Long-Term Treatment with a Compound Polysaccharide-Based Health Product (Infinitus Polysac Plus) Enhances Innate and Adaptive Immunity in Mice

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Abstract

This study aimed to investigate the effects of a compound polysaccharide-based health product (Infinitus Polysac Plus, IPP) on innate and adaptive immunity in mice. Both ex vivo/in vivo mouse models and an in vitro system using cultured mouse splenocytes were adopted for the assessment of innate and adaptive immunity. For the innate immune response, long-term IPP treatment (0.26 and 0.78 g/kg x 20 doses) enhanced the carbon clearance activity and phagocytic rate of macrophages, as well as natural killer cell activity in mice. The IPP-induced increase in natural killer cell activity was accompanied by the suppression of tumor growth in Sarcoma-180 cell-inoculated mice. For the adaptive immune response, while long-term IPP treatment increased the splenocyte index in mice, IPP incubation with mouse splenocytes in vitro potentiated their concanavalin A-stimulated proliferation. Long-term IPP treatment significantly restored the hemolytic activity of serum on sheep red blood cells and dinitrofluorobenzene-induced delayed-type hypersensitivity in sensitized and immunosuppressed mice. In conclusion, the results indicate that long-term IPP treatment produces stimulatory effects on both innate and adaptive immunity in mice.

Keywords: Compound Polysaccharide, Innate Immunity, Adaptive Immunity

1. Introduction

Polysaccharides isolated from medicinal fungi and herbs are known to possess immunomodulatory activity in both experimental and clinical settings [1,2]. It has been suggested that a mixture of polysaccharides from various medicinal fungi and herbs can produce immunomodulatory effects in a synergistic manner [3-5]. Innate and adaptive immunity are the mainstay of immune defense against microbial infections. While innate immune responses, which involve phagocytotic activities and secretion of pro-inflammatory mediators, do not require prior exposure to an antigen, adaptive immune responses, such as the eliciting of antigen-specific cell-mediated response and production of antigen-specific antibodies, are dependent on processes generated by previous exposure to an antigen [6,7]. In the present investigation, we aimed to define the immunopharmacological profile of Infinitus Polysac Plus (IPP), a Chinese herbal health product containing a mixture of biologically active polysaccharides, on innate and adaptive immunity in mice, using both ex vivo/in vivo mouse models and an in vitro system utilizing cultured mouse splenocytes. IPP has been consumed by hundreds of thousands of individuals over the past 15 years in China.

2. Materials and Methods

2.1. Chemicals, Cell Materials and Herbal Product

Concanavalin A (Con A), 2,4-dinitro-1-fluorobenzene (DNFB) and cyclophosphamide (CYC) were purchased from Sigma (St. Louis, MO). RPMI-1640 medium and fetal calf serum were obtained from Gibco™ (Grand Island, NY). MTT (3-[4, 5-dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide)-based cell proliferation kit I was purchased from Boehringer Mannheim GmbH (Germany). Chicken red blood cells (CRBC) and sheep red blood cells (SRBC) were purchased from Hemostat La-
boratories (Dixon, CA). YAC-1 cells and Sarcoma 180 cells were supplied by American Type Culture Collection (ATCC; Manassas, VA). All other chemicals were of analytical grade.

IPP was prepared by mixing the individual aqueous extract of 9 herbs, namely, Fructus Lycii [Lycium barbarum L. (Solanaceae)], Poria [Porzia cocos (Schw.) Wolf, Flos Chrysanthemi [Chrysanthemum morifolium Ramat.], Radix Codonopsis [Codonopsis pilosula (Franchet) Nannfeldt (Campanulaceae)], Lentinula [Lentitmus edodes (Berk.) Sing. (Agaricaceae)] and Tremella (Tremella fuciformis). All herbs were authenticated with reference to morphological and chemical criteria stated in Chinese Pharmacopoeia. The combined extract was dried and made into a tablet dosage form. The total polysaccharide content of IPP was 15% (w/w) as determined by a colorimetric method [8].

2.2. Animal Care

Adult female and male ICR mice were maintained under a 12-h dark/light cycle at a 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.

2.3. Animal Treatment

Female ICR mice (25 - 30 g), with 6 - 10 animals in each group, were orally treated by gavage with IPP at daily doses of 0.26 or 0.78 g/kg for 20 doses within 4 weeks (i.e., 5 doses per week). The low dose is the equivalent of a human dose (35 mg/kg/day) for IPP. Control animals received the vehicle (distilled water) only.

2.4. Innate Immunity Assays

2.4.1. Carbon Clearance Test in Vivo—Measurement of Carbon Clearance

Twenty-four hours after the last dosing with IPP, mice were injected with 0.2 mL of 5% starch solution into the abdominal cavity to stimulate the aggregation and activation of macrophages. Two hundred microlitre of 20% (v/v, in 0.9% saline) CRBC suspension were intraperitoneally injected into mice 24 hours after the starch administration. After 30 min, the restrained mice were sacrificed by cervical dislocation with metal forceps, and their abdominal cavity was opened under aseptic conditions to harvest peritoneal macrophages. The peritoneal cavity was washed with 2 mL phosphate buffered saline (PBS) and the PBS wash formed the peritoneal fluid. Then, 1 mL of peritoneal fluid was collected from each mouse and constituted the macrophage suspension. An aliquot (200 µL) of macrophage suspension was placed on a clean glass slide. The slides were then incubated at 37°C for 30 min, and then rinsed with PBS. Cells on the slide were fixed with a mixture of acetone and methanol (1:1, v/v) for 10 min, and then dried, followed by staining with Wright-Giemsa solution for 6 min and washing with distilled water. After drying, the cells on the slide were examined by light microscopy, with the counting of at least 100 macrophages in different fields. The phagocytic rate and index of macrophages were calculated as follows [9]:

\[
\text{Phagocytic rate (PR)} = \left( \frac{\text{number of macrophage ingesting CRBC}}{\text{number of total macrophage}} \right) \times 100\%
\]

\[
\text{Phagocytic index (PI)} = \left( \frac{\text{number of total ingested CRBC}}{\text{number of macrophage ingesting CRBC}} \right)
\]

2.4.2. Phagocytic Activity of Macrophages in Vivo Measurement of Phagocytic Rate and Index

Twenty-four hours after the last dosing with IPP, mice were injected with 0.2 mL of 5% starch solution into the abdominal cavity to stimulate the aggregation and activation of macrophages. Two hundred microlitre of 20% (v/v, in 0.9% saline) CRBC suspension were intraperitoneally injected into mice 24 hours after the starch administration. After 30 min, the restrained mice were sacrificed by cervical dislocation with metal forceps, and their abdominal cavity was opened under aseptic conditions to harvest peritoneal macrophages. The peritoneal cavity was washed with 2 mL phosphate buffered saline (PBS) and the PBS wash formed the peritoneal fluid. Then, 1 mL of peritoneal fluid was collected from each mouse and constituted the macrophage suspension. An aliquot (200 µL) of macrophage suspension was placed on a clean glass slide. The slides were then incubated at 37°C for 30 min, and then rinsed with PBS. Cells on the slide were fixed with a mixture of acetone and methanol (1:1, v/v) for 10 min, and then dried, followed by staining with Wright-Giemsa solution for 6 min and washing with distilled water. After drying, the cells on the slide were examined by light microscopy, with the counting of at least 100 macrophages in different fields. The phagocytic rate and index of macrophages were calculated as follows [9]:

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2.4.3. Natural Killer (NK) Cell Activity

2.4.3.1. Isolation of Splenocytes (Effector Cells)

Twenty-four hours after the last dosing with IPP, mice were sacrificed as described above. Spleens were aseptically removed from the dissected mice with scissors and forceps in cold RPMI-1640 medium (without phenol red). Then, a single cell suspension was obtained by staining with Wright-Giemsa solution for 6 min and washing with distilled water. After drying, the cells on the slide were examined by light microscopy, with the counting of at least 100 macrophages in different fields. The phagocytic rate and index of macrophages were calculated as follows [9]:

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\]
2.4.3.2. Measurement of NK Cell Activity

YAC-1 cells, which were used as target cells (T), were seeded in 96-well U-bottom culture plates at $2 \times 10^6$ cells/well in RPMI-1640 medium (without phenol red) supplemented with 5% HIFBS. Spleen cells, prepared as described above, were used as effector cells (E), and they were added at $1 \times 10^6$ cells/well to give an E:T ratio of 50:1. The cell mixture was then incubated for 24 hours at 37°C in atmospheric air containing 5% CO₂. After incubation, lactate dehydrogenase (LDH) activity in the culture medium was measured. Briefly, the cell mixture was centrifuged at $540 \times g$ for 5 min and 100 μL of the resultant supernatant was then mixed with 100 μL LDH substrate buffer. The reaction mixture was incubated at 37°C for 10 min in the dark. The reaction was terminated by adding 30 μL 1M HCl. Absorbance of the reaction mixture was measured at 570 nm. NK cell activity, which was estimated by the following equation, was expressed as the percentage of target cells being killed [10]:

$$NK \text{ cell activity (\%)} = \frac{[A_i - A_{ii} - A_{iii}](A_{iv} - A_i)}{A_{iv} - A_i} \times 100\%$$

where $A$ = absorbance value of the respective experimental sample at 570 nm,

- $i$, denotes basal LDH release from target cells;
- $ii$, denotes LDH release from a mixture of target cells and effector cells;
- $iii$, basal LDH spontaneously released from effector cells;
- $iv$, denotes total LDH from target cells.

2.4.4. Tumor Growth Inhibitory Activity Tumor Cell Inoculation

Adult male ICR mice (~30 g) were randomized into 4 groups, with at least 10 animals in each. Male instead of female mice were used according to the method of Wang et al. [11]. An aliquot (200 μL) of Sarcoma-180 cells at a cell concentration of $10^7$ cells/mL was subcutaneously injected into the right groin of mice on day 1. Twenty-four hours after the inoculation, mice were treated with IPP at daily doses of 0.26 and 0.78 g/kg for 20 days until sacrifice. Body weight and tumor weight were measured, and data were expressed as tumor weight per g body weight (i.e. tumor index).

2.5. Adaptive Immunity Assays

2.5.1. Hemolysis of Sheep Red Blood Cell (SRBC) at 50% (HC₅₀)

Aliquots (0.2 mL) of 2% sheep red blood cells (SRBC) were injected into the abdominal cavity of mice on the day of dose 18. Twenty-four hours after the last dosing with IPP, blood samples were drawn and serum samples were obtained thereafter. Serum samples were diluted at 1:(100 - 400) (v/v) with saline, 200 μL of diluted serum samples were added into glass tubes, and then 100 μL of 20% SRBC suspension and 200 μL of 6-fold diluted guinea pig complement were added into the tubes. After incubation at 37°C for 30 min, the reaction was stopped by putting the tubes in an ice bath, and the reaction mixtures were centrifuged at 960 × g for 10 min. An aliquot (200 μL) of the resultant supernatant was mixed with 600 μL of Doshi reagent (0.2 g potassium ferricyanide, 0.05 g potassium cyanide, 1.0 g sodium hydrogen carbonate in 1 L distilled water). In parallel, absorbance for 100% hemolysis was obtained from the following preparation: 100 μL 20% SRBC mixed with 400 μL red blood cell lysis buffer, and then 200 μL of the mixture was added into 600 μL Doshi reagent to develop the color for the measurement of absorbance. The absorbance at 550 nm for assay mixtures was measured and the 50% hemolytic concentration (HC₅₀) of the serum was estimated by the following equation [12]:

$$Sample \ HC_{50} = (\text{sample absorbance/}50\% \text{ hemolytic absorbance}) \times \text{dilution factor}.$$

2.5.2. Con A-Induced Blastogenesis of Mouse Splenocytes in Vitro and ex Vivo

2.5.2.1. Isolation of Mouse Splenocytes

All procedures were conducted under aseptic conditions. Splenic tissues were obtained by dissection after sacrifice as described above from female ICR mice (25 - 30 g), and isolated splenocytes were resuspended in RPMI-1640 medium supplemented with 10% HIFBS at a final concentration of $5 \times 10^6$ viable cells/mL. The viability of isolated splenocytes was determined by Trypan blue exclusion.

2.5.2.2. Ex Vivo Immunomodulatory Activity

Twenty-four hours after the last dosing with IPP, animals were sacrificed as described above, and splenic tissues were obtained under aseptic conditions for isolation of splenocytes, as described above. Mouse splenocytes ($10^6$ cells) were cultured in a medium, in the absence or presence of Con A, in a final volume of 100 μL. Con A was added at final concentrations of 0.5, 1, 2 or 4 μg/mL. Splenocyte index was also estimated by the total number of splenocytes per gram body weight.

2.5.2.3. In Vitro Immunomodulatory Activity

Isolated mouse splenocytes were cultured in a medium,
in the presence or absence of Con A, with or without IPP (dissolved in PBS), in a final volume of 100 µL in 96-well flat bottom microtiter plates. Con A (prepared in aqueous solution) was added at final concentrations of 0.5, 1, 2 or 4 µg/mL. Aliquots of IPP (10 µL) were added at 3 increasing final concentrations in the range 1.56 - 12.5 µg/mL. Control cells received 10 µL of vehicle (PBS) only. Three separate experiments were performed, and data were expressed as percent change with respect to the IPP-untreated control.

2.5.2.4. Measurement of Splenocyte Proliferation
Splenocytes were cultured for 72 hours at 37°C in a humidified atmosphere of air containing 5% CO₂. At the end of the culture period, the extent of splenocyte proliferation was determined by a colorimetric assay using MTT-based cell proliferation kit I. The extent of Con A-stimulated proliferation of isolated splenocytes was estimated by computing the area under the curve (AUC) of the graph plotting the net absorbance (mean absorbance of cells stimulated with Con A—mean absorbance of cells not stimulated with Con A) against Con A concentration. The effect of IPP on the extent of Con A-stimulated proliferation was compared with that of the IPP-untreated control, and data were expressed as percent control [13].

2.5.3. Dinitrofluorobenzene-Induced Delayed-Type Hypersensitivity Mouse Ear Swelling Assay
Animals were treated with IPP as described above. On the days of dose 17 and 18, female mice were sensitized by topical application of 25 µL of 0.5% (v/v) DNFB in acetone-olive oil (4/1, v/v) onto a designated shaved surface of the abdomen. Topical application of 0.2% DNFB on both ears did not cause a primary reaction in unsensitized mice. The immunosuppression was induced by intraperitoneal injection of CYC at 300 mg/kg three days after the last sensitization. To elicit delayed-type hypersensitivity (DTH), animals were challenged by applying 20 µL of 0.2% DNFB in a mixture of acetone and olive oil (v/v, 4/1) on the dorsal side of both ears two days after immunosuppression. The ear swelling reaction was assessed 24, 48 and 72 hours post-challenge by measuring the thickness of the ears with a micrometer. The extent of edema was estimated for each ear with reference to the difference in thickness before and after DNFB challenge. The extent of enhancement in DTH in IPP-treated animals was estimated by comparison with IPP-untreated controls [14].

2.5.4. Statistical Analysis
Data were analyzed by one-way ANOVA using SPSS statistical software. Significant differences between two groups were determined by Least Significant Difference when \( P < 0.05 \).

3. Results

3.1. Innate Immunity
IPP treatment (0.26 or 0.78 g/kg × 20) increased the phagocytic index in mice (~20% - 22%), as assessed by the carbon clearance test (Figure 1). IPP treatment significantly increased the phagocytic rate of macrophages in mice, with the maximum degree of stimulation (190%) being observed at the dose of 0.26 g/kg (Figure 2). However, no detectable changes in macrophage phagocytic index were observed.

IPP treatment at a dose of 0.78 g/kg significantly increased (129%) NK cell activity in mice (Figure 3). The increased NK cell activity resulting from IPP administration was accompanied by a significant suppression (62%) of tumor growth in mice (Figure 4).

3.2. Adaptive Immunity
Serum from SRBC-immunized mice showed an enhancement in hemolytic activity on SRBC, as evidenced by an increase in the degree of dilution of serum that caused 50% hemolysis (Figure 5). The hemolytic activity of the serum was greatly reduced in immunosuppressed mice. IPP pretreatment (0.26 or 0.78 g/kg × 20) caused a dose-dependent restoration of hemolytic activity of serum in SRBC-immunized and CYC-immunosuppressed mice, with the maximum stimulation being 56%, when compared with IPP-untreated and immunosuppressed controls.

Figure 1. Effect of long-term IPP treatment on carbon clearance activity in mice. Animals were treated with Infinitus Polysac Plus (IPP) administered orally at daily doses of 0.26 or 0.78 g/kg for 20 doses within 4 weeks. The carbon clearance test was performed and phagocytic index (α) was estimated, as described in Materials and methods. Values given are means ± S.E.M., with n = 6 - 10. *Significantly different from the IPP-untreated control (\( P < 0.05 \)).
Con A-stimulated proliferation of mouse splenocytes in vitro (Figure 7).

DNFB induced DTH in sensitized mice, as evidenced by the increase in ear thickness, with the maximum increase in the extent of edema (35 fold) occurring at 48 h post-DNFB challenge (Figure 8). DNFB-induced DTH was almost completely inhibited in immunosuppressed mice. IPP pretreatment at a dose of 0.78 g/kg partially but significantly restored the DNFB-induced DTH reaction in immunosuppressed mice, with the maximum increase in the extent of edema formation (10 fold) occurring at 24 h post-DNFB challenge at a dose of 0.78 g/kg.

IPP treatment significantly increased the splenocyte index of mice (20%) at a dose of 0.76 g/kg (Figure 6).

While IPP pretreatment did not produce any detectable changes in the extent of Con A-stimulated proliferation of mouse splenocytes ex vivo (Data not shown), it dose-dependently (1.56-12.6 µg/mL) potentiated (3-14%) the

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Figure 6. Effect of long-term IPP treatment on splenocyte index in mice. Animals were treated with IPP as described in Figure 1. The total number of splenocytes was measured, and data were expressed as splenocyte index (total number of cells/g body weight). Values given are means ± S.E.M., with n = 6. *Significantly different from the IPP-untreated control (P < 0.05).

Figure 7. Effect of long-term IPP co-incubation on Con A-induced blastogenesis in mouse splenocytes in vitro. Isolated mouse splenocytes were subjected to Con A challenge (1 - 4 µg/mL) in the absence or presence of IPP (1.56 - 12.5 µg/mL). The extent of Con A-stimulated blastogenesis was estimated, and data were expressed as percent control (compared to the Con A-stimulated group without IPP co-incubation). Values given are means ± S.E.M., with data obtained from 3 separate experiments. *Significantly different from the non-IPP co-incubated control (P < 0.05).

4. Discussion

Long-term IPP treatment at oral doses (which didn’t show any observable adverse effect in the fed mice), including an equivalent of a human dose, was found to enhance phagocytic activity in mice, as evidenced by increases in carbon clearance and phagocytic rate of macrophages. The increased rate of carbon clearance, indicative of enhanced Kupffer cell phagocytosis [15], is consistent with the activation of macrophages in IPP-treated mice. A recent study in chickens showed that the extent of phagocytosis by monocytes/macrophages in vitro correlated with the capacity in pathogen clearance and antibody production in vivo [16]. IPP treatment also stimulated NK cell activity in mice. NK cells play an important role in serving as the first line of defense against microbial infections as well as providing surveillance on neoplastic cells [17]. In this connection, the stimulation of NK cell activity by IPP treatment was associated with the suppression of tumor growth in Sarcoma 180 cell-inoculated mice. Conceivably, IPP treatment can stimulate the innate (i.e. non-specific) immune system, presumably via activation of macrophage and NK cells, and hence increase the resistance to microbial infection and growth of neoplastic cells in the body.

The adaptive (i.e., specific) immune system is responsible for the production of antigen-specific antibodies that protect against infectious agents by neutralizing viruses, destroying targets by antibody-dependent cellular cytotoxicity and complement-mediated cell lysis, etc. [18,19]. In this regard, IPP treatment was found to restore the hemolytic activity on SRBC of serum in SRBC-sensitized and CYC-immunosuppressed mice, indicative of a potentiation of adaptive humoral immunity [6]. The generation of an adaptive humoral immune response involves the functional interaction between T- and B-lymphocytes such that the antigen-activated T helper cells produce a variety of cytokines (such as interleukin-2, interleukin-6 and interferon-γ) that then cause B-lymphocytes to proliferate and differentiate into antibody-pro-
ducting plasma cells [20,21]. In this connection, IPP treatment produced an increase the splenocyte index in mice, and IPP co-incubation also potentiated Con A-stimulated splenocyte blastogenesis in vitro. Given that Con A-stimulated splenocyte blastogenesis is a measure of T-lymphocyte immunity [22], the ability of IPP to enhance Con A-stimulated splenocyte proliferation is consistent with an enhanced humoral immune response produced by IPP treatment. DTH reactions, such as chemical contact allergy as was the case for DNFB, are regarded as adaptive cell-mediated immune responses involving T helper-I type cells [23]. IPP treatment was found to restore the DNFB-induced increase in ear thickness in DNFB-sensitized and CYC-immunosuppressed mice, indicative of potentiation of adaptive cell-mediated immunity [24]. The mixture of herb-derived polysaccharides present in IPP produces immunostimulatory effects on both innate and adaptive immune systems in mice. Supplementation with polysaccharides derived from various edible fungi has previously been shown to increase the phagocytic activity of macrophages and NK cell activity in mice [2]. However, no detectable effects were produced by edible fungus polysaccharides on the hemolytic activity on SRBC and DNFB-induced DTH in mice.

In conclusion, long-term IPP treatment enhanced both innate and adaptive immunity in mice. The immunopharmacological profile of IPP provides a scientific rationale for the time-honored use of this compound polysaccharide-containing herbal health product (which is generally regarded as safe according to the high usage in Mainland China) for promoting health.

5. Acknowledgements

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6. References


