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Differential Effects of Yin- and Yang-Chinese Tonifying Herbs on Innate and Adaptive Immunity

Hoi Yan Leung, Kam Ming Ko*

Division of Life Science, Hong Kong University of Science & Technology, Hong Kong, China
Email: *bcrko@ust.hk

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Abstract

The present study investigated the effect of treatment with methanolic extracts of Yin- and Yang-Chinese tonifying herbs on concanavalin A (Con A)/lipopolysaccharide (LPS)-stimulated splenocyte proliferation (adaptive immunity) and natural killer (NK) cell activity (innate immunity) in an *ex vivo* mouse model. The results indicated that while treatment with most Yin herbal extracts potentiated the Con A/LPS-stimulated splenocyte proliferation, only Yang (but not Yin) herbal extracts stimulated NK cell activity. The differential effects of Yin- and Yang-Chinese tonifying herbs on innate and adaptive immunity are consistent with the Chinese medicine theory which depicts the Yin and Yang functional components of Zheng Qi (vital energy), with the Yang component being responsible for the first line of defense against invading microorganisms (*i.e.*, innate immunity) and the Yin oner serving as a follow-up defensive response (adaptive immunity).

Keywords

Yin, Yang, Innate Immunity, Adaptive Immunity, Natural Killer Cell Activity, Splenocyte Proliferation

1. Introduction

The immune system consists of complex and comprehensive defensive mechanisms against internal and external threats in the body. It protects the body against foreign invaders, such as bacteria and viruses, through two major complementary defensive systems: innate immunity and adaptive immunity. Both immune responses are directed to "non-self" antigens present in invading microorganisms and act to initiate a cascade of events, eliminating the invaders.

The two types of immune responses differ in the time-course and molecular/cellular mechanisms involved in their defensive action [1].

Innate immunity, also known as natural immunity, is the first line of defense against invading microorganisms. It comprises physical, chemical, and cellular components, with the latter consisting of phagocytes, basophils, natural killer cells, etc. Innate immunity is non-specifically activated by pathogen-associated molecular patterns and enables rapid responses within a few hours regardless of the nature of antigen. When innate immunity is insufficient to thwart a foreign attack, antigen-specific adaptive immunity, which consists of humoral (*i.e.*, antibody-mediated) and cell-mediated responses, can respond with a delayed time-course [2].

Chinese medicine emphasizes the importance of the functional balance of Yang and Yin in the body. According to Chinese medicine theory, Zheng Qi, also known as vital energy in the body, is generated by the interplay of Yang Qi (inherited) and Yin Qi (acquired) [3]. Zheng Qi can manifest in two functional forms: Wei Qi (protective Qi) and Ying Qi (nutritive Qi). While Wei Qi circulates outside the meridian around the surface of the body in a cyclic manner, thereby providing the first line of defense against foreign threats (*i.e.*, invading microorganisms), Ying Qi flows along a network of meridians in a regular pattern and is responsible for providing nutrients to various organs including those essential for immune function [3] [4] [5] [6] [7].

The practice of Chinese medicine uses herbs of various functional categories (Qi, Blood, Yin, and Yang) to restore the overall balance of Yin and Yang in the body [6] [7]. Previous studies in our laboratory have demonstrated that Yang herbs can enhance mitochondrial ATP generation in both *ex vivo* and *in vitro* assay systems [8] [9], whereas Yin herbs can stimulate concanavalin A (Con A)/lipopolysaccharide (LPS)-induced mouse splenocyte proliferation *ex vivo* and *in vitro*, an indirect measure of adaptive immunity [9]. Given that extracellular ATP has been shown to be involved in the activation of the innate immune response [10] [11] [12] [13], Yang herbs may act to enhance innate immunity. To test the hypothesis that Yin herbs can act on adaptive immunity and Yang herbs on innate immunity, we investigated the effect of Yin and Yang herbal extracts on innate and adaptive immune responses, which were assessed by measuring the natural killer (NK) cell activity and Con A/LPS-induced splenocyte proliferation in an *ex vivo* mouse model.

2. Materials and Methods

2.1. Reagents

Fetal bovine serum (FBS) was obtained from ThermoFisher Scientific Inc. (Waltham, MA, USA). RPMI-1640 medium (without phenol red), penicillin, streptomycin, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide), nitroblue tetrazolium chloride (NBT), lithium lactate, phenazine methosulfate (PMS), β-nicotinamide adenine dinucleotide hydrate (β-NAD) and LPS were

purchased from Sigma-Aldrich Co (St. Louis, MO, USA). Con A was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Herbal materials were obtained from Lee Hoong Kee Limited, CRCare Store, or Eu Yan Sang. All other chemicals were of analytical grade.

2.2. Herbal Extraction

Herbal materials (100 g) were cut into small pieces and soaked overnight in methanol in a ratio of 1:3 - 1:20 (weight: volume, g: mL), depending on the physical property of the herbal material. The herbal material was then heated under reflux in methanol at 60˚C for 2 h. This procedure was repeated once. The pooled methanol extracts were dried by evaporating the solvent under reduced pressure. The herbal extracts were obtained at various yields (see **Table 1**) [10].

Table 1. The percentage of yields of methanolic extract of Yang-invigorating and Yin-nourishing herbs.

	Pharmaceutical name	Chinese name	Yield (%)	Amount of herb (g) equivalent to 1 g extract
Yang-invigorating herb	Morindae Radix	巴戟天	29.9	3.34
	Cynomorii Herba	鎖陽	15.5	6.45
	Cistanches Herba	肉蓯蓉	26.0	3.85
	Epimedii Herba	淫羊藿	9.0	11.1
	Eucommiae Cortex	杜仲	12.1	8.26
	Dipsaci Radix	續斷	35.0	2.86
	Cuscutae Semen	菟絲子	4.2	23.6
	Cibotii Rhizoma	狗脊	23.0	4.35
	Drynariae Rhizoma	骨碎補	6.10	26.9
Yin-nourishing herb	Prinsepiae Semen	內仁肉	38.3	2.61
	Polygonati Odorati Rhizoma	玉竹	4.9	20.6
	Dendrobium pachyrhiza Rhizoma	石斛草	5.4	18.6
	Ligustri Lucidi Fructus	女貞子	8.0	6.68
	Asparagi Radix	天冬	7.7	13.1
	Ophiopogonis Radix	麥冬	2.1	47.2
	Oryzae Glutinosae Radix	糯稻根須	1.9	53.5
	Pholidotae Chinensis Herba	石仙桃	10.2	9.82
	Ecliptae Herba	旱蓮草	3.8	26.2

2.3. Animal Care

Adult female ICR mice were maintained under a 12-h dark/light cycle at an ambient temperature of approximately 22˚C and allowed food and water *ad libitum*. Experimental protocols were approved by the Research Practice Committee at the Hong Kong University of Science & Technology (No. 2017019).

2.4. Animal Treatment

Female ICR mice (30 g - 35 g), with 4 animals in each group, were orally treated by gavage with methanolic herbal extracts at daily doses of 1 g/kg for 3 days. Control animals received the vehicle (water) only [14].

2.5. Isolation of Splenocytes

Twenty-four hours after the last dosing with the methanolic herbal extract, mice were sacrificed by cervical dislocation using metal forceps. Splenic tissue obtained from the mice was pressed through a mesh stainless steel sieve using a glass pestle in 25 mL RPMI-1640 medium to obtain a single-cell suspension. The suspension was centrifuged at 400 ×*g* for 5 min, and the pellet was washed once with RPMI-medium. The red blood cells within the pellet were subjected to hypotonic lysis with water (4.5 mL). The lytic process was stopped by adding 0.5 mL 10X Hank's Balanced Salt Solution (HBSS: 1.4 M NaCl, 53 mM KCl, 4.4 mM KH_2PO_4, 55.6 mM Glucose and 3.36 mM Na_2HPO_4) and 5 mL RPMI-medium supplemented with 5% (v/v) heat-inactivated (HI) FBS. Following centrifugation, the pellet was resuspended in RPMI-1640 medium supplemented with 5% HIFBS for cell counting, using 0.4% trypan blue. Finally, the splenocytes were diluted to a final concentration of 1×10^7 cells/mL in RPMI-1640 medium supplemented with 5% HIFBS used as effector cells for NK cell activity. Aliquots of cells at a final concentration of 5×10^6 cells/mL in RPMI-1640 medium supplemented with 10% HIFBS were used for Con A/LPS induced splenocyte proliferation assay [15] [16].

2.6. NK Cell Activity Assay

YAC-1 cells, which were used as target cells (T), were seeded in 96-well U bottom culture plates at 2×10^4 cells/well in RPMI-1640 medium supplemented with 5% HIFBS. Splenocytes, prepared as described above, were used as effector cells (E), and were added at 1×10^6 cells/well to give an E/T ratio of 50:1. The cell mixture was then incubated for 24 h at 37˚C in atmospheric air containing 5% CO_2. Following incubation, lactate dehydrogenase

(LDH) activity in the culture medium was measured. Briefly, the cell mixture was centrifuged at 540 ×*g* for 5 min and 100 µL of the resultant supernatant was mixed with 100 µL LDH substrate buffer containing 0.32 mg/ml NBT, 50 mM lithium lactate, 0.28 mM PMS, 1.3 mM β-NAD in 0.2M Tris-HCl, pH 8.2. The reaction mixture was incubated at 37˚C for 5 min in the dark. The absorbance of the reaction mixture was measured at 600 nm after incubation. NK cell activity,

which was estimated by the following equation, was expressed as the percentage of target cells killed [15].

$$\text{NK cell activity}(\%) = \left[(\text{Aii} - \text{Ai} - \text{Aiii})/(\text{Aiv} - \text{Ai})\right] \times 100\%$$

where A = absorbance value of the respective experimental sample at 600 nm,

i, denotes basal LDH release from target cells;

ii, denotes LDH release from a mixture of target cells and effector cells;

iii, denotes basal LDH spontaneously released from effector cells;

iv, denotes total LDH from target cells.

2.7. Con A/LPS-Induced Splenocyte Proliferation *Ex vivo*

Twenty-four hours after the last dosing with the methanolic herbal extract, splenocytes were obtained as described above. Mouse splenocyte concentration was adjusted to 5×10^6 cells/mL with RMPI-medium supplemented with 10% HIFBS, and 80 μL of cell suspension was seeded in each well of a 96-well plate, in the absence or presence of Con A/LPS, in a final volume of 100 μL. Con A/LPS was added at final concentrations of 0, 0.5, 1, 2, 4, or 7.5 μg/mL. Splenocytes were then cultured for 72 h at 37˚C in a humidified atmosphere of 5% CO_2 in air. The extent of cell proliferation was then assessed.

MTT-based cell proliferation assay.

An aliquot (10 μL) of MTT (5 mg/mL in PBS) was added to each well. After 4 h of incubation, 100 μL of solubilization buffer (10% sodium dodecyl sulfate, 45% dimethylformamide, pH 4.7 adjusted by glacial acetic acid) was added, and the mixtures were incubated in 5% CO_2 at 37˚C overnight to dissolve the formed crystals. The extent of splenocyte proliferation was determined by measuring the absorbance at 600 nm using a microplate reader. The extent of Con A/LPS-stimulated proliferation of isolated splenocytes was estimated by computing the area under the curve (AUC) of a graph plotting the percentage of initial absorbance (mean absorbance of cells stimulated with Con A (or LPS)/mean absorbance of cells not stimulated with Con A (or LPS) × 100%) against Con A (or LPS) concentration. The extent of potentiation of Con A (or LPS)-stimulated splenocyte proliferation by the herbal extract treatment was estimated by comparing the untreated control and expressed as percent control [16].

2.8. Statistical Analysis

Data were expressed as mean ± SD (n = 4) and analyzed by Student's t-test (by Excel), to determine significant differences between groups at a level of $p < 0.05$.

3. Results

Methanol extraction of Yang-invigorating and Yin-nourishing herbs produced dried extracts at yields ranging from 0.8% to 52% relative to the dry weight of the raw herbs (Table 1). A previous study showed that intragastric administration of Yin extracts at 1 g/kg/day for 3 days in mice was effective in potentiating

Con A-stimulated proliferation of splenocytes *ex vivo* [14]. In the present study, to examine whether herbal extract treatments produced any stimulatory effect on innate and adaptive immunity, herbal extracts were administered at a constant dose of 1 g/kg/day for 3 days, regardless of the differences in extraction yields.

Oral treatment with methanolic extracts (1 g/kg/day for 3 days) of most of the tested Yin herbs (7 out of 9) caused the potentiation of Con A/LPS-stimulated proliferation of mouse splenocytes *ex vivo*, with the degree of stimulation ranging from 16% - 56% and 19% - 92% in Con A- and LPS-stimulated splenocytes, respectively, with the Prinsepiae Semen extract being most effective (**Figure 1** and **Figure 2**). No detectable effects were observed following treatment of mice with Pholiditae Chinensis Herba and Ecliptae Herba extracts. In contrast, treatments with all tested Yang herbal extracts did not produce any detectable effect on Con A/LPS-stimulated splenocyte proliferation.

On the other hand, most of the tested Yang herbal extracts (7 out of 9) caused an increase in splenic NK cell activity following the same oral treatment regimen in mice, with the extent of stimulation ranging from 14% - 55%, with the effect of Morinda Radix extract being most prominent *ex vivo* (**Figure 3**). No detectable effects were observed with treatment with Cibotii Rhizoma and Drynariae Rhizoma extracts. Treatment with all tested Yin herbal extracts did not produce any detectable effects on splenic NK cell activity.

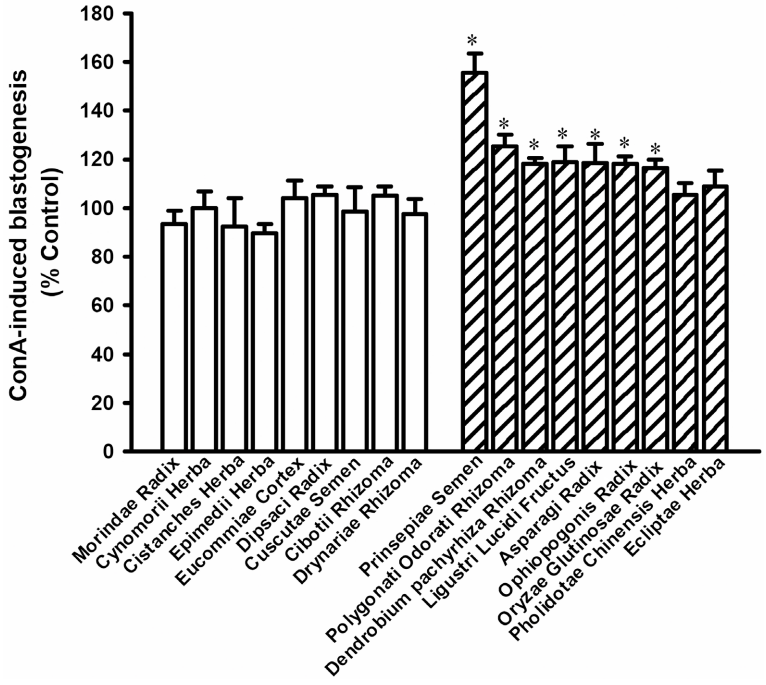

Figure 1. The effect of Yang-invigorating and Yin-nourishing methanolic herbal extracts on Con A-induced-blastogenesis. Con A-induced splenocyte blastogenesis was measured as described in Materials and Methods. Values given are percent control when compared with untreated control and expressed as mean ± SD (n = 4). The control value was 2102 ± 302 (AUC). *Significantly different from control with p-value < 0.05 (using Student's t-test).

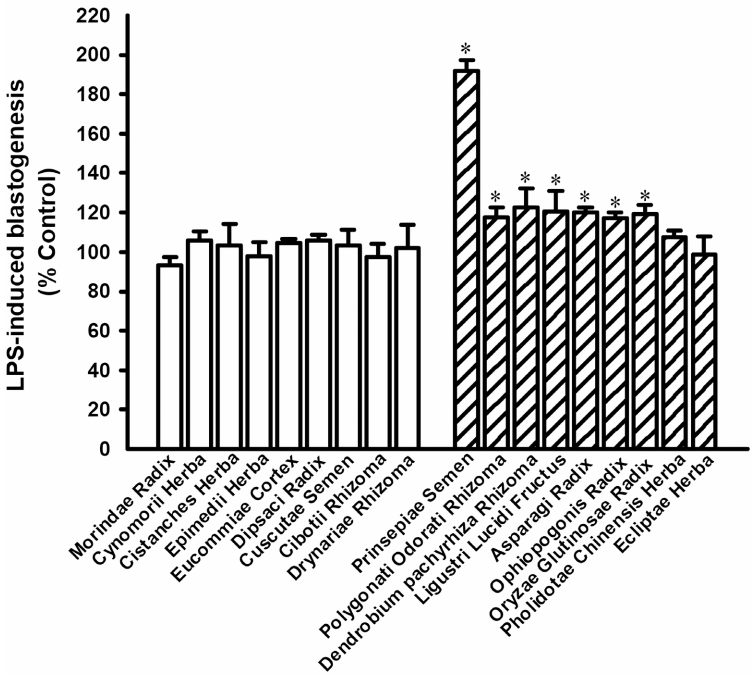

Figure 2. The effect of Yang-invigorating and Yin-nourishing methanolic herbal extracts on LPS-induced-blastogenesis. LPS-induced splenocyte blastogenesis was measured as described in Materials and Methods. Values given are percent control when compared with untreated control and expressed as mean ± SD (n = 4). The control value was 1606 ± 314 (AUC). *Significantly different from control with p-value < 0.05 (using Student's t-test).

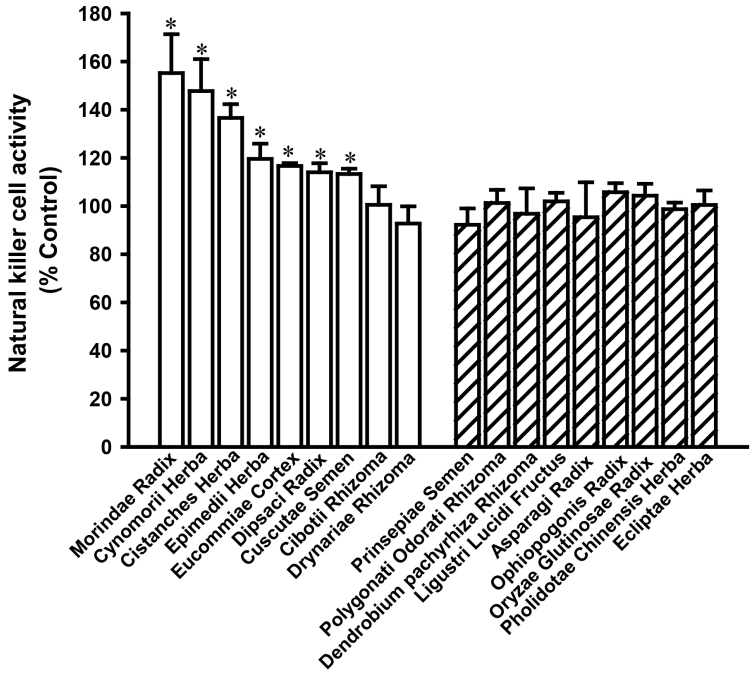

Figure 3. The effect of Yang-invigorating and Yin-nourishing methanolic herbal extracts on NK cell activity. NK cell activity was measured as described in Materials and Methods. Values given are mean ± SD (n = 4) and expressed as percent control when compared with the untreated control. The control value was 7.84 ± 1.98 (%). *Significantly different from control with p-value < 0.05 (using Student's t-test).

4. Discussion

Splenocytes isolated from mouse spleen consist of B- and T-lymphocytes, which mainly respond to antigenic stimulation by LPS and Con A, respectively [17]. An early study in our laboratory demonstrated that methanolic extracts of Yin-nourishing herbs potentiated the Con A-stimulated splenocyte proliferation both *ex vivo* and *in vitro* [9] [14]. In the present study, immunopotentiation of splenocyte proliferation by treatment with Yin herbal extracts was observed in both Con A- and LPS-stimulated splenocytes. The absence of detectable stimulatory activity induced by Pholidotae Chinensis Herba and Ecliptae Herba extracts may be due to the insufficient dosage used. In contrast, none of the Yang herbal extracts produced detectable effects on Con A/LPS-stimulated proliferation of mouse splenocytes *ex vivo*. While Yin herbs, such as Ophiopogonis Radix and Ligustri Lucidi Fructus, which have active components such as Ophiopogon polysaccharides [18] and oleanolic acid [19], respectively, in modulating the responsiveness of splenocytes upon antigen stimulation, the Yang herbs may not contain immunomodulatory chemical components such as polysaccharides and triterpenoids.

In the present study, treatment with methanolic extracts of Yang herbs was found to stimulate NK cell activity *ex vivo*. NK cells residing in splenic tissue originate in the lymphoid lineage of blood cells and participate in innate immunity [20]. NK cells exhibit cytotoxic effects through direct or indirect target recognition. In the direct pathway, the identification of targets occurs through a general signal from NK cell surface receptors that receive activating and inhibiting environmental signals [21]. As extracellular ATP is an effective stimulant of NK cell activity [11] [12] [13], it is possible that Yang herbal extract treatments may stimulate mitochondrial ATP production in splenic cells and thereby increase the extracellular ATP concentration in splenic tissue, with the resultant activation of NK cell activity. Our earlier finding supports this postulation that all tested Yang herbs stimulate mitochondrial ATP production in H9c2 cardiomyocytes [8]. In this regard, Cistanches Herba and Cynomorri Herba were found to contain chemical components such as β-sitosterol and ursolic acid, respectively, that can stimulate mitochondrial electron transport and hence ATP production [22] [23]. While the absence of detectable NK cell activity stimulatory effects of Cibotii Rhizoma and Drynariae Rhizoma extracts may be due to the insufficient dosage used in the treatments, the inability of Yin herbs to stimulate NK cell activity is likely related to the absence of chemical components that can produce an enhancing effect on ATP production. While animal testing with one dosage of herbal extracts may not provide sufficient information for reference in clinical situations, the present study aimed to demonstrate the differential effects of Yin and Yang herbs on adaptive and innate immunity. The experimental findings also suggest a means for characterizing the Yin and Yang properties of Chinese tonifying herbs using an *ex vivo* mouse model.

5. Conclusion

While treatments with Yin herbal extracts but not Yang herbal extracts could potentiate Con A/LPS-stimulated splenocyte proliferation, only Yang herbal extracts were found to stimulate splenic NK cell activity. This experimental observation is consistent with the Chinese medicine theory which depicts the Yin and Yang functional components of Zheng Qi. While the protective Qi, under Yang in nature, is responsible for innate immunity, the nutritive Qi, under Yin in nature, governs the adaptive immunity in the body. As such, Yang-invigorating herbs can therefore upregulate the NK cell activity and the Yin-nourishing herbs can potentiate adaptive immunity, as assessed by Con A/LPS-stimulated splenocyte proliferation.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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