Protective Effects of a Neutral Polysaccharide Isolated from the Mycelium of *Antrodia cinnamomea* on *Propionibacterium acnes* and Lipopolysaccharide Induced Hepatic Injury in Mice

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Mycelia of *Antrodia cinnamomea* were extracted with chloroform and hot water. A neutral polysaccharide named *ACN2a* separated from the water extract was purified using 10% CCl_3COOH, and repeated column chromatography on HW-65 and DE-52 cellulose. Its structure was determined by chemical and spectroscopic analyses. *ACN2a* was composed of Gal, Glc, Fuc, Man and GalN (in the ratio 1 : 0.24 : 0.07 : 0.026 : faint), in which an α-D-(1→6)-Gal linkage accounted for 73% of all linkages. The ratio of branch points was about 16% of the total residual numbers, and branches were attached to C-2 of galactosyl residues of the main chain. *ACN2a* had an average molecular weight of 12.9×10^5 Daltons, $[\alpha]_D^{25}=+115°$ ($c=0.44$, H_2O); $[\eta]=0.0417 dl\cdot g^{-1}$, $Cp=0.2663$ cal/(g·°C). The hepatoprotective effect of *ACN2a* was evaluated using a mouse model of hepatic injury that was induced by *Propionibacterium acnes* (*P. acnes*) and lipopolysaccharide (LPS). The administration of *ACN2a* (0.4, 0.8 g/kg/d, *p.o.*), significantly prevented increases in serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) enzyme activities in mice treated with *P. acnes*–LPS, indicating hepatoprotective activity *in vivo*.

Key words *Antrodia cinnamomea*; neutral polysaccharide; *Propionibacterium acnes*; hepato protective effect; mycelium

Antrodia cinnamomea Chang TT & WN Chou (Chinese name, niu-chang-chih or niu-chang-ku; synonym *Antrodia camphorata* Wu SH *et al.*) is a new species of the genus Antrodia (family polyporaceae, Aphyllophorales) that is parasitic on the inner cavity of the endemic species *Cinnamomum kanehirai* Hay. This endangered species has been used in Taiwan to treat food, alcohol, and drug intoxication, diarrhea, abdominal pain, hypertension, skin itching and liver cancer as a Chinese folk medicine.[1)]

The hot water extract of *A. cinnamomea* has hepatoprotective effect,[2,3)] but the responsible component remains unclear.

Here we describe the separation and purification of a neutral polysaccharide named *ACN2a* from a hot water extract of the mycelium of *A. cinnamomea*. We examined the hepatoprotective activity of the extract in mice with *Propionibacterium acnes* (*P. acnes*)–lipopolysaccharide (LPS) induced hepatotoxicity and elucidated the structure of this polysaccharide (*ACN2a*) by chemical and spectroscopic means.

Experimental

Materials *Antrodia cinnamomea* mycelia (Lot No. #C082002-1) were provided by Simpson Biotech Co. Ltd. (Taiwan). Pullulans (Shodex Standard P-82) were purchased from Showa Denko Co. Ltd. (Tokyo, Japan) and used as standard molecular weight markers.

General Optical rotation was determined in H_2O using a JASCO DIP-360 automatic polarimeter and UV absorption was measured using a Shimadzu UV-2200 UV–VIS recording spectrometer. Infrared (IR) spectra were recorded in a KBr disk or liquid film using a JASCO FT/IR-230 IR spectrometer and NMR spectra were recorded on a Varian Unity Plus 500 (^1H, 500 MHz; ^{13}C, 125 MHz) and a Varian GEMINI 300 (^1H, 300 MHz, ^{13}C, 75 MHz). A solution of polysaccharide in D_2O was measured with 1,4-dioxane as the external reference. Analysis by GC-MS proceeded on a Shimadzu GC-17A gas chromatograph equipped with a JEOL mass spectrometer. Samples were separated by thin-layer chromatography (TLC) on pre-coated silica-gel 60 F_{254} and cellulose F plates (Merck Darmstadt Germany, 0.25, 0.1 mm, respectively), and spots were detected by spraying with 10% H_2SO_4 or aniline hydrogen phthalate (AHP) and then heating at 100 °C. Carbohydrate concentrations were determined using a phenol–H_2SO_4 method.[4)]

Structural Analysis of a Neutral Polysaccharide from *Antrodia cinnamomea*

Extraction and Fractionation of Polysaccharides Freeze-dried mycelia of *A. cinnamomea* (1.5 kg) were extracted three times with 4 l of $CHCl_3$ at room temperature for 1 d, then combined extracts filtered and evaporated *in vacuo* to yield a $CHCl_3$ extract. The residue was dried and dipped into H_2O (20 volumes) at room temperature for 1 h and extracted for 2 h 3 times at 100 °C. The combined hot water extracts were concentrated to 800 and 3200 ml of EtOH was added. The mixture was stirred and left overnight in a refrigerator. The precipitates were filtered, washed with cold EtOH, dried and suspended in 10% trichloroacetic acid (TCA). The TCA-soluble fraction obtained by centrifugation (2500×***g*** for 10 min) was dialyzed against distilled water for 3 d. The nondialyzable portion was lyophilized *in vacuo* to yield 14.25 g of a brownish residue (*AC*).

Ion-Exchange Column Chromatography of *AC* *AC* (100 mg) dissolved in H_2O was eluted through a column (Whatman International Ltd., Maidstone, England. 2.0×20 cm) containing DE-52 with 60 ml of H_2O, 60 ml of 0.5 M NaCl, 60 ml of 1 M NaCl, 60 ml of 2 M NaCl, and 2 ml fractions were collected. The H_2O fraction (*ACN*) was concentrated and lyophilized (yield, 68.3 mg).

Gel Filtration of *ACN* *ACN* (68.3 mg) was dissolved in 0.2 M NaCl and eluted with the same solution through a column containing Toyopearl HW-65 (Tosoh Corp., Tokyo, Japan. 2.0×90 cm) and 5 ml fractions were collected. The fractions were separated into two portions (*ACN1*, *ACN2*; yield, 19, 49 mg, respectively) according to the elution profile determined from phenol–H_2SO_4 monitoring at 480 nm. *ACN2* was further purified by column chromatography on HW-65 under the same conditions as described above. A colorless polysaccharide was obtained (*ACN2a*; yield, 41 mg).

Estimation of Molecular Weight The average molecular weight of the polysaccharide (*ACN2a*) was estimated using HPLC. The sample was eluted through a TSK-$GMPW_{XL}$ gel filtration column (7.8×300 mm i.d., Tosoh Corp., Tokyo, Japan) with 0.2 M NaCl at a rate of 1 ml/min. Commercially available pullulans (Shodex Standard P-82) were the standard molecular markers.

Identification of Component Sugars The polysaccharide (2 mg) was dissolved in 2 ml of 2 N trifluoroacetic acid (TFA) and sealed. After hydrolysis for 1 h at 125 °C in a steam autoclave,[5)] TFA was eliminated from the reaction mixture by evaporation and then hydrolysates were reduced with $NaBH_4$ (6 mg) and trimethylsilylated with silblender-HTP (hexamethyldisilazane : trimethylchlorosilane : pyridine = 2 : 1 : 10) for GC-MS analysis (column, DB-1, J & W Scientific, 0.25 mm i.d.×30 m; column temperature, increased from 50—190 °C in increments of 5 °C/min; then maintained at

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190 °C for 12 min).

Determination of Absolute Configuration of Component Sugars The absolute configuration of component sugars was determined as described by Hara *et al.*[6] Polysaccharide (1 mg) was hydrolyzed in 2 N trifluoroacetic acid (TFA, 3 ml) at 125 °C for 1 h then TFA was removed by evaporation to yield a sugar fraction. Pyridine solutions (0.5 ml) of this fraction (2 mg) and L-cysteine methyl ester hydrochloride (3 mg) were mixed and warmed at 60 °C for 1.5 h, then dried by flashing with N_2. The dried sample was trimethylsilylated with silblender-HTP (0.4 ml) at 60 °C for 1 h. After partitioning between $CHCl_3$ (3 ml) and H_2O (3 ml), the $CHCl_3$ extract was analyzed by GC-MS (column, DB-wax, J & W Scientific, 30 m×0.25 mm; column temperature increased from 50—230 °C in increments of 10 °C/min, then maintained at 230 °C for 12 min).

Methylation Analysis The polysaccharide (5 mg) was methylated with methyl iodide according to the Anumula and Taylor method.[7] Methylated polysaccharides were hydrolyzed with 4 N trifluoroacetic acid (TFA, 8 ml) for 90 min at 125 °C in an autoclave. After TFA was removed by evaporation, the hydrolysates were converted to alditol acetates with 1 M NH_4OH containing 3 mg/ml $NaBH_4$, followed by acetylation. The partially methylated alditol acetates were analyzed by GC and GC-MS (column, SP-2330, Supelco, Bellefnte, PA, 60 m×0.25 mm, 0.20 μm film thickness. Helium was the carrier gas and the column temperature was increased from 160 to 210 °C in increments of 2 °C/min, then increased from 210 to 240 °C in increments of 5 °C/min and finally maintained at 240 °C for 14 min. Peak areas were corrected using the published molar response factors.[8] The derivatized compounds were identified by comparing their relative retention times to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol and their GC-EI-MS fragmentation patterns.

Protective Effect of a Neutral Polysaccharide (*ACN2a*) against *P. acnes*–LPS Induced Hepatotoxicity

Preparation of *P. acnes* We cultured *P. acnes* (ATCC 6919) with brain heart infusion medium (Wako Pure Chemical Industries, Ltd., Osaka, Japan), containing L-cysteine (0.03%) and Tween 80 (0.03%) under anaerobic conditions for 48 h at 37 °C. The culture was centrifuged at 5500×***g*** for 15 min at 4 °C and then washed with phosphate-buffered saline (PBS). The bacterial pellet was resuspended in 300 ml PBS and the cells were killed by heating at 80 °C for 30 min, then lyophilized to prepare a heat-killed *P. acnes* powder. Lipopolysaccharide from *Escherichia coli* 055:B5 was purchased from Sigma-Aldrich, Inc. (Steinheim, Germany). FK506 (tacrolimus hydrate) was provided by Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan).

Animals Four-week-old male ICR mice (SLC, Hamamatu, Japan) weighing 18—20 g were acclimatized for one week before being used to examine the protective effect of *ACN2a* against hepatotoxicity induced by *P. acnes*–LPS.[9,10]

Hepatoprotective Experiment The hepatoprotective activity of *ACN2a* was investigated using (1) normal control mice (untreated); (2) *P. acnes*–LPS treated controls; (3) *ACN2a* 0.2 g/kg/d (*p.o.*), (4) *ACN2a* 0.4 g/kg/d, (5) *ACN2a* 0.8 g/kg/d plus *P. acnes*–LPS and (6) FK506 (1 mg/kg *p.o.*) plus *P. acnes*–LPS.

Heat-killed *P. acnes* cells suspended in saline were injected *via* the tail vein at a dose of 0.15 mg/mouse. Seven days later, acute liver damage was induced by an intravenous injection of LPS at a dose of 0.05 μg/mouse. *ACN2a* was applied once daily through a gastric tube to the animals for 7 consecutive days. On the 8th day, LPS was injected 1 h after *ACN2a* was applied. FK506 served as a positive control drug[11] and was administered through a gastric tube 48, 36, 24, 12 and 1 h before the intravenous injection of LPS. Blood samples were collected into tubes for analysis of liver injury 6 h after LPS injection, and then these animals were sacrificed. The tubes were centrifuged at 3500×***g*** for 15 min and the sera were stored at −20 °C until ALT and AST enzyme activities, which are markers of hepatocyte injury, were determined using kits (transaminase CII-test Wako; Wako Pure Chemical Industries, Ltd. Osaka, Japan).

Results and Discussion

Structural Analysis of *ACN2a* In the course of our studies on the hepatoprotective effects of an *A. cinnamomea* mycelium extract, we found that both chloroform and water extracts had potent inhibitory effect on hepatic injury in mice.[12] Since the chloroform extract was rather toxic in mice, we investigated the water extract to find hepatoprotective substances.

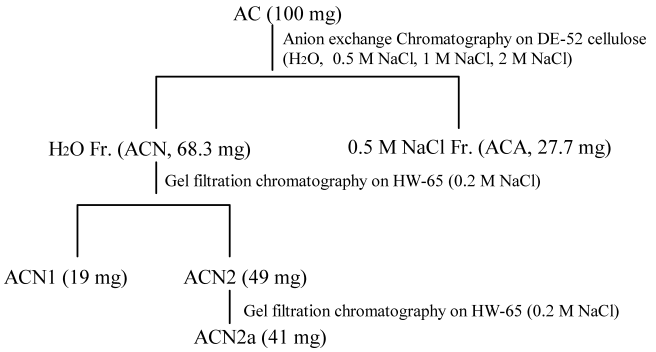

Chart 1. Fractionation of Hot Water Extract from *A. cinnamomea*

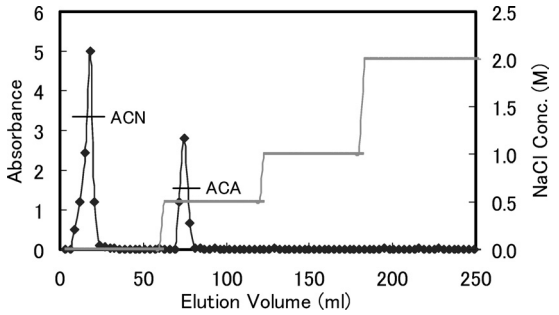

Fig. 1. Elution of Crude Polysaccharide Fraction from *Antrodia cinnamomea* mycelia by Ion Exchange Column Chromatography on DE-52

Polysaccharide was detected using phenol–H_2SO_4.

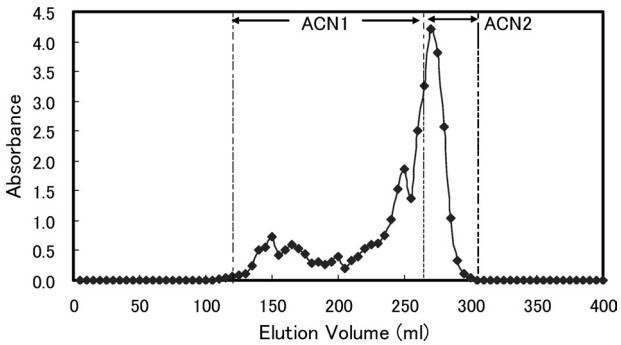

Fig. 2. Elution of *ACN* by Gel Filtration Column Chromatography on HW-65

Polysaccharide was detected using phenol–H_2SO_4.

Chart 1 shows hot water fractionation of the *A. cinnamomea* mycelium extract. The non-dialyzable portion (*AC*) of the 10% TCA-soluble fraction had hepatoprotective activity and contained polysaccharides that were positive for the phenol–H_2SO_4 reaction. Figure 1 shows *AC* fractionation by ion-exchange column chromatography on DE-52 cellulose. The most potent hepatoprotective fraction (*ACN*) was then separated by gel filtration on HW-65 to yield *ACN1* and *ACN2* (Fig. 2), the latter of which was further purified by gel filtration on HW-65 to yield a colorless polysaccharide (*ACN2a*) with hepatoprotective properties. *ACN2a* migrated as a single fraction on HPLC, at an apparent molecular weight of 12.9×10^5 Daltons. The polysaccharide had the appearance of cotton, and the following properties: $[\alpha]_D=+115.0°$ ($c=0.44$, H_2O); intrinsic viscosity $[\eta]=0.0417\ dl\cdot g^{-1}$ (measured with an Ostwald viscometer); specific heat *Cp*=

Table 1. Methylated Sugar Analysis

Methylated sugar	Molar ratio	t_R	MS main fragments (m/z)	Linkage type
2,3,4-tri-Me-Fuc	0.209	0.789	71, 89, 101, 117, 131, 161, 175	Fuc-(1→
2,3,4,6-tetra-Me-Glc	0.084	1	71, 87, 101, 117, 129, 145, 161, 205	Glc-(1→
2,4,6-tri-Me-Glc	0.026	1.31	71, 87, 101, 117, 129, 161, 233	→3)-Glc-(1→
2,3,6-tri-Me-Glc	0.157	1.489	87, 99, 101, 113, 117, 233	→4)-Glc-(1→
2,3,4-tri-Me-Gal	1	1.6	71, 87, 101, 117, 129, 161, 189	→6)-Gal-(1→
3,4-di-Me-Gal	0.276	1.881	87, 99, 129, 189	→2,6)-Gal-(1→

t_R is retention time of each component, relative to that of 1,5-*O*-acetyl-2,3,4,6-tetra-Me-Glc.

0.2663 cal/(g·°C) [measured by a differential scanning calorimeter (DSC)]. The protein content of *ACN2a* was 0.20% (measured by Bradford method[13]) and that of nitrogen was 0.12% (according to an elemental analysis). Sulfate was undetectable in *ACN2a* (measured using a barium rhodizonate method[14] and IR).

Component sugar analysis showed that the polysaccharide consisted of galactose, glucose, fucose, mannose and galactosamine (1 : 0.24 : 0.07 : 0.026 : faint), indicating that 74.9% of the sugar was galactose.

The optical rotational value of *ACN2a*, 115.0° (c=0.44, H_2O), suggested that the component sugars had an α-D- or β-L-configuration due to the isorotation rule of Hudson.[15—17] The absolute configurations of the respective sugars were L-fucose, D-galactose, D-glucose and D-mannose according to the method reported by Hara *et al.*[6]

Three characteristic absorption bands at 1153, 1080 and 1034 cm^{-1} (Furanoid forms have only two absorption bands in this region) of the FT-IR spectrum suggested the presence of pyranoid forms of sugars.[18] An absorption band at 918 cm^{-1} suggested a D-glucopyranose unit. In addition, the absorption band at 874 cm^{-1} was characteristic of mannopyranoid and galactopyranoid.[19] An NH_2 absorption band at 1637 cm^{-1} suggested aminosugars, which was also supported by the elemental analysis.

Anomeric proton signals located at a region more than δ 4.80 (δ 4.89, δ 4.91, δ 4.96) in the ^{1}H-NMR spectrum, suggested that the component sugars had an α-D-configuration. This agrees with the optical rotation estimation. Since anomeric proton signals were also observed at a region less than δ 4.80 (δ 4.74, δ 4.66), the component sugars might include some with a β-L-configuration.[20,21] A methyl proton signal was observed at δ 1.13, which was assigned to methyl protons of the fucose residue. An anomeric proton signal of this sugar was detected at less than δ 5.0 as a singlet. These findings suggested that the fucose residue had the β-L-configuration (an anomeric proton signal of α-L-fucose is observed at more than δ 5.0).[20,22]

C-4 and C-5 signals were observed in the ^{13}C-NMR spectrum at less than δ 80. This finding suggested that component sugars possessed pyranoid forms (the chemical shifts of C-4 and C-5 for furanose forms are evident in the region δ 80—85).[20,21,23] This finding agrees with the estimation from the IR spectral data. In addition, a signal was observed at δ 13.7, which was assigned to a methyl carbon of the fucose residue, suggesting that the residue was L-fucose (the C-6 signal of D-fucose appears at δ 60—65).[20] This agrees with the conclusion obtained from the ^{1}H-NMR spectra.

Methylation analysis showed that *ACN2a* was composed of terminal-fucose, 1,4-linked glucose, 1,6-linked and 1,2,6-linked galactose residues, and some terminal and 1,3-linked glucose residues (Table 1). Furthermore, 72.8% of the *ACN2a* backbone was composed of α-D-1,6-Gal (α-D-1,6-, α-D-1,2,6-), The ratio of branch points was approximately 15.8% of the total, and branches were attached to the C-2 of the galactosyl residues in the main chain.

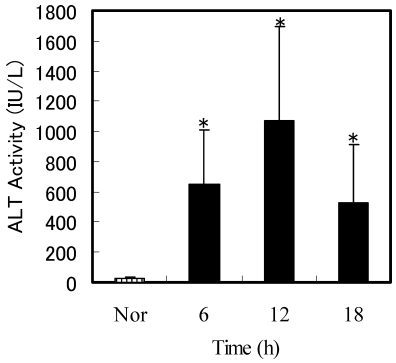

Fig. 3. Serum ALT Levels after LPS Injection

Nor, normal control; blood samples collected at 6, 12 and 18 h after intravenous injection of LPS. Results represent means±S.D. of values obtained from 10 mice per group. *p<0.05 compared with corresponding normal controls determined by Student's t-test.

Recently, Cheng *et al.* reported the isolation of anti-angiogenic polysaccharides from the *A. cinnamomea* mycelium,[24] but their preparations seem to be a mixture of high, medium and low molecular weight polysaccharides, and the compositions of sugars differ from that of *ACN2a*.

Hepatoprotective Effect Hepatitis is widespread, especially in developing countries, yet specific medicines for the treatment of this disease have not been discovered. Traditional medicine has recently received considerable focus in efforts to develop new drugs for hepatitis. Compounds that can either decrease the necrotic damage to hepatocytes *via* enhanced defense mechanisms against toxic insult or improve the repair of damaged hepatocytes are considered potentially useful in the treatment of human hepatitis.[25]

An injection of *P. acnes* followed by LPS into mice provides a useful experimental model of acute hepatic damage, but most animals die from severe liver injury within 24 h of the LPS injection.[26] We found that the optimal experimental doses of *P. acnes* and LPS were 0.15 mg and 0.05 μg/mouse, respectively. All of animals survived, and liver injury was the most severe 12 h after the intravenous injection of LPS (Figs. 3, 4). We therefore collected blood samples for liver injury analysis 6 h after LPS injection.

Figures 5 and 6 show the effect of *ACN2a* on the ALT and AST levels in serum of mice treated with *P. acnes*–LPS. The acute hepatotoxicity reaction was significantly (p<0.05) and dose-dependently suppressed in all of the animals that were

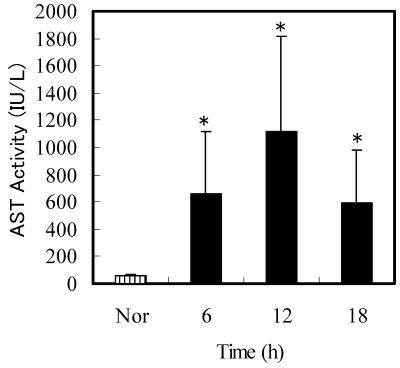

Fig. 4. Serum AST Levels after LPS Injection

Nor, normal control; blood samples collected at 6, 12 and 18 h after intravenous injection of LPS. Results represent means±S.D. of values obtained from 10 mice per group. $*p<0.05$ compared with corresponding normal controls determined by Student's *t*-test.

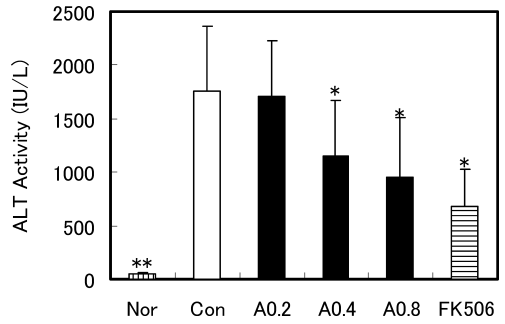

Fig. 5. Effect of *ACN2a* on Serum ALT Levels in ICR Mice with *P. acnes*–LPS Induced Liver Injury

Nor, normal control; Con, *P. acnes*–LPS; A0.8, *ACN2a* 0.8 g/kg+*P. acnes*–LPS; A0.4, *ACN2a* 0.4 g/kg+*P. acnes*–LPS; A0.2, *ACN2a* 0.2 g/kg+*P. acnes*–LPS; FK506, FK506 1 mg/kg+*P. acnes*–LPS. Results represent means±S.D. of values obtained from 10 mice per group. $*p<0.05$ and $**p<0.001$ compared with corresponding *P. acnes*–LPS controls determined by Student's *t*-test.

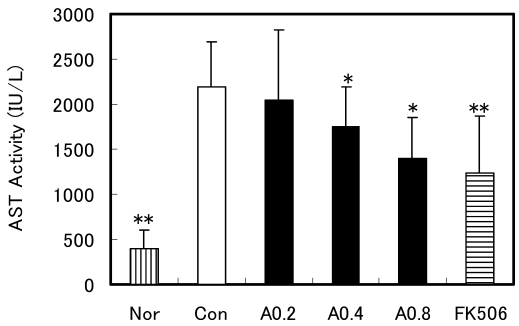

Fig. 6. Effect of *ACN2a* on Serum AST Levels in ICR Mice with *P. acnes*–LPS Induced Liver Injury

Nor, normal control; Con, *P. acnes*–LPS; A0.8, *ACN2a* 0.8 g/kg+*P. acnes*–LPS; A0.4, *ACN2a* 0.4 g/kg+*P. acnes*–LPS; A0.2, *ACN2a* 0.2 g/kg+*P. acnes*–LPS; FK506, FK506 1 mg/kg+*P. acnes*–LPS. Results represent means±S.D. of values obtained from 10 mice per group. $*p<0.05$ and $**p<0.001$ compared with corresponding *P. acnes*–LPS controls determined by Student's *t*-test.

pretreated with 0.4 and 0.8 g/kg of body weight of *ACN2a*, indicating that *ACN2a* had protective effect on *P. acnes*–LPS induced hepatic toxicity in mice.

Chart 2 shows the mechanism of the experimental model induced by *P. acnes*–LPS. The injection of *P. acnes* into mice *via* the tail vein results in monocytic infiltration of the liver, thus increasing the number of hepatic macrophages, which subsequently are activated by the intravenous injection of

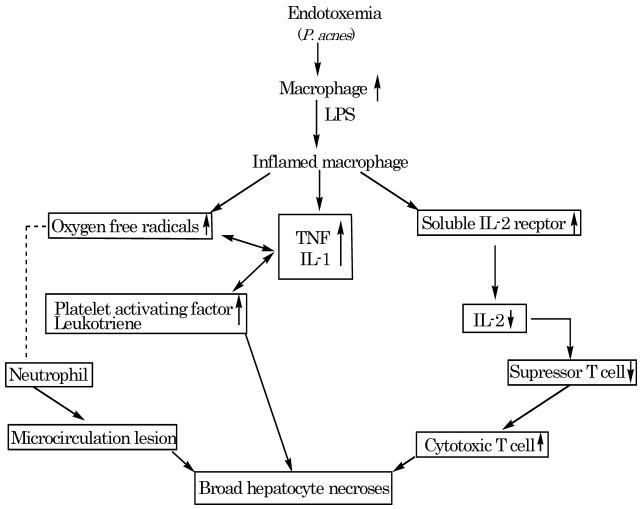

Chart 2. Possible Mechanism of *P. acnes*–LPS Induced Hepatic Toxicity

LPS. Cytokines such as tumor necrosis factor (TNF), IL-1, soluble IL-2 receptor are released from the hepatic macrophages and levels increase in the surrounding tissues.[26,27] The liver is then damaged in three ways. Tumor necrosis factor and IL-1 broadly necrotize hepatocytes *via* platelet activating factor (PAF) and leukotrienes,[28] and *via* neutrophil and microcirculation lesions, in which oxygen free radicals play a major role.[29,30] Interleukin-2 levels decrease because of binding to soluble IL-2 receptors, which results in decreasing suppressor T cells and increasing cytotoxic T cells (CTL)[31] that broadly necrotize hepatocytes.

The water extract of *A. cinnamomea* mycelia can scavenge oxygen free radicals and increase levels of IL-2.[32] We found that both crude and neutral (*ACN2a*) polysaccharides had protective effect against *P. acnes*–LPS induced hepatic toxicity in mice. *ACN2a* from *A. cinnamomea* might partly exert its hepatoprotective activity by scavenging oxygen free radicals, which would obstruct the second pathway of *P. acnes*–LPS induced hepatic toxicity, or increase levels of IL-2, resulting in decreased CTL and liver protection.

We are the first to structurally characterize a neutral polysaccharide from the mycelium of *A. cinnamomea* that has hepatoprotective activity. The backbone was composed of α-D-1,6-Gal, which comprised about 73% of the molecule. Further studies on the relationship between α-D-1,6-Gal and hepatoprotective activity are underway at our laboratory.

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