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Chemical and Biochemical Studies on Carbohydrate Esters. XII. The Phagocytic Response of the Reticuloendothelial System in Mice following Intraperitoneal Administration of Disaccharide Esters of Fatty Acids

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The effects of twenty-three disaccharide esters of fatty acids on the phagocytic response of the reticuloendothelial system (RES) were examined by measuring the intravascular clearance of colloidal carbon 24 h after a single intraperitoneal injection into ddY mice. A marked increase in the phagocytic activity was demonstrated with the following samples: i) nine lots of industrially produced sucrose-tallowates containing the mono- and higher esters of stearic and palmitic acids (*ca.* 2—4: 1) in various ratios ranging from 3: 7 to 7: 3, and ii) three laboratory-prepared samples, that is, sucrose-(di)- and -(poly)-stearates and trehalose-(di)-stearate. The phagocytic indexes shown by these agents at doses of 100 and 50 mg/kg, and, in some cases, even at 25 mg/kg were higher than, or comparable to, that obtained with 50 mg/kg of zymosan. Such a significant RES-stimulation could not be observed with other samples, *i.e.*, a lot of commercial sucrose-tallowate consisting almost entirely of monoesters and a commercial sucrose-palmitate containing the mono-, di- and polyesters, ii) laboratory-prepared samples such as sucrose- and trehalose-(mono)-stearates, and -(mono)-myristates, maltose-(mono)-, -(di)-, and -(poly)-stearates and maltose-(mono)- and -(poly)-palmitates, and iii) sucrose and stearic acid in the free form.

Keywords——carbon clearance test; phagocytic activity; reticuloendothelial system; immunopotentiator; disaccharide ester of fatty acid; structure-activity relationship

Previous studies of this series[1] have shown that various fatty acid esters of sucrose, trehalose (α,α-trehalose), maltose, and D-glucose exert cytotoxic effects against certain experimental tumors.[1,2] Recently we have also found that some of these esters possess an indirect, host-mediated antitumor effect upon solid sarcoma 180 in mice, probably due to their immunopotentiating ability.[2f] Independently of our investigations, cord-factor (α,α-trehalose-6,6′-dimycolate), and related artificial analogs have been found by many workers to exhibit diverse biological effects including antitumor and immunoadjuvant activities.[3] In addition, a synthetic maltose-tetrapalmitate has recently been demonstrated to be a nontoxic immunopotentiator with antitumor activity by Nigam *et al.*[4] It is well known that macrophages play a significant role in the host defense against neoplastic disease.[5] In this regard, the effects on the phagocytic response of the reticuloendothelial system (RES) following administration of the disaccharide esters of fatty acids to mice were tested by observing the vascular clearance of colloidal carbon in the present study.[6]

Materials and Methods

Materials——The following twenty-three esters were chosen to be examined from among the samples which had been used in our previous antitumor tests:[2] i) commercial sucrose esters; DK-esters of Lot Nos. F-50, -70, -110, and -160 (Dai-ichi Kogyo Seiyaku Co., Ltd., Kyoto, Japan) and RS-esters of Lot Nos. S-370, -570, -770, -1170, -1570, M-90, and P-1570 (Ryoto Co. Ltd., Tokyo, Japan), containing mono-, di-, and poly-tallowates (major constituents, stearates and palmitates (2—4: 1)) at various ratios with the one exception of RS-ester P-1570, which was composed almost entirely of palmitoyl esters, and ii) laboratory-prepared esters; sucrose-(mono)-, -(di)-, and -(poly)-stearates, sucrose-(mono)-myristate, trehalose-(mono)- and -(di)-

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stearates, trehalose-(mono)-myristate, maltose-(mono)-, -(di)-, and -(poly)-stearates, and maltose-(mono)- and -(poly)-palmitates, which were obtained by transesterification of the disaccharide with the methyl ester of a fatty acid, followed by column chromatographic fractionation. Before use, the test samples were confirmed to be free from endotoxin (bacterial lipopolysaccharide) according to the J.P. Pyrogen Test; endotoxin is known to be one of the most potent RES-stimulants.[7] When the *Limulus* coagulation test, a sensitive method newly developed for the detection of endotoxin,[8] was carried out by using the "Pyrogent (*Limulus* Amebocyte Lysate)" (Mallinckrodt Inc.), the samples also gave negative results. However, the negative test results did not guarantee the absence of the endotoxin, because mixtures of test samples (3 mg/ml each) and *E. coli* endotoxin (Sigma Chemical Co.; minimum concentration for gelation, 0.03 ng/ml) (0.05 ng/ml) occasionally failed to show a positive reaction in this bioassay for unknown reasons. The reference agents, stearic acid, sucrose, and zymosan ("Zymosan A" from *Saccharomyces cerevisiae*; Sigma Chemical Co.) were purchased commercially.

Methods——The carbon clearance test was conducted essentially as described by Biozzi *et al.*[9] 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 resulting supernatant was diluted in sterile, physiological saline to bring the carbon concentration to 16 mg/ml. Each test sample was dissolved (or suspended) in saline and administered intraperitoneally (*i.p.*) to five-week-old ddY mice (ten per group), weighing 20 to 25 g, at a dose of 100, 50, or 25 mg/kg. For comparison, one group of mice was injected *i.p.* with 50 mg/kg of zymosan and the mice of the control group received saline alone at the dose of 0.5 ml/mouse. After 24 h,[10] the mice were injected with the carbon suspension *via* the tail vein at a dose of 16 mg per 100 g body weight. Blood (0.02 ml/mouse) was taken from the retro-orbital venous plexus at 4 min intervals, and immediately hemolyzed by adding 3 ml of 0.1% Na_2CO_3 solution. The optical density of the hemolyzed blood sample was then read in a colorimeter at 600 nm. The phagocytic index K and the relative K-value were calculated based on 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$=(K_{\text{sample}}-K_{\text{saline}})/K_{\text{zymosan (50 mg/kg)}}-K_{\text{saline}})\times 100$.

Results and Discussion

The results obtained are shown in Table I. A marked increase in the phagocytic response of the RES was observed with the following samples: i) all the commercial sucrose-tallowates except for RS-ester M-90, and ii) laboratory-prepared sucrose-(di)- and -(poly)-stearates and trehalose-(di)-stearate. The phagocytic indexes given by them at doses of 100 and 50 mg/kg were higher than, or comparable to, that shown by 50 mg/kg of zymosan (an insoluble yeast cell-wall fraction), which is known to enhance the RES-function remarkably under conditions similar to those employed here.[8a,11] Generally, 25 mg/kg dose was found to be less effective than the higher doses, though some samples exhibited rather strong activity even at this dosage. The rest of the samples did not cause marked RES-stimulation at the doses tested.

Although the ester preparations used in this work were isomeric mixtures and not chemically homogeneous, certain probable structure–activity relationships may be deduced from the present findings. Since the sucrose-(di)- and (-poly)-stearates were both markedly effective, in contrast to the corresponding monostearate, di- and higher substitution would be favorable for this biological activity. Similarly, of ten sucrose-tallowates tested, nine, in which the total contents of di- and poly-esters exceeded 30%, were all highly effective, whereas one, RS-ester M-90, which was composed almost solely of monoesters was ineffective. The strong effects of the sucrose-tallowates should be attributable mainly to their di- and poly-stearoyl components, not to the co-existing palmitoyl esters, since the latter were contained in smaller quantities than the stearoyl esters, and, in addition, their activities proved to be greatly inferior to those of the stearates, as judged from the results with the RS-ester P-1570, which consisted principally of mono-, di-, and poly-palmitates. These observations suggest that a longer fatty acyl moiety would be advantageous for enhancement of the phagocytic activity. The inactivity of the sucrose-(mono)-myristate may be explained in terms of the mono-substitution with a shorter acyl function.

The trehalose-(di)-stearate was effective, while the trehalose-(mono)-stearate and -(mono)-myristate were both inactive. The results indicate that substitution of the sucrose residue with another non-reducing disaccharide unit, trehalose, does not cause a significant change in this

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TABLE I. The Effects of Disaccharide Esters of Fatty Acids on the Carbon Clearance Activity

Test sample: Code No.	Ester constitution	Composition (%): Mono	Di	Poly	Phagocytic index K (mean±S.E.)[a] Dose (mg/kg): 100	50	25	Relative K-value[b] Dose (mg/kg): 100	50	25	Set No. of exptl.
Industrially produced samples[c]											
DK F-50	Sucrose-(mono, di, poly)-tallowate	30	70		0.1862±0.0046	0.1543±0.0138	0.1087±0.0053	133	89	25	A
DK F-70	Sucrose-(mono, di, poly)-tallowate	40	60		0.1810±0.0050	0.1907±0.0060	0.1677±0.0067	126	140	108	A
DK F-110	Sucrose-(mono, di, poly)-tallowate	50	50		0.1736±0.0066	0.1678±0.0083	0.1592±0.0064	116	108	96	A
DK F-160	Sucrose-(mono, di, poly)-tallowate	70	30		0.1877±0.0049	0.1582±0.0067	0.1285±0.0064	125	88	51	B
RS S-370	Sucrose-(mono, di, poly)-tallowate	20	34	46	0.1787±0.0034	0.1550±0.0067	0.1269±0.0078	100	70	36	C
RS S-570	Sucrose-(mono, di, poly)-tallowate	30	37	33	0.1700±0.0040	0.1806±0.0045	0.1662±0.0076	103	119	98	D
RS S-770	Sucrose-(mono, di, poly)-tallowate	40	36	24	0.1752±0.0052	0.1782±0.0055	0.1446±0.0063	112	116	64	D
RS S-1170	Sucrose-(mono, di, poly)-tallowate	60	30	10	0.1775±0.0033	0.1759±0.0042	0.1254±0.0071	112	110	47	B
RS S-1570	Sucrose-(mono, di, poly)-tallowate	70	25	5	0.1952±0.0060	0.1613±0.0073	0.1100±0.0080	142	99	27	A
RS M-90	Sucrose-(mono, di)-tallowate	93	7	0	0.0929±0.0036	0.0895±0.0034	0.0860±0.0036	3	−1	−6	A
RS P-1570	Sucrose-(mono, di, poly)-palmitate[d]	70	25	5	0.1321±0.0051	0.1238±0.0058	0.1110±0.0053	58	47	29	A
Laboratory-prepared samples											
SS-C(mono)	Sucrose-(mono)-stearate	100	0	0	0.0870±0.0080	0.0890±0.0032	0.0804±0.0036	−2	−1	−10	B
SS-C(di)	Sucrose-(di)-stearate	0	100	0	0.1636±0.0060	0.1663±0.0092	0.1381±0.0092	112	116	71	E
SS-C(poly)	Sucrose-(poly)-stearate	0	0	100	0.1698±0.0067	0.1425±0.0073	0.1096±0.0067	103	68	27	B
SM-C(mono)	Sucrose-(mono)-myristate	100	0	0	0.1159±0.0050	0.1062±0.0050	0.0976±0.0060	22	10	−1	C
TS-C(mono)	Trehalose-(mono)-stearate	100	0	0	0.0978±0.0062	0.0982±0.0056	0.0876±0.0048	0	0	−13	C
TS-C(di)	Trehalose-(di)-stearate	0	100	0	0.1772±0.0053	0.1559±0.0062	0.1208±0.0072	112	85	41	B
TM-C(mono)	Trehalose-(mono)-myristate	100	0	0	0.1039±0.0048	0.1005±0.0063	0.1047±0.0055	7	3	8	C
MS-C(mono)	Maltose-(mono)-stearate	100	0	0	0.1210±0.0085	0.1094±0.0060	0.1111±0.0051	44	25	28	E
MS-C(di)	Maltose-(di)-stearate	0	100	0	0.1095±0.0058	0.1032±0.0056	0.1029±0.0070	25	15	15	E
MS-C(poly)	Maltose-(poly)-stearate	0	0	100	0.1086±0.0062	0.0897±0.0044	0.0921±0.0054	24	−7	−3	E
MP-C(mono)	Maltose-(mono)-palmitate	100	0	0	0.1140±0.0031	0.1087±0.0046	0.0871±0.0076	32	24	11	E
MP-C(poly)	Maltose-(poly)-palmitate	0	0	100	0.1318±0.0067	0.1357±0.0050	0.1245±0.0044	42	46	33	C
Samples used for comparison											
Sucrose					0.0790±0.0039	0.0881±0.0047	0.0766±0.0050	−24	13	−27	C
Stearic acid					0.1063±0.0054	0.0895±0.0045	0.0818±0.0027	10	−11	−20	C

a) $p<0.001$.

b) Calculated based on the following K-values:

Set No. of exptl.	A	B	C	D	E
K of zymosan (50 mg/kg)	0.1622±0.0054	0.1678±0.0062	0.1790±0.0049	0.1678±0.0062	0.1562±0.0062
K of physiologic saline	0.0904±0.0025	0.0884±0.0035	0.0982±0.0057	0.1035±0.0049	0.0938±0.0073

c) The sucrose-tallowates commonly contained stearates and palmitates in ratios of 2—4: 1, along with small amounts of myristates.

d) The sucrose-palmitate contained the stearoyl (*ca.* 15%) and myristoyl (trace) esters.

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biological activity. On the other hand, the five maltose esters tested all showed poor effects, regardless of the degree of esterification and the acyl chain-length, suggesting that replacement of the non-reducing disaccharide moiety by a reducing one results in loss of activity.

The mechanisms involved in the carbon phagocytic potential of the RES induced by the present samples are not yet clear. It is, however, apparent that the ester molecule itself is responsible for the activity, since neither sucrose nor stearic acid in the free form proved to be effective. All the disaccharide ester preparations employed in this work can act as non-ionic surfactants with detergent properties. The contribution of detergent activity to the acceleration of the carbon clearance may be excluded on the basis of the complete ineffectiveness of some of the samples tested.

Various chemical and biological agents have been reported as RES-stimulants.[5] Experimental animals treated with such agents are known to show increased rejection of tumor grafts, resistance to oncogenic viruses, reduction of metastases, and regression of established tumors.[5] Our present investigations have revealed that di- and higher esters derived from non-reducing disaccharides and long-chain fatty acids represent a new class of low-molecular-weight RES-stimulants. Further studies are, however, needed to define the correlation between the RES-stimulatory and antitumor activities of these compounds.

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