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Effects of *Phytolacca acinosa* polysaccharides I on immune function in mice

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ABSTRACT Radioactivities of [^{3}H]TdR uptaken by splenocytes and released from [^{3}H] TdR — labeled YAC-1 cell line were measured to determine the degree of lymphocyte proliferation and natural killer (NK) cell activity. Seven days after mice treated with *Phytolacca acinosa* polysaccharides I (PAP—I) 5—50 mg · kg^{-1}, the NK cell activity, and lymphocyte proliferation induced by Con A 5 μg · ml^{-1} or lipopolysaccharides 10 μg · ml^{-1} were significantly augmented. Splenocytes from mice treated with ip PAP-I 5—50 mg · kg^{-1} were incubated with Con A 5 μg · ml^{-1} for 24 h to induce interleukin-2 (IL-2) and for 40 h to induce NK cytotoxic factor (NKCF). Radioactivities of [^{3}H]TdR uptaken by CTLL-2 cell line and YAC-1 cell line were used to measure the IL-2 and NKCF activities, respectively. PAP-I enhanced the production of IL-2 and NKCF. These results suggest that PAP-I augments the immunological functions *in vivo*.

KEY WORDS *Phytolacca acinosa*; polysaccharides; concanavalin A; lipopolysaccharides; natural killer cells; interleukin-2; immunologic cytotoxicity

Phytolacca acinosa polysaccharides I (PAP-I), a kind of polysaccharides isolated from *Phytolacca acinosa* Roxb[1]. It enhanced the production of interleukin-2 (ZHENG Qin-Yue, 1989) and interleukin-3 (unpublished) *in vitro* and increased the cytotoxicity of macrophages and its production of tumor necrosis factor and interleukin-1[2]. We examined the effects of PAP-I on mitogen induced lymphocyte proliferation, NK cell activity (NKCA), production of IL-2, and natural killer cytotoxic factor (NKCF) to clarify its immunological enhancing effects *in vivo*.

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MATERIALS AND METHODS

BALB c mice, bred and maintained in our animal facilities, were used at age of 8-12 wk, weighing 19.8 ± *s* 1.8 g. IL-2-dependent murine cytotoxic T-cell line (CTLL-2) was obtained from Shanghai Medical University. Mycoplasma-free YAC-1 lymphoma cell line, gifted from Dr CHAO Xue-Tao (Department of Immunology, Second Military Medical University), was maintained in RPMI-1640 with 10% heat-inactivated fetal calf serum.

RPMI-1640 (Sigma), was supplemented with penicillin 100 IU · ml^{-1}, streptomycin 100 IU · ml^{-1}, 0.2% $NaHCO_3$, and 2-mercaptoethanol 0.1 mmol · L^{-1}. Concanavalin A (Con A) and lipopolysaccharides (LPS), from Sigma, were prepared at a concentration of 200 μg · ml^{-1} and stored at −20 C. Deoxyribonucleases (DNase) and fetal calf serum (FCS) were obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences, FCS was heat-inactivated at 56℃ for 30 min before use. DNase and trypsin (Difco) were dissolved in phosphate buffer solution (PBS) at concentrations of 200 μg · ml^{-1} and 2.4%, respectively. [^{3}H]TdR 666 GBq · mol^{-1} was obtained from Shanghai Institute of Nuclear Research, Chinese Academy of Sciences. Recombinant interleukin-2 5.8×10^5 U · mg^{-1} was kindly provided by Dr YANG Kang. PAP-I was freshly dissolved in PBS and heated at 100℃ for 30 min.

Splenocyte preparation Mice were killed by cervical dislocation 7 d after PAP-I or saline ip. The spleen was pressed through stainless steel sieves in cold RPMI-1640 under aseptic condition. Erythrocytes were removed by 0.83% NH_4Cl buffer solution, washed twice, and centrifuged (500× *g*) for 10 min. Viable cell suspension was obtained by trypan blue exclusion method and adjusted to a concentration of 5×10^6 cells/ml before use.

Lymphocyte proliferation Radioactivity of [^{3}H]TdR uptaken by splenocytes was measured to determine the degree of lymphocyte proliferation. Splenocytes 5×10^5 per well in RPMI-1640 containing 10% FCS were seeded into 96-well flat-bottom microtiter plates, and incubated in the presence or absence of Con A 5 $\mu g\cdot ml^{-1}$, LPS 10 $\mu g\cdot ml^{-1}$ for 72 h at 37℃, 5% CO_2, [^{3}H]TdR 1875 Bq per well was added 16 h before the termination of culturing. The cells were harvested on type-49 glass fiber filters. The radioactivity of the filters was counted in a FJ-2107 β scintillation counter.

NKCA assay Assay of NKCA was performed as reported earlier[3] with slight modifications. YAC-1 cells $5\times10^5\cdot ml^{-1}$ were labeled with [^{3}H]TdR 3.7 KBq $\cdot ml^{-1}$ for 2 h, washed thrice, adjusted to 10^5 cells $\cdot ml^{-1}$. YAC-1 cell suspension 100 μl was added to the 96-well flat-bottom microtiter plates and then cocultured in triplicate with effector cells at effector: target ratios (E : T) of 80 : 1, 40 : 1, and 20 : 1 for 20 h at 37℃, 5% CO_2. Trypsin 4.8 mg $\cdot ml^{-1}$ and DNase 50 $\mu g\cdot ml^{-1}$ were added 2 h before the end of culture. Cells were harvested on type-49 glass fiber filters through filtration. Radioactivity of filters were counted in a FJ-2107 β scintillation counter.

$$NKCA\% = (dpm_T - dpm_{(T+E)})/dpm_T$$

Induction of IL-2 and NKCF Splenocyte suspension ($5\times10^6\cdot ml^{-1}$) from mice treated ip with PAP-I or saline was stimulated with Con A 5 $\mu g\cdot ml^{-1}$ in 24-well flat-bottom plates for 24 and 40 h to induce IL-2 and NKCF[4], respectively. Supernatants were harvested by centrifugation ($1000\times g$) for 10 min and stored at -20℃ until use.

IL-2 assay IL-2 bioactivity was quantitated by using microassay for its ability to support the proliferation of IL-2 strictly dependent cell line CTLL-2[5]. Serial dilutions of supernatant (from 1 : 2 to 1 : 64) 100 μl was incubated with 100 μl CTLL-2 cell $1\times10^5\cdot ml^{-1}$ for 24 h at 37℃, 5% CO_2. [^{3}H]TdR 1875 Bq was added 4 h before the termination of culture. Cells were harvested and radioactivity was counted. The unit of IL-2 activity was defined as the method of AUC[6] referenced standard rIL-2 preparation.

NKCF activity assay NKCF activity was determined by radioactivity of [^{3}H]TdR uptaken by NK sensitive cell line YAC-1. YAC-1 cell suspension 100 μl, $1\times10^5\cdot ml^{-1}$ containing 10% FCS were seeded into 96-well flat-bottom microtiter plates and then incubated with NKCF supernatants with serial dilutions (1 : 2-1 : 32) for 24 h at 37℃, 5% CO_2. [^{3}H]TdR 1875 Bq per well was added 6 h before the termination of culture. Cells were harvested and radioactivity was measured.

Statistics Differences measured by *in vitro* assay were evaluated by *t* test. Each experiment was done at least 3 times. Data from the mean of three experiments or one representative experiment were shown.

RESULTS

Effect of PAP-I on lymphocyte proliferation induced by mitogen Splenocytes from BALB c mice treated with PAP-I ip 0-50 mg $\cdot kg^{-1}$ were incubated with Con A 5 $\mu g\cdot ml^{-1}$, LPS 10 $\mu g\cdot ml^{-1}$ or RPMI-1640. PAP-I could significantly augment the T, B mitogen induced lymphocyte proliferation. When the dosage of PAP-I was 20 mg $\cdot kg^{-1}$, the radioactivities of T, B mitogen-induced lymphocyte proliferation were 48 239±6 484 ($P<0.01$) and 7 442±1 105 dpm ($P<0.01$) (Tab 1).

Tab 1. Effect of *Phytolacca acinosa* Polysaccharides I (PAP-I) on lymphocytes proliferation induced by Con A 5 μg · ml⁻¹, lipopolysaccharides (LPS) 10 μg · ml⁻¹ or RPMI-1640. The data are from the mean of 4 separate wells. $\bar{x}\pm s$. *$P>0.05$, **$P<0.05$, ***$P<0.01$ *vs* 0 mg·kg⁻¹.

PAP-I/ mg·kg⁻¹	Radioactivity/dpm RPMI-1640	Con A	LPS
0	1439±312	19175±3188	1555±212
5	1538±248*	33313±8494**	1296±277*
10	5403±941***	28925±3301***	3871±659***
20	6770±2683***	48239±6484***	7442±1105***
50	10485±730***	32304±5161***	4589±731***

Effect of PAP-I on IL-2 production from splenocytes induced by Con A Splenocytes were incubated with Con A 5 $\mu g\cdot ml^{-1}$ for 24 h to induce IL-2 production. PAP-I (ip) en-

hanced IL-2 production from splenocytes in a dose-dependent manner. The IL-2 activities were 12.1±2.4, 27.0±6.4 ($P<0.05$), 60.9±2.2 ($P<0.01$), 55.7±8.7 ($P<0.01$) $U \cdot ml^{-1}$ in PAP-I 0, 10, 20, 50 $mg \cdot kg^{-1}$ groups, respectively.

Effect of PAP-I on NKCA Splenocytes from mice treated with a single ip of PAP-I 0-20 $mg \cdot kg^{-1}$ were prepared on d 7 and incubated with NK sensitive cell line YAC-1 with E:T ratios of 80:1, 40:1, and 20:1. PAP-I augmented the NKCA as the ratio elevated, when the dosage of PAP-I was 20 $mg \cdot kg^{-1}$ and the E:T ratio was 80:1, the NKCA reached 49.20 ±0.42% (Tab 2).

Tab 2. Effect of PAP-I on NK cell activity. $n=3$, $\bar{x}\pm s$, *$P>0.05$, **$P<0.05$, ***$P<0.01$ *vs* 0 $mg \cdot kg^{-1}$. **Effector : Target ratios were indicated below.**

PAP-I, $mg \cdot kg^{-1}$	Natural killer activity, % 20:1	40:1	80:1
0	14.75±1.06	18.15±1.48	32.00±4.67
5	23.80±5.37**	28.15±8.69*	36.70±0.14*
10	27.55±3.89***	29.15±4.59**	46.15±0.92***
20	32.00±0.57***	33.55±0.35***	49.20±0.42***

Effect of PAP-I on NKCF production from splenocytes induced by Con A Splenocytes from mice treated with PAP-I 0, 5, and 10 $mg \cdot kg^{-1}$ (ip, once) were prepared on d 7 and incubated with Con A 5 $\mu g \cdot ml^{-1}$ for 40 h to induce NKCF production. PAP-I augmented the NKCF production from splenocytes induced by Con A (Fig 1).

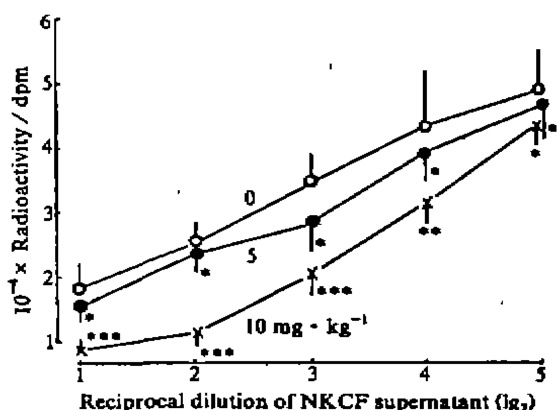

Fig 1. Effect of PAP－I on production of natural killer cytotoxic factor (NKCF) from murine splenocyte induced by Con A 5 $\mu g \cdot ml^{-1}$. n=mean of 4 separate wells, $\bar{x}\pm s$, *$P>0.05$, **$P<0.05$, ***$P<0.01$ *vs* 0 $mg \cdot kg^{-1}$.

DISCUSSION

Our present results revealed that mice treated with ip PAP-I once could significantly augment lymphocyte proliferation capability, IL-2 production. It also showed significant enhancement of IL-2 production and lymphocyte proliferation with concentration dependent fashion from splenocytes and Con A-activated splenocytes (unpublished data). These studies confirmed that PAP-I could enhance lymphocyte proliferation, IL-2 production *in vivo* and *in vitro*. We did not know why the effective dosage of PAP-I which activated macrophages to produce TNF and IL-1 in previous study of Ref 2 did not agree with that in our present study. The difference may be explained as that: the effective dosage of PAP-I on lymphocyte was different from that on macrophage. In addition, from the data of Ref 2, dosage dependent pattern was not shown, the dosage in previous study may not be proper.

In the light of the observation that IL-2 could promote natural killer cell activity[7], we investigated whether PAP-I (ip) also augment NK cell activity, the results showed that the effective dosage of PAP-I (ip) in inducing IL-2 could also promote NK cell activity in dose dependent fashion. NK cells played a role in the resistance against tumor, in the host immunity to viral and microbial infection and in the regulation of lymphoid and other hemopoietic cell production[8]. NK cell activity was

considered mainly to be regulated by interferon (IFN) and IFN inducers[9]. Aside from IFN, the NK system activators included the lymphokins, IL-2, and IL-3[10,11]. It remains unclear whether PAP-I could enhance IFN production *in vivo* and *in vitro* in this experiment. the present investigation of PAP-I augmenting IL-2 production and previous study of its inducing IL-3 production[12] may be one of the mechanisms of NK cell activity regulation.

NKCF was considered to be NK lytic mediators and could lead to the development of a model for probing the mechanism of NK cell mediated cytotoxcity[13]. Our experiments confirmed that PAP-I ip could enhance the production of NKCF, and in these dosages, PAP-I ip could also augment NK cell activity to lysis YAC-1 cell. From the results we could see the parallel between NK cell activity and NKCF production. Our data provided a further evidence that PAP-I was an immunopotentiator and might have important implications in the host response to infection and neoplastic diseases.

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商陆多糖 I 对小鼠免疫功能的影响

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摘要　小鼠一次 ip 商陆多糖 I (PAP-I) 5-50 mg·kg⁻¹，7 d 后取脾脏，用[³H]TdR 参入法及[³H]TdR 标记的 YAC-1细胞[³H]TdR 释放法检测淋巴细胞转化及 NK 细胞活性，PAP-I 能显著促进 Con A，LPS 诱导的淋巴细胞转化，增强 NK 细胞活性．用 Con A 5 μg·ml⁻¹和上述脾细胞培养24及40 h，分别诱导 IL-2及 NKCF，PAP-I 显著促进 IL-2及 NKCF 产生，结果提示 PAP-I 可增强小鼠免疫功能．

关键词　商陆；多糖类；刀豆球蛋白 A；脂多糖类；自然杀伤细胞；白细胞介素-2；免疫细胞毒性