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Effects of *Achyranthes bidentata* polysaccharides on interleukin-1 and tumor necrosis factor-alpha production from mouse peritoneal macrophages

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ABSTRACT *Achyranthes bidentata* polysaccharides (ABP), extracted from the root of *Achyranthes bidentata*, induced interleukin-1 (IL-1) synthesis as well as tumor necrosis factor-α (TNF-α) synthesis and secretion from thioglycolate-primed mouse peritonesl macrophages *in vitro*. ABP 100—800 $\mu g \cdot ml^{-1}$ enhanced both synthesis and release of IL-1 when stimulated by lipopolysaccharides (LPS) (5 $\mu g \cdot ml^{-1}$), but had no significant influences on synthesis and release of TNF-α induced by LPS (10 $\mu g \cdot ml^{-1}$). Studies on IL-1 and TNF-α production induced by ABP (200 $\mu g \cdot ml^{-1}$) alone or plus LPS showed that peak levels of IL-1 release reached at 24 h and that of TNF-α release at about 2 — 6 h after incubation. Peritoneal macrophages from mice ip ABP 25 and 50 $mg \cdot kg^{-1} \cdot d^{-1} \times 5$ d prnduced much more IL-1 than those from control group. Perinoneal macrophages from ip ABP 100 $mg \cdot kg^{-1} \cdot d^{-1} \times 6$ d alone released more TNF-α *vs* control group, and showed a synergetic action with LPS (10 $\mu g \cdot ml^{-1}$), which was as strong as the positive control agent BCG. These results provide an explanation for the immunopotentiating effect of ABP.

KEY WORDS *Achyranthes bidentata*; polysaccharides; macrophages; interleukin-1; tumor necrosis factor; lipopolysaccharides

A number of macrophage functions such as *phagocytosis*, *lysosomal enzyme activity*, cytocidal activity, and production of cytokines were modulated by some polysaccharides from different sources[1-3]. *Achyranthes bidentata* is a Chinese traditional tonic herb. The bioactive polysaccharides isolated from its root is called *Achyranthes bidentata* polysaccharides (ABP). ABP possessed not only immunopotentiating effects but also antitumor activity in mice[4]. In this paper, the effects of ABP on IL-1 and TNF-α production from murine peritoneal macrophages were studied *in vitro* and *in vivo* to explain its mechanism at cytokine levels.

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MATERIALS AND METHODS

ABP A water-soluble white powder ($m = 1.34$ kDa, glucose : mannose = 2 : 1 in molar ratio)[5], was prepared by Prof TIAN Geng-Yuan (Shanghai Institute of Organic Chemistry).

Mice Inbred ICR mice (3 months old, $21.5 \pm s$ 1.3 g, for IL-1 and TNF-α production) and BALB c mice (6—8 wk old, for IL-1 activity assay) were purchased from the Shanghai Animal Center, Chinese Academy of Sciences.

Cell line L929 cell line (tumorigenic murine fibroblast) was kindly supplied by Prof QIAN Ding-Hua (College of Pharmacy, Shanghai Second Military Medical University).

Reagents Concanavalin A (Con A) and lipopolysaccharides (LPS) from *E coli* were purchased from Sigma Co. Dactinomycin was purchased from Fluka Co. Crystal violet was obtained from Shanghai Chongmin Chemical Reagent Factory, and [^{3}H]TdR was obtained from Shanghai Institute of Nuclear Sciences. Medium RPMI 1640 was purchased from Gibco Co. All RPMI 1640 containing media were supplemented with HEPES buffer 10 $mmol \cdot L^{-1}$, penicillin 100 $IU \cdot ml^{-1}$, streptomycin 100 $\mu g \cdot ml^{-1}$, *L*-glutamine 2 $mmol \cdot L^{-1}$, 2-mercaptoethanol 50 $\mu mol \cdot L^{-1}$, and 10% newborn bovine serum, pH 7.2.

Peritoneal macrophages (PMΦ) Mouse peritoneal lavage with cold RPMI 1640 medium was collected from the ICR mice injected ip 10% (wt/vol) thioglycolate broth (1 ml/mouse) 4 d before harvest (*in vivo* test, mice were not given ip thioglycolate broth), and resuspended in RPMI 1640 medium at a

concentration of 2×10^6 cells·ml^{-1}. The cell suspension (1 ml) was seeded to 24-well culture plate. After incubation for 2 h at 37℃ in a 5% CO_2 atmosphere, nonadherent cells were removed by washing twice with RPMI 1640 medium. The cells were used as PMΦ.

Production of IL-1 by PMΦ

1 *In vitro* study To the PMΦ monolayer cells in 24-well tissue culture plate, RPMI-1640 medium, various concentrations of ABP or/and LPS (5 μg·ml^{-1}) were added to each well (final volume per well was 1 ml). Then the plate was incubated at 37℃ in air with 5% CO_2 for 24 h. After incubation, all the supernatants containing extracellular IL-1 were collected and clarified by centrifugation ($600\times g$, 10 min). The remaining cells were washed and disrupted by 3 freeze-thawing cycles in 1 ml RPMI 1640 media and the soluble fraction containing intracellular IL-1 was obtained by centrifugation ($600\times g$). The two kinds of sample supernatants were stored at −25℃ until assay for IL-1 activity.

2 *In vivo* study Mice were treated with ip ABP 25 and 50 mg·kg^{-1}·d$^{-1}\times5$ d. PMΦ were prepared on d 6 and cultured with LPS (5 μg·ml^{-1}) for extracellular IL-1 production.

IL-1 assay IL-1 activity was measured by thymocyte proliferation assay[4]. Suspension of thymocytes (2×10^5 cells per well) taken from BALB/cA mice was distributed over a flat-bottomed 96-well microtiter plate. To the cell suspension, 50 μl of PMΦ culture supernatant were added. The cultures were then incubated in the presence of a suboptimal concentration of Con A (5 μg·ml^{-1}) for 72 h at 37℃ in a 5% CO_2 incubator. These cultures were added with [^{3}H]TdR (9.25 kBq/well) for the final 6 h of incubation period and were harvested onto glass fiber filters. IL-1 activity was expressed as dpm/2×10^5 thymocytes.

Induction of TNF-α by PMΦ

1 *In vitro* study To the PMΦ monolayer cells in 24-well culture plate, RPMI 1640 medium, various concentions of ABP alone or plus LPS (10 μg·ml^{-1}) were added to each well yielding a final volume of 1 ml. The plate was reincubated for 4 h at 37℃ in a humidified 5% CO_2 atmosphere. At the end of incubation, all the supernatants containing extracellular TNF-α were harvested by centrifugation ($600\times g$, 10 min). The remaining cells were covered with 1 ml fresh RPMI 1640 medium, frozen and thawed 3 times. These supernatants containing intracellular TNF-α were collected by centrifugation ($600\times g$, 10 min). All samples were stored at −25℃ until assay.

2 *In vivo* study Mice were treated with ip ABP 25, 50, and 100 mg·kg^{-1}·d$^{-1}\times6$ d. In positive control group, BCG was ip once 200 mg·kg^{-1}. On d 7, PMΦ were prepared and cultured alone or with LPS (10 μg·ml^{-1}) for extracellular TNF-α production.

TNF-α activity assay The TNF-α activity in different samples were determined by using the cytotoxicity assay against L929 cells as described previously[11]. L929 cells (5×10^4 cells/100 μl) were seeded into the flat-bottom 96-well microtiter plates and incubated 24 h at 37℃ in 5% CO_2 atmosphere. Culture medium was removed. TNF-α supernatant (100 μl) and RPMI 1640 medium containing dactinomycin D (final concentration 1 μg·ml^{-1}) were added to each well. Plates were reincubated for 20 h. The cells stained with 0.5% crystal violet (containing 3.2% formaldelyde isotonic solution) for 15 min. Plates were rinsed and dried. The residue was dissolved in 47.5% EtOH (100 μl/well) which was measured at 490 nm on an ELISA autoreader. TNF-α activity was calculated: % cytotoxicity = (A control − A test)/A control.

RESULTS

IL-1 production from PMΦ treated with ABP PMΦ were cultured with ABP 200 μg·ml^{-1} (in preliminary experiment, this concentration enhanced IL-1 production) alone or plus LPS 5 μg·ml^{-1} for 2—72 h. The supernatants were collected and assayed. The levels of IL-1 induced by ABP alone or ABP plus LPS reached peaks at 24 h and then declined gradually (Fig 1). Hence, in subsequent experiments, IL-1 activity was usually detected at 24 h after ABP treatment.

Effects of ABP on extracellular and intracellular IL-1 production from PMΦ PMΦ were treated with ABP 50—800 μg·ml^{-1} for 24 h. Extracellular IL-1 production was not affected, but ABP 200—800 μg·ml^{-1} augmented intracellular IL-1 synthesis. ABP (100—800 μg·ml^{-1}) promoted both extracellular and intracellular IL-1 from PMΦ when induced

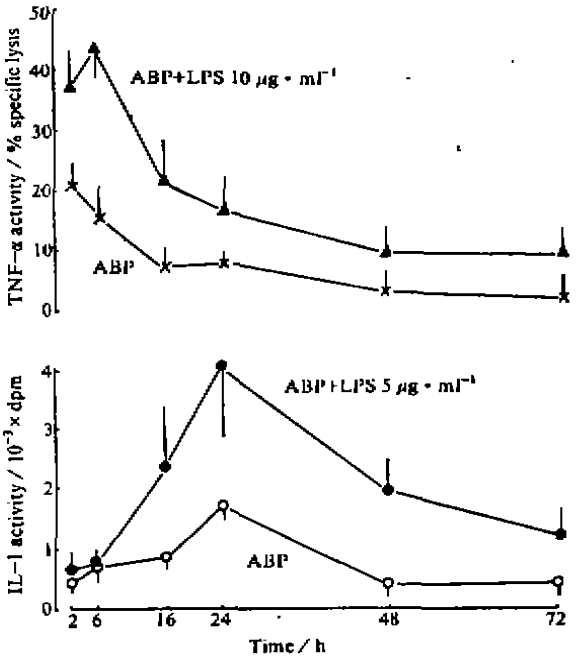

Fig 1. Extracellular IL-1 and TNF-α production from mouse peritoneal macrophages induced by ABP (200 μg·ml⁻¹)±LPS *in vitro*. $n=3$, $\bar{x}\pm s$.

by LPS (5 μg·ml^{-1}) and that the stimulating effect of ABP on IL-1 synthesis was more potent than that on IL-1 secretion (Tab 1).

Effect of ip ABP on extracellular IL-1 production from PMΦ induced by LPS Mice were treated with ip ABP 25 and 50 mg·kg^{-1}·d^{-1}×5 d, and PMΦ were collected and assayed for extracellular IL-1 activities. ABP enhanced the extracellular IL-1 production induced by LPS (5 μg·ml^{-1}) (Tab 2).

TNF-α production from PMΦ stimulated with ABP PMΦ were cultured with ABP (200 μg·ml^{-1}) alone or plus LPS (10 μg·ml^{-1}, suboptimal concentration) at 2—72 intervals. The levels of TNF-α stimulated with ABP alone reached the peak at 2 h and then dropped. The levels of TNF-α induced by ABP and LPS reached the maximum at 6 h after incubation (Fig 1). For convenience, a 4-h time point was chosen for evaluating the amounts of TNF-α produced by PMΦ after various treatment schedules.

Tab 1. Extracellular and intracellular IL-1 activities *after* ABP±LPS *mice peritoneal macrophages in vitro*. $n=3$, $\bar{x}\pm s$. $^{*}P>0.05$, $^{**}P<0.05$, $^{***}P<0.01$ *vs* control.

ABP μg·ml^{-1}	LPS μg·ml^{-1}	$10^3\times$dpm/2×10^4 thymocytes Extracellular	Intracellular
0	—	137±15	117±11
50	—	129±20*	126±24*
100	—	135±27*	134±29*
200	—	141±24*	238±37***
400	—	132±31*	228±26***
800	—	138±23*	223±22***
0	5	51±8	170±22
50	5	70±11*	208±18*
100	5	94±17**	426±38***
200	5	105±12***	404±77***
400	5	129±21***	483±37***
800	5	148±12***	408±29***

Tab 2. Effect of ip ABP on extracellular IL-1 production from murine peritoneal macrophages induced by LPS (5 μg·ml^{-1}). $n=3$, $\bar{x}\pm s$. $^{***}P<0.01$ *vs* saline.

Dose mg·kg^{-1}·d^{-1}×d	IL-1 activity dpm/2×10^5 thymocytes
Saline ×5	1 339±240
ABP 25×5	5 142±742***
ABP 50×5	2 824±306***

Effects of ABP on extracellular and intracellular TNF-α production *in vitro* PMΦ were treated with ABP (50—800 μg·ml^{-1}) for 4 h. TNF-α activities in extracellular and intracellular supernatants were assessed. ABP (50 — 800 μg·ml^{-1}) increased extracellular TNF-α production in a concentration-dependent manner, and that the intracellular TNF-α evels were elevated by ABP (400, 800 μg·ml^{-1}) (Tab 3). But ABP had no significant influences on extracellular and intracellular amounts of TNF-α induced by LPS

(10 μg·ml^{-1}).

Tab 3. Effects of ABP±LPS on extracellular and intracellular TNF-α production from mouse peritoneal macrophages *in vitro*. $n=3$, $\bar{x}\pm s$. $^{*}P>0.05$, $^{**}P<0.05$, $^{***}P<0.01$ *vs* control.

ABP μg·ml^{-1}	LPS μg·ml^{-1}	TNF-α activity/% Extracellular	Intracellular
0	—	17.6±2.9	4.4±2.3
50	—	23.5±1.5**	8.9±2.1*
100	—	25.5±1.5**	10.1±2.7*
200	—	31.4±3.0***	8.9±2.7*
400	—	31.6±2.1***	11.5±2.5**
800	—	33.3±2.6***	15.6±1.2***
0	10	37.3±2.3	20.0±2.2
50	10	39.2±3.2*	20.1±2.1*
100	10	39.4±1.0*	22.2±3.3*
200	10	37.4±2.5*	15.6±3.8*
400	10	36.7±3.1*	20.0±1.1*
800	10	39.8±3.2*	17.8±2.4*

Effect of ip ABP on extracellular TNF-α production from PMΦ After mice were treated with daily ip ABP, PMΦ were prepared and cultured in the presence or absence of LPS (10 μg·ml^{-1}) for 4 h. Then the supernatants were collected and TNF-α activity was measured. ABP 100 mg·kg^{-1} enhanced extracellular TNF-α production from PMΦ without LPS stimulation, and ABP 100 mg·kg^{-1} also elevated the extracellular TNF-α production induced by LPS markedly (Tab 4). BCG exerted a significant increase effect on extracellular TNF-α production induced by LPS.

DISCUSSION

Through comparison of the time course between IL-1 and TNF-α production *in vitro*, it was found that the peak level of IL-1 was reached at 24 h but that of TNF-α at 2—6 h after incubation. The discrepancy may be related to different mechanisms of IL-1 and TNF-α production in peritoneal macrophages.

Tab 4. Effect of ip ABP±LPS (10 μg·ml^{-1}) on production of extracellular TNF-α from mouse peritoneal macrophages. $n=3$, $\bar{x}\pm s$. $^{*}P>0.05$, $^{***}P<0.01$ *vs* saline.

	Dose mg·ml^{-1}·d^{-1}×d	TNF-α activity/% without LPS	with LPS
—	Saline×6	14.9±1.5	25.3±3.1
ABP	25×6	16.4±1.7*	19.4±2.6*
ABP	50×6	16.5±0.9*	26.9±3.0*
ABP	100×6	25.7±3.6***	35.4±1.5***
BCG	200×1	16.9±2.7*	39.5±3.2***

Primed macrophages were reported to have expressed high levels of TNF-α mRNA, and induction with triggering agents (such as LPS) resulted in immediate synthesis and release of TNF-α[8]. The peak time of TNF-α production different from that of IL-1 may be closely involved in the preexistent TNF-α mRNA in cytoplasm.

The full activation of macrophages needs 2 stages — priming and triggering. Macrophages at different stages of activation exhibit different physiologic capacities, reflecting their potential to execute distinct complex functions. Only fully activated macrophages possess the ability to produce cytokines such as IL-1 and TNF-α[9]. *In vitro* experiments, ABP could induce synthesis of IL-1 as well as secretion and synthesis of TNF-α from thioglycolate-primed macrophages. These results suggested that ABP may have properties of triggering agents. In addition, ABP *in vitro* has an evident synergistic effect with LPS in IL-1 synthesis and secretion from thioglycolate-primed macrophages too, but has no effect on extracellular and intracellular TNF-α production induced by LPS. The differences of ABP on IL-1 and TNF-α production stimulated by distinct suboptimal LPS may result from different subpopulations of macrophages which produce distinct cytokines. It would be of interest to investigate the

interaction of ABP with LPS on both IL-1 and TNF-α production further.

In *in vivo* experiments, the levels of release of IL-1 and TNF-α induced by the triggering agent LPS from peritoneal macrophages obtained from mice treated with ip ABP were substantially elevated. The effect of ABP on TNF-α release was as potent as that of the well-known priming agent BCG. These results indicate that ABP also exerts a priming effect on macrophages. Taking all these findings together, it was suggested that ABP may not only be a priming agent but also be a triggering agent on macrophages. This nature of ABP closely resembled IFN-γ[9].

IL-1 and TNF-α are important cytokines with diverse biological functions, and play key roles in immune responses[10,11]. The enhancement of ABP on both IL-1 and TNF-α production not only provided an explanation for the mechanism of its immunomodulating action, but also suggested the possible application of this novel immunopotentiating agent to a wider range of disorders such as in neoplasms and chronic viral infections.

Though both dosages enhanced IL-1 production very significantly, it seemed that 25 $mg \cdot kg^{-1}$ was more potent than 50 $mg \cdot kg^{-1}$. From our experience, the dose-response relationship of polysaccharides are not so evident as other compounds, it always shows an optimal dosage which gives the highest effects.

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332-336 (13)

牛膝多糖对小鼠腹腔巨噬细胞产生白细胞介素-1和肿瘤坏死因子的影响

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摘要 牛膝多糖(ABP)诱导小鼠腹腔巨噬细胞合成IL-1及合成与分泌TNF-α；对LPS 5 $\mu g \cdot ml^{-1}$诱生的IL-1合成与释放有促进作用；但对LPS 10 $\mu g \cdot ml^{-1}$诱生的TNF-α无影响. ABP 200 $\mu g \cdot ml^{-1}$刺激产生的IL-1达峰时间在作用后24 h；TNF-α则为其后2—6 h. ABP 25，50 $mg \cdot kg^{-1}$ ip提高LPS诱导的IL-1产生，100 $mg \cdot kg^{-1}$ ip促进TNF-α生成，作用强度与卡介苗相当.

关键词 牛膝；多糖；巨噬细胞；白细胞介素-1；肿瘤坏死因子；脂多糖