LIGNIFIED NATURAL PRODUCTS AS POTENTIAL MEDICINAL RESOURCES. I POTENTIATION OF HEMOLYTIC PLAQUE-FORMING CELL PRODUCTION IN MICE

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We have found that water-soluble extracts of lignified natural products are strong potentiaters of hemolytic plaque-forming cell production in mice. Apparently, the active components involved belong to a lignin family. Consequently, study of medicinal potential, especially immunopotentiating capacity, of lignified materials is important.

KEYWORDS lignified material; lignin; pine cone; plaque-forming cell

Before 1980, little attention was paid to the biological activity of lignified materials, one of the commonest families of natural products in the plant kingdom. Few papers and patents on antiviral and antitumor activities had appeared.[1] By 1989, reports began to appear of the anti-HIV (human immunodeficiency virus) activity of water soluble lignin in a culture medium of Lentinus edodes mycelia,[2] and lignin sulfonate in waste from the pulp industry.[3] Since 1987, in a series of studies of pine cone (PC) extracts, we reported that non-dialyzable high-molecular weight fractions of hot water and alkaline extracts from pine cones had various kinds of biological activity: anti-HIV,[4] anti-herpes simplex virus,[5] anti-influenza virus,[6] antitumor,[7] and antimicrobial.[8] In more mechanistic studies, we found that PC extracts promote the production of macrophage differentiation-inducing factor(s),[7] granulocytic cell iodination,[9] and splenocyte proliferation.[10] Spectral analyses of the bioactive fractions revealed that each fraction contained, mainly, a lignin-related structure.[5,6,11] This prompted further studies, beyond the PC research, of the biological activity of water-soluble, high-molecular weight natural lignified materials. In the present study, lignified materials obtained by successive extractions with hot water and 4% NaOH were assayed for their activity in stimulating the production of hemolytic plaque-forming cells (PFC) in mice. The materials examined included three kinds of wood chips from the coniferous slash pine (Pinus caribaea) and douglas fir (Pinus douglasii), and from tallow wood (broadleaved) and two Basidium parasites (lignified shelf fungi), in addition to the bioactive fractions from the pine cone.[7] PSK, an anticancer protein-bound polysaccharide from Coriolus versicolor,[12] was tested as a positive control.

Materials Wood chips of slash pine (SP), douglas fir (DF), and tallow wood (TW) were the gifts of Mr. Hiroshi Nakatsuka, Head of Seishi-kogyo Shikenjo, Shizuoka prefecture. Two lignified shelf fungi, Ganoderma lucidum Karst. (reishi, GL) and Ume tree parasite (baikisei, UP) were from stocks of the Institute of Cancer Research, Kanebo Co. Each of these lignifed materials (100 g) was ground into small pieces and extracted for three 20-hr periods with 1.0 l methanol each. The residue was extracted with ethanol in the same way

Table I. Production of Hemolytic Plaque-Forming Cells (PFC) in Spleen of CDF_1 Mice Pretreated with Extracts from Lignified Materials and Immunized with Sheep Red Blood Cells (% Ratio vs. Control)

Fraction	PFC/spleen (PFC/1.6 x 10^5cells)		
	5 mg/kg	10 mg/kg	20 mg/kg
SP(H_2O)	**220**±42 (144±21)	**278**±44 (163±21)	**407**±19 (**212**±19)
Before dialyzed	118±19 (116±17)	169±34 (123±21)	**230**±10 (184±20)
SP(4%NaOH)		127±14 (124±9)	137±37 (109±26)
DF(H_2O)	83±5 (65±4)	98±12 (71±7)	139±16 (92±9)
DF(4%NaOH)		97±14 (96±19)	124±29 (106±20)
TW(H_2O)	94±14 (79±10)	130±10 (113±17)	101±7 (115±2)
TW(4%NaOH)		109±19 (107±16)	101±39 (90±28)
GL(H_2O)	82±12 (74±7)	92±10 (75±10)	121±12 (92±12)
GL(4%NaOH)		190±34 (159±26)	152±46 (144±59)
UP(H_2O)	123±11 (104±13)	181±12 (112±5)	197±16 (117±12)
UP(4%NaOH)		164±42 (143±28)	113±13 (115±10)
PC(H_2O)		196±52 (172±55)	**297**±102 (193±62)
Fr.V	**299**±96 (**393**±127)	**223**±57 (**333**±89)	**403**±125 (**554**±144)
PC(4%NaOH)			
Fr.VI	76±16 (83±23)	106±23 (139±33)	115±68 (115±60)
Fr.VI(HCl-Diox)		160±47 (154±39)	**222**±33 (**242**±18)
Alkali lignin	101±17 (71±13)	134±16 (105±14)	106±10 (102±11)
PSK	110±17 (86±13)	119±16 (103±15)	156±22 (140±18)
Control	100 (100)	100 (100)	100 (100)

Abbreviations of the fractions are given in **Materials**. Values over 200% PFC of the control are underlined bold-faced. The standard error follows each value. The observed PFC's for the control slightly varied in every experimental runs; 24.0±5.3/160,000 spleen cells and 54,000±13,000/spleen in one control experiment.

as the methanol extraction. The residue was then extracted three times with 1.0 l of hot water for 3 h each time. The hot-water extracts were dialyzed against running water for 12 h, and then lyophilized: SP(H_2O), 1.4 g; DF(H_2O), 1.8 g; TW(H_2O), 2.9 g; GL(H_2O), 2.5 g; and UP(H_2O), 1.1 g. The residues from the hot-water extraction were extracted three times with 1.0 l of 4% NaOH at room temperature for 20 h, and dialyzed against running water for 12 h, then lyophilized: SP(4%NaOH), 1.2 g; DF(4%NaOH), 2.6 g; TW(4%NaOH), 4.8 g; GL(4%NaOH), 9.8 g; UP(4%NaOH), 14.5 g. Fr. V of the hot water extract of pine cones (PC) of Pinus parviflora Sieb. et Zucc. was purified by absorption on a DEAE cellulose column, then eluted with 0.15 M NaOH.[7] Fr. VI of PC(4%NaOH) was a 4% NaOH extract of the pine cone, followed by acid-precipitation.[7] Commercial alkali lignin was purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo), and PSK was supplied by Kureha Chem. Co. (Tokyo).

Hemolytic Plaque-Forming Cell (PFC) Production CDF_1 mice (6-week-old male) were injected i.p. (5 in a group) with an extract dissolved in 0.2 ml saline daily from day 1 to day 5. On day 3, 5 x 10^7 sheep red blood cells (SRBC) were injected i.v. into each test mouse. The mice were sacrificed on day 7, the spleens were extirpated, and the spleen cells were prepared as a suspension at a concentration of 2 x 10^6 cells/ml of Eagle's MEM. The assay mixture consisted of 0.4 ml of the above spleen cell suspension, 0.05 ml of packed SRBC-Eagle's MEM (1:1), and 0.05 ml of guinea pig serum. PFC was assayed with 0.1 ml of the assay mixture in a Cunningham chamber by 1 h incubation at 37°C.[13] The results are

shown in Table I. Among the lignified materials examined in the present study, the hot water extract of slash pine, SP(H_2O), noticeably stimulated the PFC production as effectively as Frs. V and VI of the pine cone. The PFC potentiating ability of SP(H_2O) was far superior to that of PSK which is known as an enhancer of PFC production and a potent antitumor agent.[12)]

Molecular Species of Active Principle All the extracts examined here were non-dialyzable. The hot water extract of slash pine, SP(H_2O), was more effective than the undialyzed sample, indicating that the dialyzed low-molecular components may be ineffective. IR and UV spectral analyses indicated that all extracts examined were mainly complexes of benzenoid units and sugar moieties with attached COOH groups. All extracts gave an ESR signal at g=2.003, indicating that a polyphenolic structure may be involved in the molecules. So the extracts may involve lignin structures conjugated with hemicelluloses, and also, possibly, hemicelluloses including uronic acids. Note that dioxane-soluble Fr.VI(HCl-Diox), which is considered to be a typical lignin, was effective in promoting PFC production. This fraction is not regarded as a single species of hemicellulose. Therefore, it is probable that a certain lignin family of high-molecular weight substances may be responsible for potentiating PFC production, i.e., immune response in host animals, although the possibility is not completely eliminated that a trace of contaminant(s) may be responsible for the biological activity concerned. In conclusion, lignified natural products, most of which are not yet analyzed, are definitely worth attention as promising medicinal resources.

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