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Antitumor activity and immuno-potentiating actions of *Achyranthes bidentata* polysaccharides

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ABSTRACT *Achyranthes bidentata* polysaccharides (ABP), isolated from the root of *Achyranthes bidentata* Blume, 50 $mg\cdot kg^{-1}$ ip or 250 $mg\cdot kg^{-1}$ ig to ICR mice inhibited the growth of sarcoma 180. ABP 50 and 100 $mg\cdot kg^{-1}$ ip prolongated the survival days of mice bearing Ehrlich carcinoma. ABP 50－800 $\mu g\cdot ml^{-1}$ did not exert direct cytotoxic effect *in vitro* on S180 cells, but enhanced the cytotoxicity of peritoneal macrophages against S180 cells. ABP 50 $mg\cdot kg^{-1}$ ip $\times$17 d or 250 $mg\cdot kg^{-1}$ ig $\times$ 16 d promoted the plaque forming cells (PFC) response to sheep red blood cells (SRBC) and serum IgG level as well as splenocyte proliferation induced by mitogen Con A or LPS in tumor-induced immunodeficient mice. ABP also elevated the NK cell activity and serum TNF content in mice bearing S180. These results indicated that the antitumor effect of ABP may be related to its potentiating effect on both specific and nonspecific host immunological responses.

KEY WORDS *Achyranthes bidentata*; polysaccharides; antibody-producing cells; IgG; lymphocyte transformation; natural killer cells; tumor necrosis factor

Immmunotherapy employing various biological response modifiers (BRM) has become a new area of tumor therapy. This mobilizes the natural defense factors against tumors. Compared with the conventional chemotherapy, it yields less side effects. Some plant polysaccharides show antitumor activities[1]. The antitumor effects of most polysaccharides are due to their immunomodulating action, not direct cytotoxic action[1－3].

Achyranthes bidentata Blume is a traditional Chinese herb with tonic actions. *Achyranthes bidentata* polysaccharides (ABP) are isolated from its root. Compared with other polysaccharides (eg, lentinan, krestin, and schizophyllan), the molecular mass of ABP is much smaller and its chemical structure is now identified[4]. In the present study, the antitumor activity and enhancement of immunologic functions of ABP including natural killer (NK) cells activity and tumor necrosis factor (TNF) content in mice bearing sarcoma 180 were investigated.

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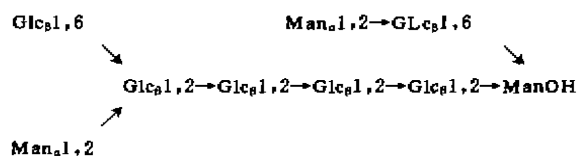

Achyranthes bidentata polysaccharides (ABP)

MATERIALS

ABP A water-soluble white powder (m = 1.34 kDa, glucose : mannose = 2 : 1 in molar ratio)[4], was provided by Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences.

Inbred ICR mice, 3 months old, weighing 20.8 $\pm$ s 1.1 g, were purchased from Shanghai Animal Center, Chinese Academy of Sciences.

YAC-1 cell line (a Moloney leukemia virus-induced mouse T-lymphoma cell) was kindly supplied by Dr WANG Qiu－Da (Shanghai Institute of Cell Biology, Chinese Academy of Sciences). L929 cell line (tumorigenic murine fibroblast) was kindly provided by Prof QIAN Ding－Hua (College of Pharmacy, Second Military Medical University). The two cell lines were subcultured with 10% new bovine serum RPMI 1640 medium.

Concanavalin A (Con A), lipopolysaccharides (LPS) and deoxyribonucleases (DNase) were purchased from Sigma Co. Trypsin was purchased from

Difco Co. Dactinomycin was purchased from Fluka Co. *Bacillus Calmette-Guerin* (BCG) was obtained from Shanghai Institute of Biological Products, Chinese Ministry of Public Health. Crystal violet (AR) was purchased from Shanghai Chongming Chemical Reagent Factory.

Medium RPMI 1640 was purchased from Gibco Lab. All RPMI 1640 media were supplemented with HEPES buffer 10 mmol L^{-1}, penicillin 100 IU ml^{-1}, streptomycin 100 $\mu g \cdot ml^{-1}$, *L*-glutamine 2 mmol·L^{-1}, 2-mercaptoethanol 50 $\mu mol \cdot L^{-1}$, and 10% newborn bovine serum, pH 7.2.

METHODS AND RESULTS

Effect of ABP on S180 cell growth *in vitro* S180 cells were harvested aseptically from ICR mice ascites 7 d after inoculation. After washing twice with RPMI 1640 medium, S180 cells were resuspended to a final density of 2×10^6 cells·ml^{-1}. S180 cell suspension (200 μl) and 10 μl of various concentrations of ABP were dispensed into each well of a 96-well flat bottomed microtiter plate, which was then incubated at 37℃ in a humidified atmosphere of 5% CO_2 for 24 h. The S180 cell growth was expressed by the incorporated radioactivity of [^{3}H]TdR (9.25 kBq / well) which was added to each well 6 h before harvesting. ABP 50—800 $\mu g \cdot ml^{-1}$ did not show any influence on the growth of S180 cells (Tab 1). This finding indicated that ABP had no direct cytotoxic action on S180 cells.

Effect of ABP on the cytotoxicity of peritoneal macrophages (MΦ) against S180 cells MΦ were collected from the peritoneal cavity of ICR mice 4 d after ip 10% (wt/vol) thioglycollate broth (1 ml/mouse). The cells were washed twice and resuspended in RPMI 1640 medium. S180 cells were prepared as mentioned above. The cell suspension 100 μl and 10 μl of ABP (each concentration in triplicate) were added to each well of 96- well microtiter plates at ratio of effector:target (E:T) cell 10:1 or 25:1. After an 18—h incubation

Tab 1. Effects of *Achyranthes bidentata* polysaccharides (ABP) on Sarcoma 180 cell growth and cytotoxicity of murine peritoneal macrophages (MΦ) against S180 cells *in vitro*. $n=3$ experiments. $\bar{x} \pm s$. $^aP>0.05$. $^bP<0.05$. $^cP<0.01$ *vs* control.

ABP $\mu g \cdot ml^{-1}$	S180 proliferation $10^{-2} \times$dpm	Cytotoxicity of MΦ (%) MΦ:S180 (10:1)	MΦ:S180 (25:1)
0	1307±48	12.8±8.1	15.8±6.3
50	1307±273^a	5.7±2.8^a	10.7±5.6^a
100	1344±158^a	15.3±3.9^a	18.7±1.1^a
200	1325±346^a	22.1±6.2^a	28.0±8.6^a
400	1318±324^a	29.1±8.4^a	38.5±9.8^b
800	1360±308^a	55.6±7.8^c	73.9±2.6^c

(37℃ in a humidified atmosphere with 5% CO2) each well was added with 9.25 kBq of [^{3}H]TdR. Cells were harvested 6 h later onto glass fiber filter and [^{3}H]TdR incorporation was determined by liquid scintillation. The % cytotoxicity of MΦ=(Control—Treatment) / Control × 100 % (dpm). ABP 800 $\mu g \cdot ml^{-1}$ (E:T=10:1) and 400—800 $\mu g \cdot ml^{-1}$ (E:T=25:1) promoted concentration-dependently the cytotoxicity of MΦ against S180 cells (Tab 1).

Antitumor activity of ABP and its effect on serum antibody IgG, plaque forming cells (PFC) and lymphocyte proliferation in mice bearing S180 ICR mice were randomly divided into 8 groups. S180 ascites (0.2 ml, about 1×10^6 cells) were injected sc into the right axilla of tumor-bearing groups on d 0. ABP was injected ip (50, 100 $mg \cdot kg^{-1}$) for 17 d or ig (250, 500 $mg \cdot kg^{-1}$) for 16 d. Mice were sensitized by ip SRBC 0.2 ml (3×10^8 cells). After 5 d the mice were bled to obtain serum for IgG determination. The spleens were excised for PFC counting and lymphocyte proliferation assay. The tumors were excised and weighed. ABP ip (50 $mg \cdot kg^{-1}$) or ig (250 $mg \cdot kg^{-1}$) exerted an inhibitory effect on tumor growth, the inhibition ratios were 43.6

and 50.5%, respectively (Tab 2).

Tab 2. Inhibitory effect of ABP on S180 in mice *in vivo*. $\bar{x} \pm s$. [a]$P > 0.05$. [b]$P < 0.05$ *vs* normal saline (NS).

	Dose/ $mg \cdot kg^{-1} \cdot d^{-1}$ ×d	Route	Mice begin/ end	Tumor weight/ mg	Inhibition/ %
	NS×17	ip	8/5	1775±730	0
ABP	50×17	ip	8/7	1001±220[b]	43.6
ABP	100×17	ip	8/6	1634±413[a]	7.9
	NS×16	ig	10/9	2263±1163	0
ABP	250×16	ig	10/8	1121±939[b]	50.5
ABP	500×16	ig	10/8	2010±1563[a]	11.2

ABP was injected ip (50, 100 $mg \cdot kg^{-1}$) into mice for 4 d prior to Ehrlich carcinoma (EC) ip inoculation (1×10^6 EC cells/0.2 ml per mouse). After inoculation, ABP was injected ip for 7 d. Two doses of ABP prolongated the survival days of EC-bearing mice 85% and 65%, respectively (Tab 3).

Tab 3. Effect of ip ABP on prolongating survival time of mice bearing Ehrlich carcinoma (EC). $\bar{x} \pm s$. [c]$P < 0.01$ *vs* normal saline (NS).

	Dose/ $mg \cdot kg^{-1} \cdot d^{-1}$ ×d	Mice begin /end	Survival time /d	Life prolongation /%
	NS×11	15/0	19.3±0.8	
ABP	50×11	8/2	35.8±17.9[c]	85
ABP	100×11	8/1	31.8±13.8[c]	65

IgG contents were determined by single immunodiffusion method. PFC was measured by improved Cunningham method[6]. Lymphocyte proliferation assay was based on the method previously described[8] with some modifications: Briefly, spleen cell suspension was prepared at concentration of 2×10^6 viable cells $\cdot ml^{-1}$. To the 96-well micro-culture plate, 200 μl of the cell suspension, mitogens at sub-optimal concentrations (Con A 5 $\mu g \cdot ml^{-1}$, LPS 10 $\mu g \cdot ml^{-1}$) were added quadruply. After incubation for 66 h at 37 ℃ in a humidified 5% CO_2 incubator, 9.25 kBq of [^{3}H]TdR was added to each well. The plate was incubated for another 6 h and the cells were collected on glass fiber filters by a multiple sample collector. The incorporated radioactivity was determined by liquid scintillation counter. Serum IgG levels, PFC counts, and lymphocyte proliferation induced by Con A or LPS in S180-bearing mice were lower than those of normal mice (Tab 4). ABP 50 $mg \cdot kg^{-1}$ ip or 250 $mg \cdot kg^{-1}$ ig enhanced these depressed immune parameters toward normal range values. ABP exhibited an optimal dosage (ip 50 mg $\cdot kg^{-1}$, ig 250 $mg \cdot kg^{-1}$) on antitumor and immunopotentiating activities (Tab 2—4).

Enhancement of ABP on natural killer cell activity of S180-bearing mice S180 cells were inoculated on d 0. Beginning on d 1, ABP was injected ip into ICR mice at 50 and 100 $mg \cdot kg^{-1}$ for 7 d. On d 8, the spleen was excised. Splenocyte suspensions were prepared at 2×10^6 viable cells $\cdot ml^{-1}$ in RPMI 1640 media, then plated in a flask and allowed to adhere for 2 h at 37 ℃ in 5% CO_2 atmosphere. After incubation, the nonadherent cells were resuspended at 1×10^7 cells $\cdot ml^{-1}$ in RPMI 1640 media as effectors. YAC—1 target cells (1×10^6 cells $\cdot ml^{-1}$) were labelled with [^{3}H]TdR 370 kBq $\cdot ml^{-1}$ for 2 h at 37 ℃ and washed thrice with RPMI 1640 medium. Finally, the cell concentration was adjusted to 1×10^5 live cells $\cdot ml^{-1}$. Target cells (0.1 ml) were seeded to 96-well flat-bottom microtiter plates. RPMI 1640 (0.1 ml) media in blank group, 2.5% Triton X 100 (0.1 ml) in maximal release group, and 0.1 ml effector cells (E:T = 100:1) in ABP group were added to each well in triplicate. After incubation for 7.5 h at 37 ℃ in humidified 5% CO_2 atmosphere, the culture cells were treated with 0.15% trypsin

Tab 4. Effects of ABP on the immune function of mice bearing S180 *in vivo*. $\bar{x} \pm s$. $^{b}P<0.05$, $^{c}P<0.01$ *vs* normal saline (NS). $^{d}P>0.05$, $^{e}P<0.05$ $^{f}P<0.01$ *vs* S180 group.

	Dose/ $mg \cdot kg^{-1} \cdot d^{-1}$ $\times d$	Route	IgG/ $g \cdot L^{-1}$	PFC/ 10^6 cells	[^{3}H]TdR uptake $10^{-2} \times dpm$	
					Con A 5 $\mu g \cdot ml^{-1}$	LPS 10 $\mu g \cdot ml^{-1}$
Normal	NS×17	ip	28 ± 9	453 ± 38	27.3 ± 5.1	
S180	NS×17	ip	19 ± 5^{b}	290 ± 67^{b}	17.6 ± 2.2^{b}	
S180+ABP	50×17	ip	40 ± 10^{f}	478 ± 75^{e}	25.4 ± 2.8^{e}	
S180+ABP	100×17	ip	25 ± 9^{e}	325 ± 54^{d}	20.3 ± 3.7^{d}	
Normal	NS×16	ig	32 ± 5	2017 ± 354	829 ± 101	152 ± 33
S180	NS×16	ig	19 ± 5^{c}	410 ± 120^{f}	108 ± 17^{c}	82 ± 7^{c}
S180+ABP	250×16	ig	27 ± 4^{f}	610 ± 68^{e}	219 ± 35^{f}	293 ± 5^{f}
S180+ABP	500×16	ig	22 ± 9^{d}	470 ± 62^{d}	103 ± 20^{d}	91 ± 7^{d}

and 0.0125% DNase for 30 min. Then the cells were collected onto glass fiber papers and detected by liquid scintillation. Results were expressed[7]: % Specific release = (Blank − Test)/(Blank − Maximum) × 100% (dpm). The NK cell activity of S180-bearing mice was greatly suppressed. ABP 50 and 100 mg $\cdot kg^{-1}$ ip augmented the suppressed NK activity and even exceeded that of the normal mice (Tab 5).

Tab 5. Enhancement of ip ABP on natural killer cell activity of S180-bearing mice. $n=4$, $\bar{x} \pm s$. $^{a}P>0.05$; $^{b}P<0.05$ *vs* normal group. $^{f}P<0.01$ *vs* S180 group.

	Dose/ $mg \cdot kg^{-1} \cdot d^{-1} \times 7d$	Natural killer cell activity/%
Normal	NS	43.5 ± 1.4
S180	NS	22.5 ± 8.0^{b}
S180+ABP	50	49.7 ± 6.6^{af}
S180+ABP	100	62.2 ± 7.2^{bf}

Effect of ABP on serum tumor necrosis factor alpha (TNF-α) production induced by LPS in S180-bearing mice S180 ascites (1×10^6 cells per 0.2 ml) were transplanted sc into the right axilla of ICR mice on d 0. ABP was injected ip (25, 50, 100 mg $\cdot kg^{-1}$) for 7 d. BCG as a positive control agent was injected ip once (200 mg $\cdot kg^{-1}$) on d 1. On d 8, 90 min after ip of LPS (0.1 mg $\cdot kg^{-1}$), mice were exsanguinated. Blood was centrifuged (400 × *g*, 10 min) and TNF-α-containing serum collected. Sera were stored at −20 ℃ until use. The levels of serum TNF-α were determined by cytotoxic assay against L929 cells as described previously[8]. L929 cells (5×10^5 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 and 100 μl of TNF-α containing serum were added to each well. RPMI 1640 medium (100 μl) containing actinomycin D was added to each well yielding a final concentration of 1 $\mu g \cdot ml^{-1}$. Plates were reincubated for 20 h, medium removed and cells stained with 0.5% crystal violet (containing 3.2% formaldehyde isotonic solution) for 15 min. Plates were rinsed and dried. The residue was dissolved in 47.5% alcohol (100 μl/well) and was measured at 490 nm on ELISA autoreader. TNF-α activity was calculated: % Cytotoxicity = $(A_{control} - A_{test})/A_{control} \times 100\%$. The ability of TNF-α production of S180-bearing mice was stronger than that of normal mice; and that ABP 50 mg

· kg^{-1} ip increased the production of serum TNF-α in tumor-bearing mice, which was as strong as the potent macrophage stimulatant BCG, but ABP 25 and 100 mg · kg^{-1} ip had less effect on TNF-α production in tumor-bearing mice.

Tab 6. Effect of ip ABP on serum tumor necrosis factor alpha (TNF-α) production of S180-bearing mice. $n=3$, $\bar{x}\pm s$. [b]$P<0.05$ *vs* normal group. [d]$P>0.05$, [e]$P<0.05$ *vs* S180 group.

	Dose/ mg·kg⁻¹·d⁻¹×7d	Serum TNF-α activity specific lysis/%
Normal	NS×7	7.2±3.9
S180	NS×7	16.4±2.6[b]
S180+ABP	25×7	16.9±1.0[d]
S180+ABP	50×7	25.6±2.6[e]
S180+ABP	100×7	22.1±4.3[d]
S180+BCG	200×1	29.2±4.2[e]

DISCUSSION

The present study demonstrated that ABP inhibited the growth of S180 tumor in mice, and that ABP prolonged the survival time of EC-bearing mice. Immuno-responses such as PFC counts, serum total IgG contents, lymphocyte proliferation induced by mitogens and NK cell activity of S180-bearing mice were much lower than those of normal mice. Previous reports[9,10] also showed that both nonspecific and specific immune functions of tumor-bearing mice were depressed markedly. In addition, our results indicated that serum TNF-α production induced by LPS in S180-bearing mice was significantly high as compared with normal mice. This finding was coincident with previous study[11].

ABP alone did not affect the [^{3}H]TdR uptake by S180 cells *in vitro* suggesting that the antitumor action of ABP was not a direct cytotoxic effect. ABP ip or ig promoted the humoral and cellular immune responses and enhanced the tumor related NK activity and TNF-α production which revealed that ABP ameliorated host immunological defense functions of tumor-bearing mice. ABP also augmented the cytotoxicity of peritoneal macrophages against S180 cells *in vitro*. Taking all these together, it is considered that the antitumor activity of ABP may be due to potentiating the host immune defense mechanisms and that it may become a hopeful BRM in cancer immunotherapy.

Not the whole large molecule of polysaccharides was necessary for its biological activities. Goldman[12] inferred that more than one "bio-active centers" might exist in molecule of polysaccharides. ABP provided a good clue for studying the struture-activity relationships of polysaccharides.

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牛膝多糖的抗肿瘤活性及其免疫增强作用

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A **摘要** 牛膝多糖(ABP) 50 mg·kg^{-1} ip 或250 mg·kg^{-1} ig 显著抑制小鼠移植性肉瘤S180生长，提高荷瘤小鼠低下的血清IgG含量和抗体形成细胞数量及脾淋巴细胞增殖反应. ABP ip 还提高荷瘤鼠NK细胞活性及LPS诱生的血清TNF-α产生. ABP 50-800 μg·ml^{-1} 体外对S180细胞无直接细胞毒作用，但能增强MΦ对S180的杀伤作用. 提示ABP抗肿瘤作用与其增强宿主免疫功能有关.

关键词 牛膝；多糖；抗体形成细胞；免疫球蛋白G；淋巴细胞转化；自然杀伤细胞；肿瘤坏死因子

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Synergistic effect of oridonin and cisplatin on cytotoxicity and DNA cross-link against mouse sarcoma S180 cells in culture

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ABSTRACT Oridonin (Ori) is an active principle isolated from *Rabdosia rubescens*. The cytotoxic effect of the combination of Ori and cisplatin was tested by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliuim bromide] colorimetric assay. IC_{50} of cisplatin to cultured S180 cells in 24 h was 9.38 μg·ml^{-1}. When the cells were treated with cisplatin plus Ori 0.5 and 1 μg·ml^{-1}, the IC_{50} were 1/3.4 and 1/6.7, respectively, of that with cisplatin alone. Modified alkaline elution was used to detect the DNA interstrand cross-link and DNA-protein cross-link in S180 cells induced by the 2 drugs. A greater amount of DNA cross-link was detected when the cells were treated with cisplatin plus Ori than with cisplatin alone ($P < 0.05$). After lysis by proteinase K, a reduction in DNA cross-link was seen, which suggested that the drugs could produce both kinds of DNA cross-link.

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KEY WORDS cisplatin; oridonin; drug combinations; cytotoxins; DNA; cross-linking reagents

Oridonin (Ori) is a diterpenoid compound isolated from *Rabdosia rubescens* (hemsl). It has been used alone or in combination with other drugs to treat human cancers, especially