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Studies on the Water-soluble Constituents of Lichens. IV.[1] Effect of Antitumor Lichen-glucans and Related Derivatives on the Phagocytic Activity of the Reticuloendothelial System in Mice[2]

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Two representative preparations of antitumor lichen-polysaccharides, that is, GE-3, a partially *O*-acetylated pustulan-type β-D-glucan isolated from *Gyrophora esculenta* Miyoshi, and UR-1-1, a lichenan-like β-D-glucan obtained from *Usnea rubescens* Stirt., were tested to examine their effects on the phagocytic response of the reticuloendothelial system (RES) in mice. The phagocytic activity was evaluated by measuring the vascular clearance of colloidal carbon 24 h after a single intraperitoneal administration of a test sample at a dose of 100, 50, or 25 mg/kg. At the dose of 100 mg/kg, GE-3 exerted a marked RES-stimulating effect, which was comparable with that given by 50 mg/kg of zymosan: the effect appeared to decrease with decreasing dose. On the other hand, UR-1-1 failed to enhance the phagocytic activity at any of the three doses tested. In addition, it appeared that introduction of either *O*-lauroyl or *O*-carboxymethyl functions into the GE-3 molecule caused considerable loss of activity.

Keywords——antitumor polysaccharide; lichen; *Gyrophora esculenta* Miyoshi; *Usnea rubescens* Stirt.; lichenan; pustulan; phagocytic response; reticuloendothelial system; carbon clearance test

Various lichen-polysaccharides have been reported to exhibit a non-cytotoxic, host-mediated antitumor effect against sarcoma 180 implanted in mice, when they were administered intraperitoneally (*i.p.*).[3] Among them, the following two types of linear polysaccharides were especially effective: partially *O*-acetylated pustulan-type β-D-glucan consisting exclusively of (1→6)-glycosidic linkages[3a-d] and lichenan-type β-D-glucan containing (1→3)- and (1→4)-linkages in the ratio of *ca.* 3: 7.[3a,d] A number of antitumor polysaccharides have also been isolated from other natural sources.[4,5] Although the exact mechanism involved in the antitumor action of polysaccharide is not yet clear, there are several lines of evidence to indicate that the activity may be due to potentiation of certain immune responses in the host.[5,6] In this regard, it is of interest that zymosan (an insoluble yeast cell-wall fraction) and the glucan component thereof have been shown to enhance markedly the phagocytic activity of the reticuloendothelial system (RES),[7] whereas, according to Chihara, lentinan and CM-pachymaran (antitumor glucans of fungal origin) were devoid of such an ability.[5,6a,b]

This work was undertaken to examine the RES-stimulating effects of the two representative antitumor lichen-glucans mentioned above. Preparations employed as test samples were GE-3 (acetylated pustulan-type glucan)[3a,b] and UR-1-1 (lichenan-type glucan),[3d] which had been obtained from *Gyrophora esculenta* Miyoshi and *Usnea rubescens* Stirt., respectively. In addition, three *O*-lauroylated derivatives of GE-3, that is, GE-3-L lots 3, 4, and 7,[1] having lauroyl contents of 3.3, 12.0, and 42.2%, respectively, and an *O*-carboxymethyl derivative of GE-3, that is, CM-GE-3,[1] with a degree of substitution (DS) of 0.85 were also adopted as test samples.

The phagocytic activity was evaluated in the carbon clearance test by the conventional method of Biozzi *et al.*[8] The sample to be tested was dissolved or suspended in physiological

saline and administered to ddY mice by a single *i.p.* injection at a dose of 100, 50, or 25 mg/kg: the mice of the control group received saline alone. In each experiment, zymosan was used as a positive reference agent at the dose of 50 mg/kg. The vascular clearance of colloidal carbon was measured 24 h after the treatment to give the phagocytic index, K. The result was also expressed as the relative K-value, which was calculated based on the formula given in "Experimental," to indicate the effect relative to that caused by zymosan.

Experimental

Materials——All the preparations tested were stocked materials which had been produced in our previous studies. Before use, they were confirmed to be free from endotoxins by the IXth J.P. Pyrogen Test. Zymosan ("Zymosan A" from *Saccharomyces cerevisiae*) was purchased from Sigma Chemical Co.

Method——The carbon clearance test was performed essentially as described by Biozzi *et al.*[8] For preparation of the carbon suspension, Pelikan C11/1431a ink (Günther Wagner, Hanover, Germany) was centrifuged at 3000 rpm for 15 min to remove the large aggregates and the resultant supernatant was diluted with saline to bring the carbon concentration to 16 mg/ml. Each test sample was dissolved (or suspended) in sterile, physiological saline and injected *i.p.* into five-week-old ddY mice (ten per group), weighing 20 to 25 g, at a dose of 100, 50, or 25 mg/kg. One group of mice was administered *i.p.* 50 mg/kg of zymosan. The mice of the control group received saline alone at the dose of 0.5 ml/mouse. After 24 h, the carbon suspension was injected into the mice *via* the tail vein at a dose of 16 mg per 100 g body weight. Blood (0.02 ml/mouse) was drawn with a capillary from the retro-orbital venous plexus at 4 min intervals after the carbon injection, and hemolyzed immediately by adding 3 ml of 0.1% Na_2CO_3 solution. The optical density of the hemolyzed blood sample was then read at 600 nm. The phagocytic index K and the relative K-value were calculated by means of the following equations, respectively: $K=(\log C_1-\log C_2)/(T_2-T_1)$ where C_1 and C_2 are the carbon concentrations at times T_1 and T_2, respectively; Relative K-value$=(K_{\text{sample}}-K_{\text{saline}})/(K_{\text{zymosan}}-K_{\text{saline}})\times 100$.

Results and Discussion

All the results obtained in the carbon clearance test are shown in Table I.

TABLE I. The Effects of Lichen-glucans and Related Derivatives on Carbon Clearance Activity

Test sample	Dose (mg/kg)	Phagocytic index (K) (mean ± S.E.)[a]	Relative K-value[b]
Exptl. 1			
GE-3 (acetylated pustulan-type glucan)	100	0.1684±0.0044	86.8
	50	0.1552±0.0088	70.5
	25	0.1291±0.0081	38.2
Zymosan	50	0.1790±0.0049	100.0
Control (saline)		0.0982±0.0057	
Exptl. 2			
UR-1-1 (lichenan-type glucan)	100	0.1038±0.0067	0.4
	50	0.0955±0.0042	−12.4
	25	0.0772±0.0085	−40.9
GE-3-L, lot 3 (lauroyl content, 3.3%)	100	0.1329±0.0068	45.7
	50	0.1343±0.0085	47.9
	25	0.1060±0.0068	3.8
GE-3-L, lot 4 (lauroyl content, 12.0%)	100	0.1231±0.0098	30.4
	50	0.1127±0.0061	14.3
	25	0.0908±0.0026	−19.7
GE-3-L, lot 7 (lauroyl content, 42.2%)	100	0.1305±0.0080	41.9
	50	0.0996±0.0067	−6.0
	25	0.1039±0.0038	0.6
CM-GE-3 (DS 0.85)	100	0.1031±0.0049	−0.6
	50	0.1161±0.0061	20.3
	25	0.0930±0.0043	−16.3
Zymosan	50	0.1678±0.0062	100.0
Control (saline)		0.1035±0.0049	

a) $p<0.001$.
b) The value was calculated based on the equation given in "Experimental."

A marked increase in the phagocytic response was demonstrated to occur following single *i.p.* administration of GE-3 at the dose of 100 mg/kg, the resulting *K*-value being comparable to that given by 50 mg/kg of zymosan. The effect of GE-3 appeared to decrease with decreasing dose. In contrast, UR-1-1 did not stimulate the phagocytic activity at any of the three doses tested; it tended to act rather suppressively. The results suggest that the acetylated pustulan-type glucans, which are found commonly and exclusively in Umbilicariaceae lichens,[3a,d,9] exert an RES-stimulating effect, while the lichenan-type glucans, which are present widely in various species of lichens,[3a,d,9] do not possess such an ability.

Since bacterial lipopolysaccharides (endotoxins) are well known as potential RES-stimulants,[7a,10] we presumed that an enhanced phagocytic activity might be gained by introducing lipophilic moieties into the GE-3 molecule. In order to test this possibility, three lots of GE-3 laurates (GE-3-L lots 3, 4, and 7) having various lauroyl contents were used as test samples in this work. However, none of them showed improved activity; on the contrary, there was a considerable loss of activity, regardless of the degree of substitution.

Although GE-3 is readily soluble in hot water, it is practically insoluble in cold water. In the hope of obtaining an RES-stimulant with higher water-solubility, a carboxymethyl derivative of GE-3 (CM-GE-3) was tested, but it proved to be ineffective.

The ineffectiveness of UR-1-1 in the present bioassay suggested that the antitumor activity of the lichenan-type glucan could not be explained in terms of stimulation of the RES, and that, in this sense, the glucan resembles lentinan and CM-pachymaran.[5,6a,b] Hitherto, various chemical and biochemical agents have been reported to possess both antitumor and RES-stimulatory activities: animals treated with them show increased rejection of tumor grafts, resistance to oncogenic viruses, reduction of metastases, and regression of established tumors.[5,11] Examples of such agents among polysaccharides are yeast-glucan,[7] dextran,[12] and lipopolysaccharides.[7a,10] We now propose that the partially *O*-acetylated pustulan-type glucan of *G. esculenta* represents a new example of an antitumor polysaccharide with RES-stimulating effect; it appears probable that analogous polysaccharides occurring in related lichens may provide additional examples.[3b-d] The exact mechanism by which the RES-stimulants reported previously increase resistance to tumors is still uncertain.[5] Although many studies by earlier workers have stressed that the macrophage, an integral component of the RES, plays an important role in defense against neoplastic disease,[11] there are some indications to the contrary.[13] At this time, we are unable to correlate the RES-stimulating effect of the acetylated pustulan with its antitumor activity, and further studies are necessary.

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References and Notes

1) Part III: Y. Nishikawa, K. Yoshimoto, R. Horiuchi, K. Michishita, M. Okabe, and F. Fukuoka, *Chem. Pharm. Bull.*, **27**, 2065 (1979).
2) Presented at the 54th Annual Meeting of the Japanese Biochemical Society, Sendai, September, 1981.
3) *a*) F. Fukuoka, M. Nakanishi, S. Shibata, Y. Nishikawa, T. Takeda, and M. Tanaka, *Gann*, **59**, 421 (1968); *b*) Y. Nishikawa, T. Takeda, S. Shibata, and F. Fukuoka, *Chem. Pharm. Bull.*, **17**, 1910 (1969); *c*) Y. Nishikawa, M. Tanaka, S. Shibata, and F. Fukuoka, *ibid.*, **18**, 1431 (1970); *d*) Y. Nishikawa, K. Ohki, K. Takahashi, G. Kurono, F. Fukuoka, and M. Emori, *ibid.*, **22**, 2692 (1974); *e*) K. Takahashi, T. Kon, I. Yokota, and S. Shibata, *Carbohydr. Res.*, **89**, 166 (1981).
4) R.L. Whistler, A.A. Bushway, P.P. Singh, W. Nakahara, and R. Tokuzen, "Advances in Carbohydrate Chemistry and Biochemistry," Vol. 32, ed. by R.S. Tipson and D. Horton, Academic Press, New York, 1976, p. 235.
5) G. Chihara, "Gan To Menekizokyo," Kodansha, Tokyo, 1980.

6) *a*) Y.Y. Maeda, J. Hamuro, Y.O. Yamada, K. Ishimura, and G. Chihara, "Immunopotentiation," (Ciba Foundation Symposium 18 (new series)), ed. by G.E.W. Wolstenholme and J. Knight, Elsevier, Excepta Medica, North-Holland, Amsterdam, 1973, p. 259; *b*) Y.Y. Maeda and G. Chihara, *Int. J. Cancer*, **11**, 153 (1973); *c*) R. Tokuzen, *Cancer Res.*, **31**, 1590 (1971).
7) *a*) G. Lemperle, *J. Reticuloendothel. Sco.*, **3**, 385 (1966); *b*) I.C. Diller, Z.T. Mankowski, and M.E. Fisher, *Cancer Res.*, **23**, 201 (1963); *c*) R.F. Kampschmidt and H.F. Upchurch, *J. Reticuloendothel. Soc.*, **5**, 510 (1968); *d*) N.R. Di Luzio, J.C. Pisano, and T.M. Saba, *ibid.*, **7**, 731 (1970); *e*) S.J. Riggi and N.R. Di Luzio, *Amer. J. Physiol.*, **200**, 297 (1961).
8) *a*) G. Biozzi, B. Benacerraf, and B.N. Halpern, *Brit. J. Exp. Pathol.*, **34**, 441 (1953); *b*) T.P. Stossel and Z.A. Cohn, "Methods in Immunology and Immunochemistry," ed. by C.A. Williams and M.W. Chase, Academic Press, New York, 1976, p. 292.
9) C.F. Culberson, "Chemical and Botanical Guide to Lichen Products," The University of North Carolina Press, Chapel Hill, 1969, p. 88.
10) *a*) D. Mizuno, O. Yoshioka, M. Akamatu, and T. Kataoka, *Cancer Res.*, **28**, 1531 (1968); *b*) B. Benacerraf and M.M. Sebestyen, *Fed. Proc.*, **16**, 860 (1957).
11) M.H. Levy and E.F. Wheelock, "Advances in Cancer Research," Vol. 20, ed. by G. Klein and S. Weinhouse, Academic Press, New York, 1974, p. 131.
12) *a*) G. Biozzi, B.N. Halpern, B. Benacerraf, C. Stiffel, and D. Mouton, *Compt. Rend. Soc. Biol.*, **150**, 317 (1956); *b*) R. Bomford and C. Moreno, *Brit. J. Cancer*, **36**, 41 (1977).
13) *a*) P. Ralph, M. Ito, H.E. Broxmeyer, and I. Nakoinz, *J. Immunol.*, **121**, 300 (1978); *b*) Z.A. Cohn, *ibid.*, **121**, 813 (1978); *c*) A. Temple, G. Loewi, P. Davies, and A. Howard, *Immunology*, **24**, 655 (1973).

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Solubility of a New Polymorph of Phenobarbital obtained by Crystallization in the Presence of Phenytoin[1)]

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Physicochemical properties of some polymorphs of phenobarbital were studied. A new polymorph, form III-Ph, with a slightly changed crystal habit was obtained by addition of phenytoin at the time of recrystallization.

The transition temperature and the heat of transition between form III-Ph and form III, and between form III and form II were determined to be 46°C and 0.201 kcal/mol, and 77°C and 0.404 kcal/mol, respectively, by solubility measurement. As the dissolution rates of form III-Ph and form III were higher than that of form II, they are expected to show better bioavailability than form II.

Keywords——phenobarbital; polymorph; phenytoin; allobarbital; recrystallization; dissolution behavior; heat of transition; heat of solution

Many pharmaceuticals can exist in different polymorphic forms and the differences in molecular arrangements can affect the physicochemical properties and bioavailability. One method to alter the properties of a crystal is to change the crystal habit of an original compound by recrystallizing it in the presence of a small amount of a compound which is structurally very similar to the base material.[2–4)]

In the case of phenobarbital, more than thirteen different polymorphic forms have been reported.[5–7)] In the present study, phenobarbital was recrystallized in the presence of a similar compound. As a result, form III-Ph was obtained by addition of 6% phenytoin, and this form showed a higher dissolution rate than forms II and III. The dissolution behavior