Chemical and biological characterization of a polysaccharide biological response modifier from *Aloe vera* L. *var*. *chinensis* (Haw.) Berg.

M.Y.K. Leung[2], C. Liu[3], L.F. Zhu[4], Y.Z. Hui[5], B. Yu[5], and K.P. Fung[1,2,3]

[2]Institute of Chinese Medicine, Chinese University of Hong Kong, Shatin, N.T., Hong Kong, People's Republic of China; [3]Department of Biochemistry, Chinese University of Hong Kong, Shatin, N.T., Hong Kong, People's Republic of China; [4]South China Institute of Botany, Chinese Academy of Sciences, Guangzhou, People's Republic of China; and [5]Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences, Shanghai, People's Republic of China

Three purified polysaccharide fractions designated as PAC-I, PAC-II, and PAC-III were prepared from *Aloe vera* L. *var*. *chinensis* (Haw.) Berg. by membrane fractionation and gel filtration HPLC. The polysaccharide fractions had molecular weights of 10,000 kDa, 1300 kDa, and 470 kDa, respectively. The major sugar residue in the polysaccharide fractions is mannose, which was found to be 91.5% in PAC-I, 87.9% in PAC-II, and 53.7% in PAC-III. The protein contents in the polysaccharide fractions was undetectable. NMR study of PAC-I and PAC-II demonstrated the polysaccharides shared the same structure. The main skeletons of PAC-I and PAC-II are β-(1→4)-D linked mannose with acetylation at C-6 of manopyranosyl. The polysaccharide fractions stimulated peritoneal macrophages, splenic T and B cell proliferation, and activated these cells to secrete TNF-α, IL-1β, INF-γ, IL-2, and IL-6. The polysaccharides were nontoxic and exhibited potent indirect antitumor response in murine model. PAC-I, which had the highest mannose content and molecular weight, was found to be the most potent biological response modifier of the three fractions. Our results suggested that the potency of aloe polysaccharide fraction increases as mannose content and molecular weight of the polysaccharide fraction increase.

Introduction

Cancer is treated conventionally by radiotherapy, surgery, chemotherapy, immunotherapy, molecular targeting, or a combination of these methods (Mehren *et al*., 2003; Niethammer *et al*., 2002; Rosenberg, 2001; Ross, 1999; Weichselbaum *et al*., 2001). Recently there is increasing interest of using complementary and alternative medicine (CAM) for the palliative care of cancer patients worldwide (Cohen *et al*., 2002; Jatoi *et al*., 2003; Nahin, 2002; Oh and Small, 2002; Pongnikorn *et al*., 2003; White, 2002;

Yoshimura *et al*., 2003). Immunomodulatory polysaccharide represents one of the many biological response modifiers (BRMs) for cancer palliative treatment. BRMs can enhance or restore the host immunity against tumor progression (Chirigos *et al*., 1987; Toko and Fujimoto, 1989).

Polysaccharide BRMs can be prepared from bacteria, fungi, and plants. Bacterial polysaccharide BRMs can be membrane proteoglycan from *Klebsiella pneumoniae* (Sironi *et al*., 1990), cell wall lipopolysaccharide (LPS) from Gram-negative bacteria (Blackstock *et al*., 2000; Heinzelmann *et al*., 2000), and exopolysaccharide from *Paenibacillus jamilae* CP-7 (Ruiz-Bravo *et al*., 2001). Fungal polysaccharide BRMs are mainly derived from cell wall or cytoplasmic reserve (Domer *et al*., 1988; Liu *et al*., 1993) and botanical polysaccharide BRMs are mainly pectic substances in origin (Ebringerova *et al*., 2003; Guo *et al*., 2000; Nose *et al*., 1998; Yaneva *et al*., 2002).

The polysaccharides may have phosphate, acetyl group, carboxylic group, peptide, and lipid attached. The sugar composition of the polysaccharides might contain variable amounts of glucose, mannose, galactose, arabinose, and xylose. The linkages of the polysaccharides can be α-, β-, (1→2), (1→3), (1→4), or (1→6)-linkages. The polysaccharides are thus very diverse in their components and linkages except those derived from fungi and aloe. In fungi, the majority polysaccharide BRMs are β-D-glucans and those from yeast are α-D-mannan and glucomannan. Polysaccharide BRMs derived from aloe are mainly mannose polymer with β-(1→4)-D linkage.

Although a lot of polysaccharide BRMs have been reported, there is little information regarding to the structure and potency relationship of the polysaccharides. Such relationship study is hindered by the very complex sugar compositions and linkages of the polysaccharides. However, aloe polysaccharides having simple sugar composition and linkage are ideal models for the studies of the structure and potency relationship. The simplicity of the polysaccharides makes the structure determination feasible, and then the studies between structure and potency can be performed. In this study, three purified polysaccharide fractions were prepared from *Aloe vera* L. *var*. *chinensis* (Haw.) Berg. and were characterized chemically and biologically to delineate the effect of sugar content and molecular weight on the potency of the polysaccharide fractions.

Results

Purification of polysaccharides in PAC50

Polysaccharides in PAC50 (see *Materials and methods*) were further separated by gel filtration. PAC50 was resolved into

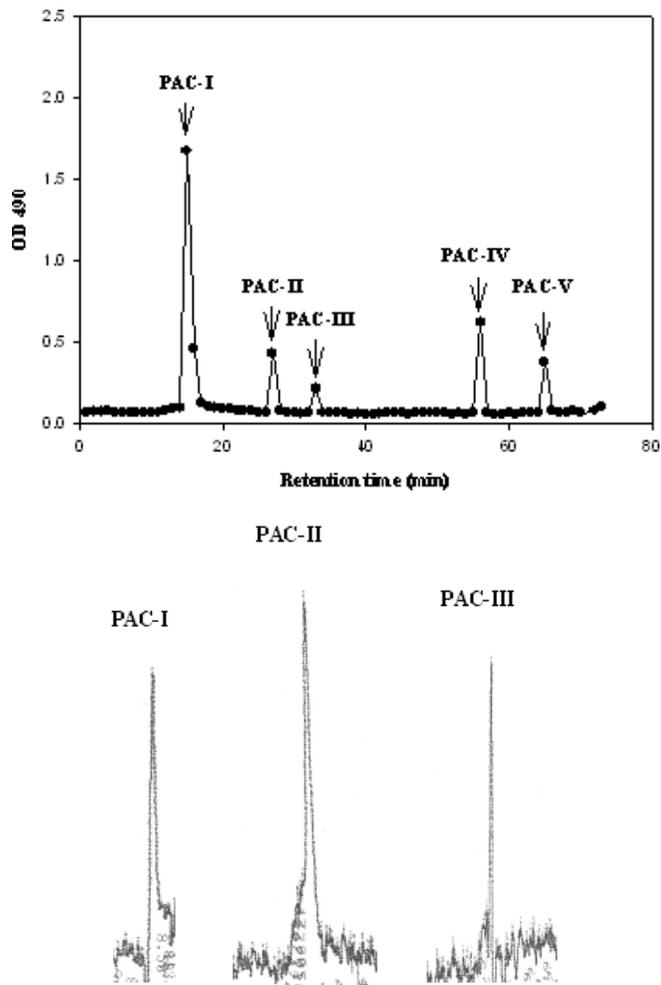[1]To whom correspondence should be addressed; e-mail: kpfung@cuhk.edu.hk

Fig. 1. Purification of PAC50. (**A**) PAC50 was resolved into five peaks. PAC-I was the largest peak and represented 60% of PAC50. PAC-II represented 10% and PAC-III represented 5%. The molecular weights of PAC-I, PAC-II, and PAC-III were 10,000 kDa, 1300 kDa, and 470 kDa according to dextran standards. (**B**) Capillary electropherograms of PAC-I, PAC-II, and PAC-III. Sample of 20 µl at 200 mg/ml eluant (H_3BO_3-KOH, pH 10) was electrophoresed at 200 V. Polysaccharide was detected by monitoring at optical density 254. Only one major peak was observed in each sample, and the peak was close to symmetry.

five distinct peaks by gel filtration high-performance liquid chromatography (HPLC) (Figure 1A). The yields of the fractions, calculated according to their polysaccharides, were 60%, 10%, 5%, 16%, and 9%. According to dextran standards, the molecular weight (MW) of PAC-I, PAC-II, PAC-III, PAC-IV, and PAC-V were 10,000 kDa, 1300 kDa, 470 kDa, 20 kDa, and 10 kDa. The purity of HPLC-separated polysaccharides were monitored by capillary electrophoresis (CE). There existed only one major peak in each capillary electropherogram of PAC-I, PAC-II, and PAC-III (Figure 1B), and each peak was close to symmetry.

Composition analysis

The purified polysaccharide fractions contained nearly exclusively carbohydrate. The percentage of carbohydrate

in PAC-I, PAC-II, and PAC-III were 98.30, 98.07, and 95.63. The protein content was undetectable in these polysaccharides. PAC-I and PAC-II shared similar monosaccharide composition and had about 90% mannose, 5–6% galactose, some glucose, and some arabinose. PAC-III had about 54% mannose, 33% galactose, 13% arabinose, and trace amounts of glucose (Figure 2).

NMR spectroscopy

The ^{13}C nuclear magnetic resonance (NMR) and ^{1}H NMR spectra of PAC-I and PAC-II were basically indistinguishable (Figure 3 and Figure 4). The ^{13}C NMR of PAC-I and PAC-II were found to be very similar to that of galactomannan. The NMR spectra of methyl glycosides and legume-seed galactomannans were used as references (Grasdalen and Painter, 1980). The carbon resonance of monosaccharide unit in polysaccharide is almost same as the corresponding free monosaccharide with the same configuration and substitution pattern. Hydroxyl group of C-4 of mannopyranosyl in PAC-I is glycosylated as there is no resonance of corresponding unglycosylated C-4 (66–68 ppm). In fact, the chemical shifts of C-4 and C-5 in PAC-I accorded with that of the galactomannan NMR references. C-6 of mannopyranosyl in PAC-I resonated at 64.5 ppm and 62.5 ppm. The hydroxyl group of C-6 in PAC-I was not glycosylated as glycosylated C-6 has chemical shift of 69.6 ppm. However, C-6 in PAC-I might be acetylated as acetylation caused the corresponding carbon resonance downfield by 1.8 ppm. The presence of acetyl group was confirmed by proton NMR (Figure 4) and infrared spectrum (unpublished data). Chemical shift in the anomeric region indicated that anomeric configuration was beta for mannopyranosyl. C-1 of mannopyranosyl in PAC-I had chemical shift of 102.2 ppm, which was similar to that of galactomannan. The ^{13}C NMR and ^{1}H NMR spectra of PAC-III were too complicated for structural interpretation (Figure 3C and Figure 4C).

In vitro and in vivo splenic lymphocytes proliferation

All the polysaccharide fractions stimulated the proliferation of both splenic T and B cells *in vitro*. The stimulation demonstrated a dose-dependent pattern (Figure 5A and 5B). The polysaccharide fractions were potent B cell stimulators and were less potent in T cell proliferation stimulation. PAC-I was the strongest stimulator and was followed by PAC-II and then PAC-III. The polysaccharide fractions when injected IP stimulated splenic lymphocyte proliferation 3 days after injection. PAC-I was the strongest stimulator and then PAC-II. PAC-III was the weakest (Figure 5C). The results accorded with *in vitro* observations. Endotoxin is a cell surface component of Gram-negative bacteria and is a potent B-lymphocyte stimulator. The stimulatory effect of PAC polysaccharides that was due to the contamination of LPS (endotoxin) was excluded by *Limulus* amebocyte lysate (LAL) test. The coagulation result of LAL test was negative and indicated that there was undetectable amount of endotoxin in PAC-I, PAC-II, and PAC-III preparation.
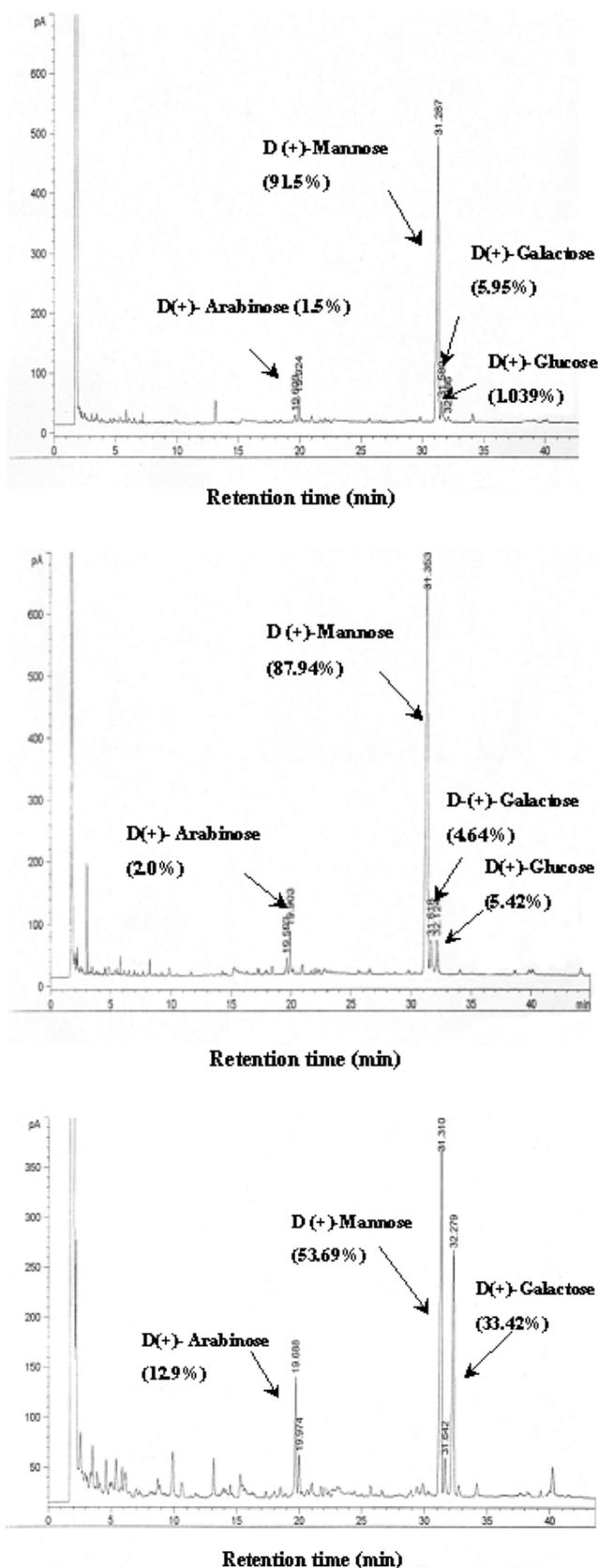
Fig. 2. Gas chromatograms of alditol acetates derived from sugar residues of PAC-I (**A**), PAC-II (**B**), and PAC-III (**C**). Polysaccharides were hydrolyzed and acetylated for GC-MS analysis. PAC-I and PAC-II were mannose rich, and PAC-III was mannose and galactose rich.

Elicitation of cytokines secretion

The polysaccharide fractions stimulated peritoneal macrophage to secrete interleukin (IL)-1β and tumor necrosis factor (TNF)-α, and stimulated splenic lymphocytes to secrete IL-2, IL-6, and interferon (INF)-γ. The stimulation followed a dose-dependent pattern and stimulation saturated at 500 mg/ml polysaccharide concentration. Cytokine level in culture medium of lymphocyte treated with polysaccharide at 500 mg/ml were tabulated in Table I. The polysaccharides when injected IV into mice triggered substantial secretion of serum TNF-α (Table I).

In vivo *antitumor activity*

Only PAC-I and PAC50 mediated significant regression of Sc-180 tumor *in vivo*. PAC-I caused a 58% reduction in tumor size and PAC50 caused a 50% reduction. PAC-II and PAC-III were unable to cause significant tumor regression (Figure 6). However, all the polysaccharide caused no significant regression of Hep-G2 tumor in nude mice (unpublished data).

Discussion

The molecular weight of PAC-IV and PAC-V determined by HPLC were lower than the specified molecular weight cutoff (MWCO) of the membrane (50 kDa) used for the preparation of PAC50. The specified MWCO of the membrane was for globular protein but not thread-like polysaccharide. The MWCO of the same membrane for polysaccharide was lower. Furthermore, the column used had very large fractionation range (10–1,000 kDa), so that the estimation of molecular weight of small molecules might not be accurate.

CE results of PAC-I, PAC-II, and PAC-III suggested the purified polysaccharide fractions might still contain some impurities. However, the purity was good enough for chemical and biological study. Protein or peptide, which can be immunostimulatory, was undetectable in PAC-I, PAC-II, and PAC-III. The result accorded with elemental analysis. Elemental analysis demonstrated that there existed no nitrogen in PAC-I, PAC-II, and PAC-III (unpublished data). Methylation analysis of the polysaccharides had been attempted. However the sizes of the polysaccharide fractions were too large for all the free hydroxyl groups to be methylated completely. The ^{13}C NMR spectrum of PAC-I was found to be very similar to that of legume-seed galactomannan (Grasdalen and Painter, 1980). Legume-seed galactomannan has manopyranosides in the main skeleton linked together by β-(1-4)-D-linkage and galactopyranoside attached at C-6 of the skeletal manopyranosides.

PAC-II, which had nearly the same composition and NMR profiles as PAC-I, had the same main skeletal structure as PAC-I but smaller in size. PAC-I had molecular weight 7.8 times that of PAC-II. The deduced structure of the main linkage of PAC-I and PAC-II is illustrated in Figure 7. The main skeleton of PAC-I and PAC-II was β-(1→4)-D-linked mannopyranosyl units. The manopyranosyl units in the polymer were O-acetylated at carbon six. The composition and linkages of PAC-III were more
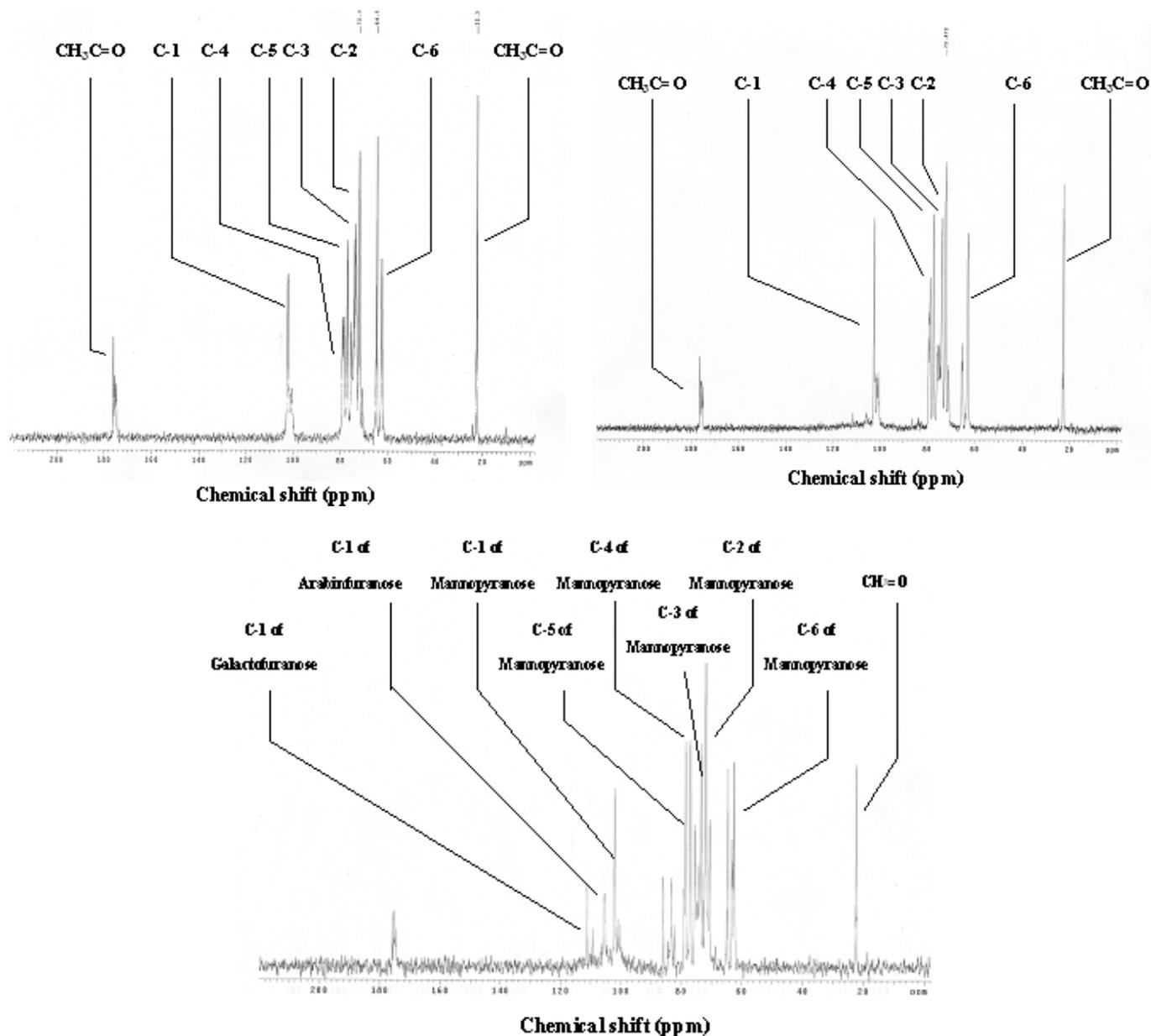
Fig. 3. ^{13}C NMR spectra of PAC-I (**A**), PAC-II (**B**), and PAC-III (**C**). Polysaccharide was dissolved in deuteriated water at 50 mg/ml. The NMR spectra were recorded by INOVA-600. The chemical shifts (δ) of C-1, C-2, C-3, C-4, C-5, and C-6 were 102.2, 72.1, 73.5, 78.5, 77.7, 62.5, and 64.5 ppm. The spectra of PAC-I and PAC-II were nearly the same. The NMR spectrum of PAC-III was too complicated for structural interpretation.

complicated than PAC-I and PAC-II. The possibility of impurities in PAC-III that contributed to the complex NMR spectra of PAC-III was rather low. CE results indicated that the purity of PAC-III was comparable to that of PAC-I and PAC-II.

All the PAC polysaccharide fractions tested were potent immunostimulators or BRMs. Immunomodulatory activities of the polysaccharide fractions, which were due to the contamination of endotoxin, was excluded by the result of the LAL test. The polysaccharide fractions stimulated peritoneal macrophage and splenic T and B cell proliferation, and activated these cells to secrete TNF-α, IL-1β, INF-γ, IL-2, and IL-6. We focused on these cytokines because they

are commonly used in tumor immunotherapy (Iwashita *et al.*, 2003; Kuninaka *et al.*, 2000; Rhines *et al.*, 2003; Terlikowski, 2002).

Both PAC-I and PAC50 mediated a significant regression of Sc-180 *in vivo* (Figure 6). The *in vivo* tumor regression properties of the aloe BRMs might be due to direct toxicity of the polysaccharide fractions, tumor-sensitive toxic metabolic derivatives of the polysaccharide fractions, or immunostimulatory properties of the polysaccharide fractions. The direct toxicity of the polysaccharide fractions was excluded because antitumor effect was only observed *in vivo* but not *in vitro* (unpublished data). The polysaccharide fractions mediated no tumor regression in nude mice,
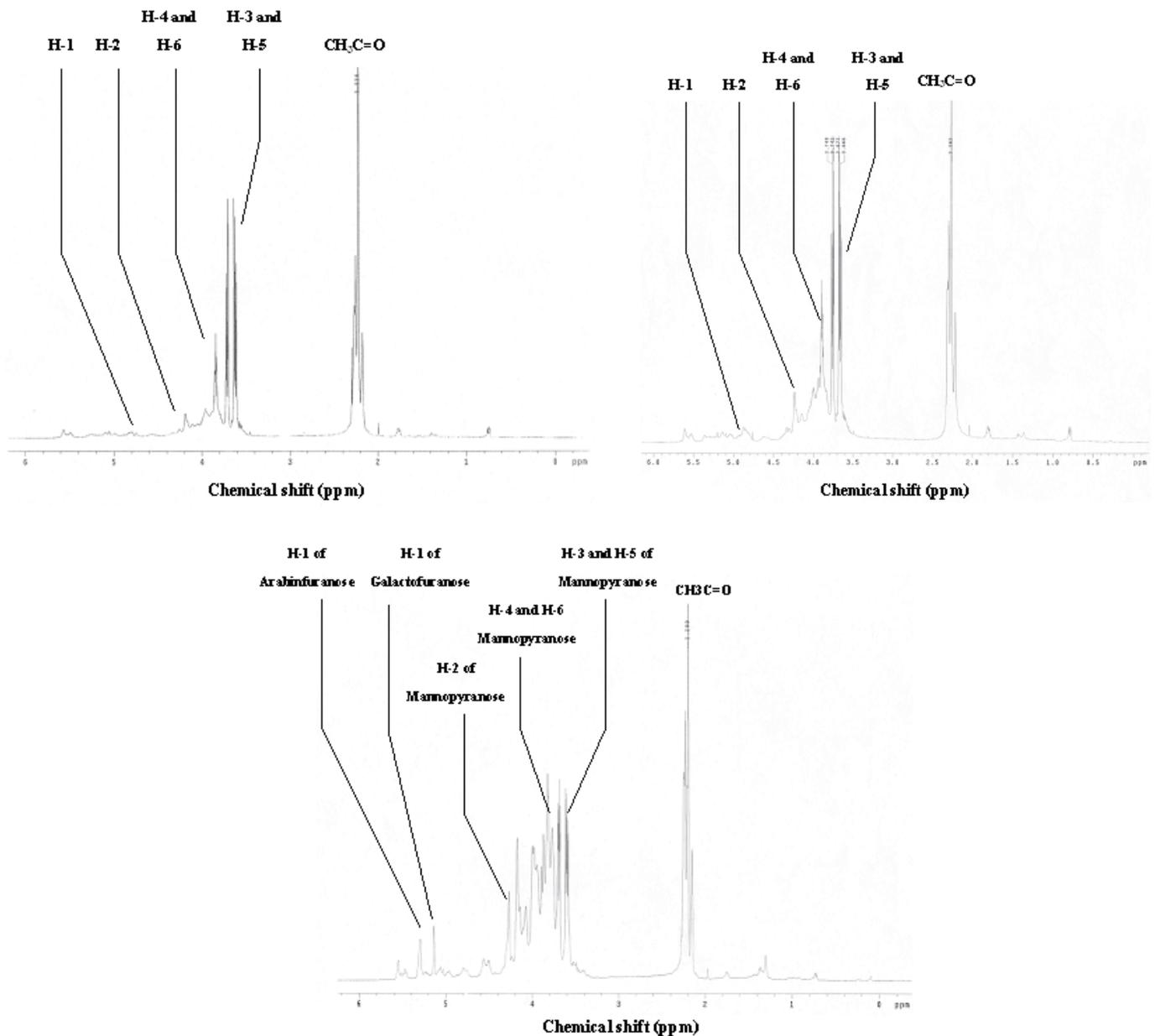
Fig. 4. ^{1}H NMR spectra of PAC-I (**A**), PAC-II (**B**), and PAC-III (**C**). Polysaccharide was dissolved in deuteriated water at 50 mg/ml. The NMR spectra were recorded by INOVA-600. Resonance at 2.21 ppm was very strong and indicated the presence of an acetyl group. The spectra of PAC-I and PAC-II were nearly the same. The NMR spectrum of PAC-III was too complicated for structural interpretation.

which suggested that aloe polysaccharides were not metabolized to tumor-sensitive toxic substances. Therefore, aloe BRMs must mediate tumor regression *in vivo* by the activation of host immune effector mechanisms.

Similar to PAC-I and PAC-II, β-(1→3)-D-glucans have simple monosaccharide composition and linkage. The *in vivo* antitumor properties of fungal β-(1→3)-D-glucans are initiated by the binding of the glucans to β-glucan receptor, such as dectin-1 (Brown and Gordon, 2001; Taylor *et al*., 2002), on immune cells. The target receptor for aloe BRMs might be mannose receptor (MR) on macrophages and dendritic cells. Aloe polysaccharides might bind to MR of dendritic cells and macrophages and lead to

activation of immunity. The stimulation of B cells by PAC polysaccharides might through another mechanism, because the presence of MR on B cells was not reported. The potent B cell stimulatory effect of PAC polysaccharides *in vitro* might be related to its highly repeating structures, which cross-link surface IgM of polysaccharide-responding B cells in a multivalent fashion. The cross-linking activates the B cells to proliferate (Anderson and Blomgren, 1971; Dorries *et al*., 1974).

The mechanism of activation of immunity by β-glucan is well established. Lesson from β-glucan study may provide clues to the immune activation by mannose-rich polysaccharide at a molecular level. Dectin-1 is a pattern

505

recognition receptor. Dectin-1 cooperates with Toll-like receptors (TLRs), and many other surface receptors for the recognition of different microbial products such as fungal cell wall, lipopolysaccaharide, lipoprotein, flagellin, and bacterial DNA (Gantner *et al.*, 2003; Underhill, 2003). The repertoire of receptors that recognize microbial products determines the immune effector mechanism to be activated. Zymosan particle, which is composed mainly of β-glucan, mannan, mannoproteins, and chitin, is recognized by dectin-1, TLRs, and CD14. The receptors work together to enhance immune response to the particles. Dectin-1 can trigger phagocytosis and reactive oxygen production directly. TLRs induce signaling through NF-κB results in the secretion of inflammatory cytokines. The TLR induction is enhanced by dectin-1. Mannose-rich polysaccharide may also collaborate with TLRs for the activation of immunity.

Altogether, mannan from aloe has a simple composition, and the linkage is an excellent model for the study of structure and activity relationship. PAC-I and PAC-II prepared from *A. vera* L. *var. chinensis* (Haw.) Berg. has potent BRMs. The polysaccharide fractions mediated indirect tumor regression *in vivo* through the activation of antitumor effector cells and the secretion of antitumor cytokines. The skeletons of the polysaccharide fractions were β-(1→4)-D-linked mannose with O-acetylation at C-6. High molecular weight and high mannose content may be essential for high antitumor potency of aloe polysaccharide.
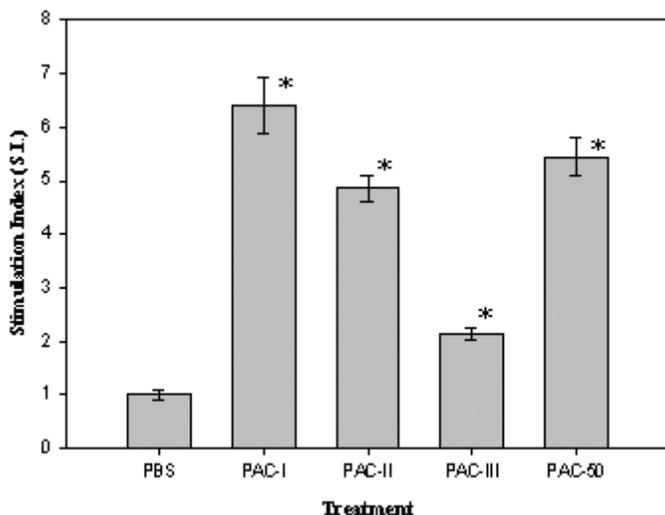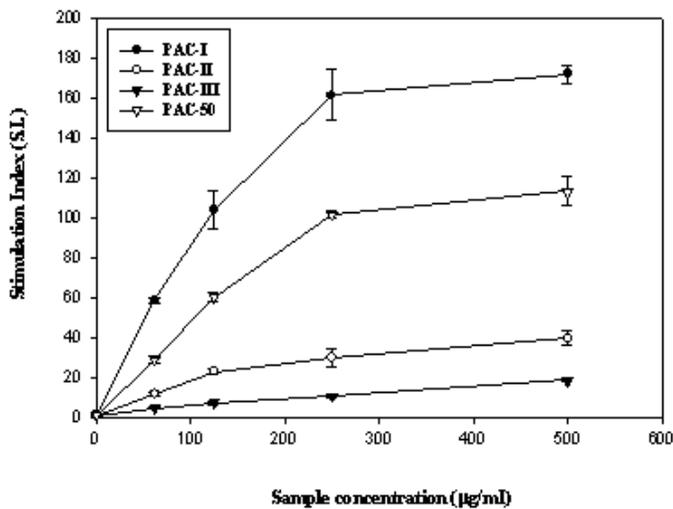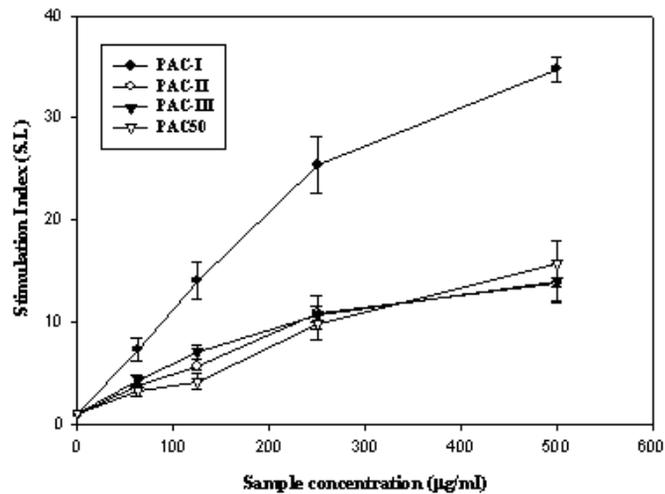
Materials and methods

Fractionation of A. vera L. var. chinensis (Haw.) Berg.

A. vera L. *var. chinensis* (Haw.) Berg., a variant of *A. vera* (*barbadensis* Miller), was supplied by South China Institute of Botany, Chinese Academy of Sciences, China. Fleshy layers of aloe leaves were collected and blended to viscous watery gel. Insoluble fibers were removed by centrifugation ($865 \times g$ for 10 min). The viscous supernatant was allowed to incubate at 4°C overnight and then centrifuged at $5000 \times g$ for 20 min to remove precipitate. The supernatant was loaded onto a column of XAD-4 resin (Rohm and Haas, Philadelphia, PA) with dimension of $5 \text{ cm} \times 15 \text{ cm}$. Collected eluate, which contained the crude polysaccharide, was fractionated by membrane with MWCO of 50 kDa. Membrane-retained fraction was

Fig. 5. The stimulatory effect of PAC polysaccharides on the proliferation of murine splenic lymphocytes. PAC polysaccharides stimulated the proliferation of murine splenic T lymphocytes (**A**) and B lymphocytes (**B**) in a dose-dependent manner *in vitro*. PAC-I was the most potent stimulator for both T and B cell stimulation. Stimulation due to PAC-I was significantly higher than that due to PAC-II and PAC-III. PAC-II and PAC-III had similar potency on T cell stimulation. However PAC-II was more potent than PAC-III on B cell stimulation. LPS of 5 μg/ml was used as positive control in B lymphocyte proliferation and Conacanavalin A of 1 μg/ml was used as positive control in T lymphocyte proliferation. The simulation index of LPS was 133 ± 8, and the simulation index of Conacanavalin A was 283 ± 45. PAC polysaccharides exhibited mitogenic activity on murine splenic lymphocytes *in vivo* (**C**). Female albino BALB/c mice were injected IP with PBS, PAC50, PAC-I, PAC-II, or PAC-III. Splenic lymphocytes were prepared from treated mice 3 days after injection. PAC-I was a stronger stimulator than PAC-II, followed by PAC-III. An asterisk indicates lymphocyte proliferation was significantly higher than that of PBS-treated control. SI ratio is calculated by dividing the radioactive count of sample-treated lymphocytes with radioactive count of medium-treated lymphocytes. Values are means $\pm$ SD ($n = 5$ for each group).

Table I. Elicitation of cytokine secretion by PAC polysaccharides at 500 mg/ml

Cytokine	Cell	Cytokine level (pg/ml cultured medium)						
		Medium	PAC-I	PAC-II	PAC-III	PAC50	ConA (1 μg/ml)	LPS (5 μg/ml)
TNF-α	Peritoneal macrophage	1 ± 0	1432 ± 47	1072 ± 15	1144 ± 29	1219 ± 76	NA	1529 ± 78
IL-1β	Peritoneal macrophage	3 ± 2	944 ± 44	589 ± 29	578 ± 51	412 ± 37	NA	1211 ± 54
IL-2	T lymphocyte	2 ± 0	23.0 ± 0.8	20.8 ± 0.8	22.6 ± 1.5	15.0 ± 0.1	51.4 ± 3.0	NA
IL-6	T and B lymphocytes	2 ± 1	515 ± 4	434 ± 41	548 ± 5	351 ± 8	507 ± 37	NA
INF-γ	T lymphocyte	1 ± 0	264 ± 2	288 ± 11	327 ± 14	27 ± 3	382 ± 32	NA
TNF-α	Macrophage	2 ± 1 (PBS IV injected)	746 ± 44	528 ± 43	145 ± 2	230 ± 5	NA	1473 ± 67 LPS (10 μg/ml) IV injected

All the PAC polysaccharide fractions induced significant secretion of cytokines when compared with medium control. TNF-α is the serum TNF-α level determined at 1.5 h after IV injection of PBS or PAC polysaccharides. Concanavalin A (ConA) and LPS were used as positive controls. NA, undetectable.
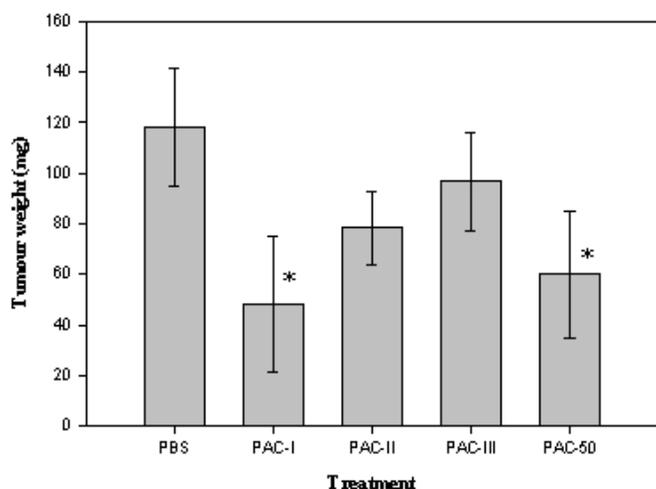

Fig. 6. Effect of PAC polysaccharides on the growth of Sc-180 *in vivo*. Sarcoma 180 cells (10^7 cells per ml PBS of 50 μl) were injected SC into the right groin of male ICR albino mice. Mice were then injected IP with PAC polysaccharides (1 mg/ml, 0.2 ml) or PBS for 10 consecutive days. PBS was used as a negative control. On day 21, tumor weight was determined. An asterisk indicates that tumor growth was suppressed significantly when compared with PBS-treated control. Values are means ± SD ($n = 10$ for each group).

concentrated with a thin film evaporator LZ-005 at 70°C (Xia Mei Yi Liao Jixie Chang, China) and then lyophilized. The fraction was designated PAC50.

Protein content determination

Bradford micro-protein assay (Bradford, 1976) was used to determine protein content. Bovine serum albumin (Sigma, St. Louis, MO) was used as protein standard. Filtered Bradford reagent (BioRad, Hercules, CA) (0.2 ml) was reacted with 0.8 ml sample at 1 mg/ml at room temperature for 15 min. Absorbance of the reaction mixture was measured at optical density 595.
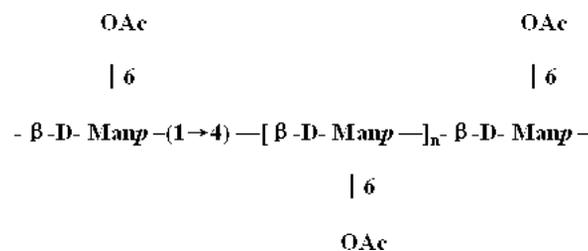

Fig. 7. The deduced linkage of PAC-I and PAC-II.

Carbohydrate content determination

Dubois method (Dubois *et al*., 1956) was used to measure carbohydrate content. D-mannose was used as standard. A sample of 400 μl at 500 μg/ml was mixed with 200 μl 6% phenol solution and 1 ml concentrated sulfuric acid. The mixture was reacted for 20 min at room temperature. Absorbance of the reaction mixture was measured at optical density 490.

HPLC of PAC50

PAC50 was dissolved in 0.1 M NaCl at 5 mg/ml. Five milliliters of PAC50 were loaded onto a Gel filtration column (Jordi Aqueous GPC Glucose Bound 10,000 Å 5 U SS column; Alltech, Nicholasville, KY) with dimensions of 22 mm × 500 mm. NaCl (0.1 M) was the mobile phase, and the flow rate was maintained at 3 ml/min. Dextrans of different molecular weights were used as standard. The eluate was collected as 3-ml fractions. The carbohydrate content in each fraction was determined by Dubois's method.

CE

Honda's method (Honda *et al*., 1989) was used to determine the purity of column separated polysaccharides. Sample of 20 μl at 200 mg/ml eluant (H_3BO_3-KOH, pH 10) was applied to Quanta 400E (Waters, Milford, CT) CE system

and electrophoresed at 20 kV. Polysaccharide was detected by measuring at optical density 254.

Monosaccharide composition analysis

Monosaccharide composition analysis followed the method of Wozniewski *et al.* (1990). A polysaccharide fraction of 3 mg was hydrolyzed with 10 ml 2 M aqueous trifluoroacetic acid in a sealed tube at 120°C for 1 h. The hydrolysate was concentrated with rotary evaporator under reduced pressure at 60°C, and remaining traces of trifluoracetic acid was removed by coevaporating with methanol (3 × 10 ml). The concentrated hydrolysate was dried over P_2O_5 in vacuum overnight. The dried hydrolysate was dissolved in 5 ml dH$_2$O, and 10 mg sodium borohydride (NaBH$_4$) was then added. The mixture was stirred at room temperature for 18 h. Excess NaBH$_4$ was removed by acetic acid. The mixture was then concentrated and dried under reduced pressure. Dried residue was dissolved in 6 ml anhydrous pyridine. Acetic anhydride (6 ml) was added, and the mixture was stirred overnight at room temperature. After incubation, the mixture was coevaporated with toluene (3 × 10 ml) at 55°C under reduced pressure and finally dried in vacuum. Dried residue was dissolved in minimal amount of chloroform for gas chromatography–mass spectrometry (GC-MS) analysis with a GC analyzer (Varian VISTA 6000, Palo Alto, CA). The GC column was OV-17 with dimension of 0.2 mm × 300 mm (Agilent Technologies, Wilmington, DE). The injector and column temperature was 250°C.

^{13}C NMR and ^{1}H NMR spectroscopy

Samples were dissolved in deuteriated water at 50 mg/ml for both ^{13}C NMR and ^{1}H NMR (Agrawal, 1992). ^{1}H NMR spectra were recorded with an INOVA-600 spectroscopy at 30°C in D$_2$O, and chemical shifts were referred to HOD. ^{13}C NMR spectra were recorded with an INOVA-600 spectroscopy at 30°C in D$_2$O, and chemical shifts were referred to Me$_4$Si.

LAL coagulation test

The LAL test is commonly employed to determine the presence of LPS or endotoxin (lipopolysaccharide) in herbal preparation. The Pyrotell (Associates of Cape Cod) testing reagent was used according to instructions. Pyrogen-free water and LPS (0.05 ng/ml) from *Escherichia coli* of serotype 0127:B8 (Sigma) were used as controls.

In vitro *lymphocyte blast transformation assay*

Splenic lymphocytes were prepared from female albino BALB/c mice by Ficoll-Plaque centrifugation. Three spleens were ground with a sterilized sieve and a 2.5-ml syringe plunger in 10 ml RPMI-1640 medium (Invitrogen, Carlsbad, CA). The ground cell suspension was transferred to a 50-ml centrifuge tube, and volume was adjusted to 20 ml with medium. Equal volume of Ficoll was then added to the bottom of the cell suspension. The tube was centrifuged at $2200 \times g$ for 20 min. Cells at interface were splenic lymphocytes and were collected. T cells were prepared by B cells depleting column (Cedarlane Labs, Hornby, Ontario, Canada), and B cells were prepared by T

cells depleting column (Cedarlane Labs, Carlsbad, CA). Prepared T and B cells were resuspended in medium supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA) at 5×10^6 cells/ml. Cell suspension of 100 μl was distributed per well of a 96-well plate. Cells were incubated with filter-sterilized polysaccharide at 37°C for 48 h. Cell proliferation was monitored by thymidine incorporation.

In vivo *lymphocyte blast transformation assay*

Polysaccharide fraction of 0.2 ml at 5 mg/ml phosphate buffered saline (PBS) was injected IP into female albino BALB/c mice. Spleens were obtained from mice on 3 days after sample administration. Splenic lymphocytes were prepared as described. Cell suspension of 100 μl at 5×10^6 cells/ml was distributed per well of a 96-well plate. Cell proliferation was monitored by thymidine incorporation at 48 h after incubation.

In vitro *IL-2, IL-6, and INF-γ secretion*

Splenic lymphocytes were prepared from female albino BALB/c mice. Cell suspension of 100 μl at 5×10^6 cells/ml concentration was plated per well of a 96-well plate. Cells were incubated with filter-sterilized polysaccharide at 37°C for 48 h. After the incubation, 100 μl medium was removed from each well, and the level of IL-2, IL-6, and INF-γ in removed medium was assayed by enzyme-linked immunoasorbent assay (ELISA) kit (Pharmingen, San Diego, CA).

In vitro *IL-1β and TNF-α secretion*

Thioglycollate broth (Difco Laboratories, Detroit, MI) of 1.5 ml at 0.3% concentration was injected IP per male BALB/c mouse. Three days after injection, the peritoneal cavity was washed with 20 ml PBS. Cells in the peritoneal wash were washed twice with RPMI-1640 medium. Cell concentration was adjusted to 10^8 cells/ml medium. Cell suspension of 100 μl was added per well of a 96-well plate. The cells were incubated at 37°C for 3 h. After incubation, nonadherent cells were removed by washing. The adherent cells, which were peritoneal macrophages, were incubated with filter-sterilized polysaccharide at 37°C for 48 h. After the incubation, 100 μl medium was removed from each well. The level of IL-1β and the level of TNF-α in removed media were assayed by murine IL-1β and TNF-α ELISA kit (Pharmingen), respectively.

In vivo *TNF-α secretion*

Polysaccharide of 0.2 ml at 500 mg/ml was injected IV per male BALB/c mouse. LPS at 10 μg/ml was used as positive control. Blood was collected 1.5 h after polysaccharide administration by heart puncture. Collected blood was clotted at room temperature and then centrifuged at $4000 \times g$ for 30 min. Serum was collected for TNF-α measurement by murine TNF-α ELISA kit (Pharmingen).

In vivo *antitumor assay*

Sarcoma 180 (Sc-180) cells (ATCC) of 50 μl at a cell concentration of 10^7 cells per ml PBS were injected SC into the right groin of male ICR albino mice (10 mice/group) on day 1. Mice were then injected IP with 0.2 ml polysaccharide

fraction at 1 mg/ml PBS or 0.2 ml PBS (PBS as negative control) for 10 consecutive days from day 1. On day 21, mice were euthanized, and solid tumors were excised for weight determination.

Statistical analysis

Student's *t*-test was used for the statistical analysis of results at a level of significance of $p < 0.05$.

Abbreviations

BRM, biological response modifier; CAM, complementary and alternative medicine; CE, capillary electrophoresis; ELISA, enzyme-linked immunosorbent assay; GC, gas chromatography; HPLC, high-performance liquid chromatography; IL, interleukin; INF, interferon; LAL, *Limulus* amebocyte lysate; LPS, lipopolysaccharide; MR, mannose receptor; MS, mass spectrometry; MWCO, molecular weight cutoff; NMR, nuclear magnetic resonance; PBS, phosphate buffered saline; TLR, toll-like receptor; TNF, tumor necrosis factor.

References

Agrawal, P.W. (1992) NMR spectroscopy in the structural elucidation of oligo-saccharides and glycosides. *Phytochemistry*, **31**, 3307–3330.

Anderson, B. and Blomgren, H. (1971) Evidence of thymus-independent humoral antibody production in mice against polyvinylpyrrolidone and *E. coli* lipopolysaccharide. *Cell. Immunol.*, **2**, 411–424.

Blackstock, R., McElwee, N., Neller, E., and Shaddix-White, J. (2000) Regulation of cytokine expression in mice immunized with cryptococcal polysaccharide, a glucuronoxylomannan (GXM), associated with peritoneal antigen-presenting cells (APC): requirements for GXM, APC activation, and interleukin-12. *Infect. Immun.*, **68**, 5146–5153.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein-dye binding. *Anal. Biochem.*, **72**, 248–254.

Brown, G.D. and Gordon, S. (2001) Immune recognition. A new receptor for beta-glucans. *Nature*, **413**, 36–37.

Chirigos, M.A., Schlick, E., and Budzynski, W. (1987) Immune response by biological response modifiers. *Cancer Detect. Prev. Suppl.*, **1**, 385–397.

Cohen, I., Tagliaferri, M., and Tripathy, D. (2002) Traditional Chinese medicine in the treatment of breast cancer. *Semin. Oncol.*, **29**, 563–574.

Domer, J., Elkins, K., Ennist, D., and Baker, P. (1988) Modulation of immune responses by surface polysaccharides of Candida albicans. *Rev. Infect. Dis.*, **10**(suppl 2), S419–S422.

Dorries, R., Schimpl, A., and Wecker, E. (1974) Action of dextran sulfate as a direct and general B cell mitogen. *Eur. J. Immunol.*, **4**, 230–233.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., and Smith, F. (1956) Colorimetric method for the determination of sugars and related substances. *Anal. Chem.*, **28**, 350–356.

Ebringerova, A., Kardosová, A., Hromádková, Z., and Hríbalová, V. (2003) Mitogenic and comitogenic activities of polysaccharides from some European herbaceous plants. *Fitoterapia*, **74**, 52–61.

Gantner, B.N., Simmons, R.M., Canavera, S.J., Akira, S., and Underhill, D.M. (2003) Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2. *J. Exp. Med.*, **197**, 1107–1117.

Grasdalen, H and Painter, T. (1980) NMR studies of composition and sequence in legume-seed galactomannans. *Carbohydr. Res.*, **81**, 59–66.

Guo, Y., Matsumoto, T., Kikuchi, Y., Ikejima, T., Wang, B., and Yamada, H. (2000) Effects of a pectic polysaccharide from a medicinal herb, the roots of *Bupleurum falcatum* L. on interleukin 6 production of murine B cells and B cell lines. *Immunopharmacology*, **49**, 307–316.

Heinzelmann, M., Polk, H.C. Jr., Chernobelsky, A., Stites, T.P., and Gordon, L.E. (2000) Endotoxin and muramyl dipeptide modulate

surface receptor expression on human mononuclear cells. *Immunopharmacology*, **48**, 117–128.

Honda, S., Iwase, S., Makino, A., and Fujiwara, S. (1989) Simultaneous determination of reducing monosaccharides by capillary electrophoresis as the borate complexes of N-2-pyridylglycanines. *Anal. Biochem.*, **176**, 72–77.

Iwashita, Y., Goto, S., Tahara, K., Kawano, K., Chen, C.L., and Kitano, S. (2003) Potent stimuli combined with lipopolysaccharide and IFNgamma may improve immunotherapy against HCC by increasing the maturation and subsequent immune response of the dendritic cells. *Cancer Biother. Radiopharm.*, **18**, 1–6.

Jatoi, A., Ellison, N., Burch, P.A., Sloan, J.A., Dakhil, S.R., Novotny, P., Tan, W., Fitch, T.R., Rowland, K.M., Young, C.Y., and Flynn P.J. (2003) A phase II trial of green tea in the treatment of patients with androgen independent metastatic prostate carcinoma. *Cancer*, **97**, 1442–1446.

Kuninaka, S., Yano, T., Yokoyama, H., Fukuyama, Y., Terazaki, Y., Uehara, T., Kanematsu, T., Asoh, H., and Ichinose, Y. (2000) Direct influences of pro-inflammatory cytokines (IL-1beta, TNF-alpha, IL-6) on the proliferation and cell-surface antigen expression of cancer cells. *Cytokine*, **12**, 8–11.

Liu, W.K., Ng, T.B., Sze, S.F., and Tsui, K.W. (1993) Activation of peritoneal macrophages by polysaccharopeptide from the mushroom, *Coriolus versicolor*. *Immunopharmacology*, **26**, 139–146.

Mehren, M.V., Adams, G.P., and Weiner, L.M. (2003) Monoclonal antibody therapy for cancer. *Annu. Rev. Med.*, **54**, 343–369.

Nahin, R.L. (2002) Use of the best case series to evaluate complementary and alternative therapies for cancer: a systematic review. *Semin. Oncol.*, **29**, 552–562.

Niethammer, A.G., Xiang, R., Becker, J.C., Wodrich, H., Pertl, U., Karsten, G., Eliceiri, B.P., and Reisfeld, R.A. (2002) A DNA vaccine against VEGF receptor 2 prevents effective angiogenesis and inhibits tumour growth. *Nat. Med.*, **8**, 1369–1375.

Nose, M., Terawaki, K., Oguri, K., Ogihara, Y., Yoshimatsu, K., and Shimomura, K. (1998) Activation of macrophages by crude polysaccharide fractions obtained from shoots of *Glycyrrhiza glabra* and hairy roots of *Glycyrrhiza* uralensis *in vitro*. *Biol. Pharm. Bull.*, **21**, 1110–1112.

Oh, W.K. and Small, E.J. (2002) Complementary and alternative therapies in prostate cancer. *Semin. Oncol.*, **29**, 575–584.

Pongnikorn, S., Fongmoon, D., Kasinrerk, W., and Limtrakul, P.N. (2003) Effect of bitter melon (*Momordica charantia* Linn) on level and function of natural killer cells in cervical cancer patients with radiotherapy. *J. Med. Assoc. Thai.*, **86**, 61–68.

Rhines, L.D., Sampath, P., DiMeco, F., Lawson, H.C., Tyler, B.M., Hanes, J., Olivi, A., and Brem, H. (2003) Local immunotherapy with interleukin-2 delivered from biodegradable polymer microspheres combined with interstitial chemotherapy: a novel treatment for experimental malignant glioma. *Neurosurgery*, **52**, 872–880.

Rosenberg, S.A. (2001) Progress in human tumour immunology and immunotherapy. *Nature*, **411**, 380–384.

Ross, G.M. (1999) Induction of cell death by radiotherapy. *Endocr. Relat. Cancer*, **6**, 41–44.

Ruiz-Bravo, A., Jimenez-Valera, M., Moreno, E., Guerra, V., and Ramos-Cormenzana, A. (2001) Biological response modifier activity of an exopolysaccharide from *Paenibacillus jamilae* CP-7. *Clin. Diag. Lab. Immunol.*, **8**, 706–710.

Sironi, M., Sica, A., Riganti, F., Licciardello, L., Colotta, F., and Mantovani, A. (1990) Interleukin-6 gene expression and production induced in human monocytes by membrane proteoglycans from *Klebsiella pneumoniae*. *Int. J. Immunopharmacol.*, **12**, 397–402.

Taylor, P.R., Brown, G.D., Reid, D.M., Willment, J.A., Martinez-Pomares, L., Gordon, S., and Wong, S.Y. (2002) The beta-glucan receptor, dectin-1, is predominantly expressed on the surface of cells of the monocyte/macrophage and neutrophil lineages. *J. Immunol.*, **169**, 3876–3882.

Terlikowski, S.J. (2002) Local immunotherapy with rhTNF-alpha mutein induces strong antitumor activity without overt toxicity. *Toxicology*, **174**, 143–152.

Toko, T. and Fujimoto, S. (1989) Augmentation of anti-tumor immunity in low-responder mice by various biological response modifiers: analysis of effector mechanism. *Jpn. J. Cancer Res.*, **80**, 1212–1219.

Underhill, D.M. (2003) Toll-like receptors: networking for success. *Eur. J. Immunol.*, **33**, 1767–1775.

Weichselbaum, R.R., Kufe, D.W., Advani, S.J., and Roizman, B. (2001) Molecular targeting of gene therapy and radiotherapy. *Acta Oncol.*, **40**, 735–738.

White, J.D. (2002) Complementary and alternative medicine research: a National Cancer Institute perspective. *Semin. Oncol.*, **29**, 546–551.

Wozniewski, T., Blaschek, W., and Franz, G. (1990) Isolation and structure analysis of a glucomannan from the leaves of *Aloe arborescens var.* Miller. *Carbohyd. Res.*, **198**, 387–391.

Yaneva, M.P., Botushanova, A.D., Grigorov, L.A., Kokov, J.L., Todorova, E.P., and Krachanova, M.G. (2002) Evaluation of the immunomodulatory activity of *Aronia* in combination with apple pectin in patients with breast cancer undergoing postoperative radiation therapy. *Folia Med.*, **44**, 22–25.

Yoshimura, K., Ichioka, K., Terada, N., Terai, A., and Arai, Y. (2003) Use of complementary and alternative medicine by patients with localized prostate carcinoma: study at a single institute in Japan. *Int. J. Clin. Oncol.*, **8**, 26–30.