1 assay (Boehringer Mannheim, Mannheim, Germany). The lymphocytes were cultured at a density of 10 000 cells per well into 96-well microtitre plates (Costar, Cambridge, Massachusetts, US) and incubated at the above conditions for 72 h. The optical density of various groups of cells were read at 450 nm wavelength on a spectrophotometer (Bio-Tek Universal Microplate Reader, ELX800, Winooski, Vermont, US).

Animal trials Female Sprague-Dawley rats were bred and maintained in the University of Hong Kong (HKU) Animal Unit. SD rats were housed under constant environmental conditions (photoperiod, temperature, air humidity, food)[5]. At weight of 250 g, 40 rats were fed with 4 mg purified KY88 daily. For the control group, 30 rats were given the same amount of saline instead. Animals were monitored everyday in terms of behaviour and excretions and they were

weighed once a week throughout a 35-d observation period.

Measurement of cytokines: IL-1b, IL-2, IL-4, IL-6, TNF-b, and IFN Previous experiments have shown cluster and colony formation of lymphocytes as well as a dose-dependent production of GM-CSF with KY88[14]. The culture medium was collected at zero and 72 h after the treatment for measurement of cytokines by ELISA kits according to the manufacturer's instructions (Roche, Germany). The standard group solutions 50 mL and cell culture supernatant were pipeted into 96-well plates. The plates were incubated for 2 h at 350 rpm and washed with washing buffer thrice. The wells were dried out of water and 200 mL of substrate tetramethylbenizidine were added into each well in dark at room temperature for 20 min. At the end, the plates were read at 450 nm wavelength. The levels of these cytokines in test samples were obtained by comparison with the standard curve generated from standards. Each sample was analyzed in triplicates. The measured cytokines included interleukin-1β (IL-1β), IL-2, IL-4, IL-6, tumor necrosis factor-α (TNF-α), and interferon-γ (IFN). All units of these cytokines were ng/L.

Statistical analysis All values were expressed as mean and standard error of mean (SEM) unless otherwise stated. Analysis was done using the SPSS for window release 6.0 (SPSS Inc, USA). The parameters were evaluated with a Paired Samples *t*-test. A *P* value of 0.05 or less was taken as statistically significant.

RESULTS

This capsule had been tested for the presence of heavy metals and micro-organisms, which was confirmed to be above the international safety level and free from micro-organisms, respectively. Of all the SD rats tested, their body weight did not show any significant difference compared with the control group. The proliferation of peripheral blood lymphocytes after stimulation with PHA and KY88 was increased using Cell Proliferation Reagent, WST1 assays. Analysis of colony counting and mean colony area showed that the colony formation was increased after adding KY88. The production of GM-CSF was in a dose-dependent manner and increased with a higher concentration of KY88[14].

The interval changes of the levels of IL-1β, IL-2, IL-4, IL-6, IFN, and TNF-α are shown Fig 1, 2. Analysis of our data suggested that the stimulation of PHA, but not KY88 induced significant increases of IL-2, IL-4, and IFN production. The differences between PHA and control groups after three-day culture were significant (All *P*<0.01). IL-1 and IL-6 levels in the superna-

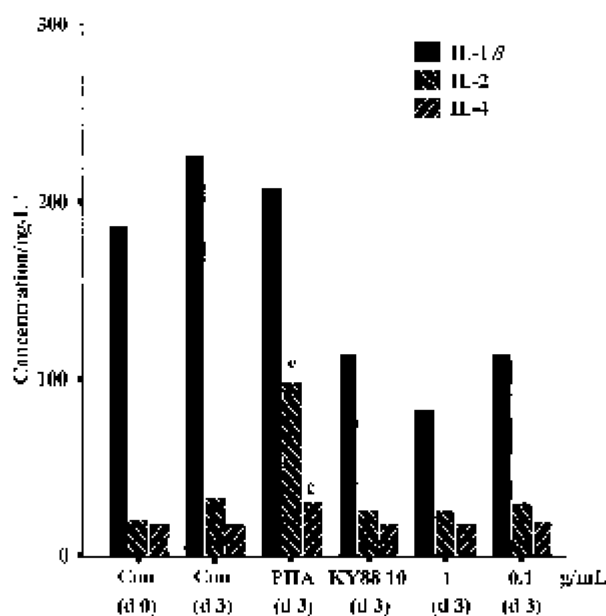

Fig 1. Production of IL-1b, IL-2, and IL-4 of *in vitro* cultured lymphocytes with the treatment of PHA (10 g/mL) and different concentrations of KY88. All units of these cytokines were ng/L. ^cP<0.01 compared with levels of the control group after three-day culture.

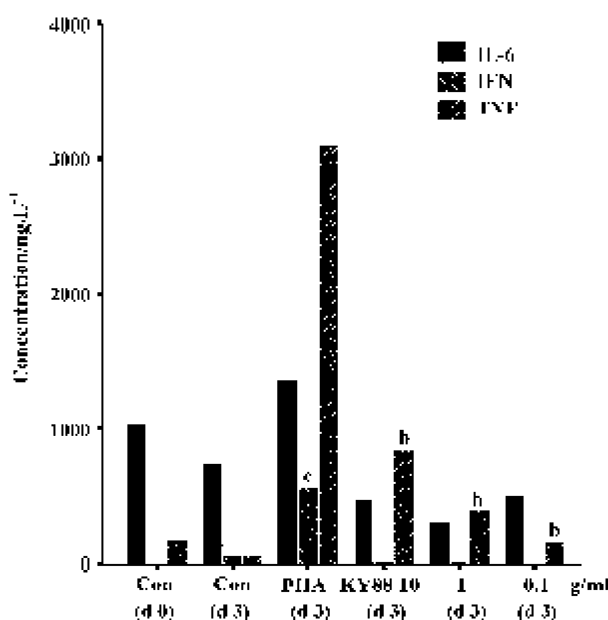

Fig 2. Production of IL-6, TNF-a, and interferon-g (IFN) of *in vitro* cultured lymphocytes with the treatment of PHA (10 g/mL) and different concentrations of KY88. All units of these cytokines were ng/L. ^bP<0.05, ^cP<0.01 compared with levels of the control group after three-day culture.

tant of cultured human lymphocytes did not show significant variations between PHA, KY88, and control groups. TNF-α levels increased after the treatment with PHA and KY88. The mean TNF-α levels were (855±251), (399±145), and (176±49) ng/L after the treatment with KY88 at the concentrations of 10, 1, and 0.1 g/mL for 3 d, respectively. However, the level in the control group without specific treatment was only (68±4) ng/L. The difference between KY88 and control groups was significant ($P<0.05$).

DISCUSSION

The compound formulation approach for chronic hepatitis aims to eliminate the HBV virus, strengthen the immune system, and induce regeneration of liver. Our previous experiments have shown that KY88 is able to stimulate lymphocyte proliferation *in vitro*. This present study confirms that the compound can induce the production of cytokines, especially TNF-α and may regulate the immune system[14].

Cytokines are a system of network. *In vitro* culture of lymphocytes has shown that TNF-α has been shown to stimulate M-CSF gene expression and monocyte proliferation[19]. Our experiments showed that the stimulation of peripheral lymphocytes with KY88 could up-regulate the expressions of TNF-α as well as GM-CSF[14]. There were statistical differences in the expressions of IL-2, IL-4, IL-6, TNF-α, and IFN between PHA and KY88. These results indicated that different mitogens might induce the proliferation of peripheral lymphocytes in different mechanisms, or the effects on lymphocyte proliferation induced by the KY88 are different from those caused by PHA.

Cytokines play important roles in the process of inflammation and anti-tumor immunology. TNF-α increment was present after the treatment of human lymphocytes with KY88. Other experiments showed that the production of cytokines increased with a longer period of incubation[18]. TNF-α plus chemotherapy have been shown to produce complete remission in some of transit melanoma and soft tissue sarcoma[15]. Clinically, the compound containing bupleurum, the source of KY88, is used as alternative medicine in the treatment of cancers and other immunocompromised diseases. Like other herbal medicines, KY88 may play a role in the regulation of the immune system[4-13].

Our study first showed the possible mechanism of the KY88 in the regulation of immune system. Knowledge on this subject is limited, although KY88 is believed to be effective against hepatitis B virus. The increment of TNF-α and GM-CSF is linked to the host responses to inflammation and tumor. This study suggests a potential new area of research into the treatment of inflammation and cancer. Our results justify further studies, such as randomized clinical trials and isolation of effective components in the KY88.

REFERENCES

1 Puente N, Valmori D, Ruegg C. The alternatively spliced domain TnFnIII A1A2 of the extracellular matrix protein tenascin-C suppresses activation-induced T lymphocyte proliferation and cytokine production. J Immunol 2001; 167: 6431-40.

2 Rosenberg SA. Identification of cancer antigens: impact on development of cancer immunotherapies. Cancer J Sci Am 2000; 6 Suppl 3: S200-S207.

3 Cheever MA, Greenberg PD, Fefer A, Gillis S. Augmentation of the anti-tumor therapeutic efficacy of long-term cultured T lymphocytes by *in vivo* administration of purified interleukin 2. J Exp Med 1982; 155: 968-80.

4 Dong Y, Kwan CY, Chen ZN, Yang MM. Antitumor effects of a refined polysaccharide peptide fraction isolated from *Coriolus versicolor*: *in vitro* and *in vivo* studies. Res Commun Mol Pathol Pharmacol 1996; 92: 140-8.

5 Dong Y, Yang MM, Kwan CY. *In vitro* inhibition of proliferation of HL-60 cells by tetrandrine and coriolus versicolor peptide derived from Chinese medicinal herbs. Life Sci 1997; 60: L135-40.

6 Kidd PM. The use of mushroom glucans and proteoglycans in cancer treatment. Altern Med Rev 2000; 5: 4-27.

7 Li XY. Immunomodulating Chinese herbal medicines. Mem Inst Oswaldo Cruz 1991; 86 Suppl 2: 159-64.

8 Mao XW, Archambeau JO, Gridley DS. Immunotherapy with low-dose interleukin-2 and a polysaccharopeptide derived from *Coriolus versicolor*. Cancer Biother Radiopharm 1996; 11: 393-403.

9 Ng TB. A review of research on the protein-bound polysaccharide (polysaccharopeptide, PSP) from the mushroom *Coriolus versicolor* (Basidiomycetes: Polyporaceae). Gen Pharmacol 1998; 30: 1-4.

10 Ooi VE, Liu F. Immunomodulation and anti-cancer activity of polysaccharide-protein complexes. Curr Med Chem 2000; 7: 715-29.

11 Qian ZM, Xu MF, Tang PL. Polysaccharide peptide (PSP) restores immunosuppression induced by cyclophosphamide in rats. Am J Chin Med 1997; 25: 27-35.

12 Wang HX, Liu WK, Ng TB, Ooi VE, Chang ST. Immunomodulatory and antitumor activities of a polysaccharide-peptide complex from a mycelial culture of Tricholoma sp, a local edible mushroom. Life Sci 1995; 57: 269-81.

13 Yang QY, Hu YJ, Li XY, Yang JC, Liu JX, Liu TF, *et al.* A new biologic response modifier substance - PSP. In: Yang OY, Kwok CY, editors. PSP International Symposium. Hong Kong: Fudan University Press; 1993. p56-72.

14 Chow LWC, Loo TY, Sham JST. Effects of a herbal compound containng bupleurum on human lymphocytes. Hong Kong Med J 2001; 7: 408-13.
15 Lejeune FJ, Ruegg C, Lienard D. Clinical applications of TNF-alpha in cancer. Curr Opin Immunol 1998; 10: 573-80.
16 Wallach D, Varfolomeev EE, Malinin NJ, Goltsev YV, Kovalenko AV, Boldin MP. Tumor necrosis factor receptor and Fas signaling mechanisms. Annu Rev Immunol 1999; 17: 331-67.
17 Aidoo A, Feuers RJ, Lyn-Cook LE, Bishop ME, Casciano DA. Characterization of rat lymphocyte primary culture for the development of an *in-vitro* mutagenesis assay: effect of interleukin-2 and 2-mercaptoethanol on the activities of intermediary metabolism enzymes and cell proliferation. Cell Biol Toxicol 1996; 12: 79-87.
18 Chang CP, Chang JY, Wang FY, Chang JG. The effect of Chinese medicinal herb *Zingiberis rhizoma* extract on cytokine secretion by human peripheral blood mononuclear cells. J Ethnopharmacol 1995; 48: 13-9.
19 Kamanna VS, Pai R, Bassa B, Kirschenbaum MA. Activation of mesangial cells with TNF-alpha stimulates M-CSF gene expression and monocyte proliferation: evidence for involvement of protein kinase C and protein tyrosine kinase. Biochim Biophys Acta 1996; 1313: 161-72.