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Lethal and Adjuvant Activities of Cord Factor (Trehalose-6,6′-dimycolate) and Synthetic Analogs in Mice

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Mycobacterial glycolipid, trehalose-6,6′-dimycolate (TDM), and its analogs, 6,6′-di-*O*-triacontanoyl-α,α-trehalose [TD(L30)], 6,6′-bis-*O*-(2-tetradecylhexadecanoyl)-α,α-trehalose [TD-(B30)], 6,6′-bis-*O*-(3-hydroxy-2-tetradecyloctadecanoyl)-α,α-trehalose [TD(BH32)], 6,6′-bis-*O*-(2-docosyl-3-hydroxyhexacosanoyl)-α,α-trehalose [TD(BH48)], 6,6′-bis-*O*-(3-tetradecanoyloxy-tetradecanoyl)-α,α-trehalose [TD(D28)], 6,6′-dideoxy-6,6′-bis(mycoloylamino)-α,α-trehalose [TDNM] and 6,6′-dideoxy-6,6′-bis(3-hydroxy-2-tetradecyloctadecanoylamino)-α,α-trehalose [TDN(BH32)], were synthesized, and their lethal and adjuvant activities were examined. TDM and TDNM were lethal when injected intravenously into mice at a dose of 150 μg as 9% oil-in-water emulsion, but the other analogs showed no lethal toxicity to mice at the same dose. The cytolytic activity of mouse peritoneal macrophages against tumor cells was potentiated by intraperitoneal injection of TDM and its synthetic analogs. TDM and TD(BH32) also stimulated nonspecific host resistance against Sendai virus infection in BALB/c mice.

Keywords——cord factor; trehalose dimycolate (TDM); toxicity; adjuvant activity; macrophage activation; virus infection

Cord factor, a glycolipid purified from mycobacterial lipid fraction, has been shown to have various biological properties: (i) lethal toxicity to mice,[1] (ii) enhancement of circulating antibody formation,[2] (iii) stimulation of macrophages,[3] (iv) stimulation of nonspecific host resistance to bacterial infection,[4] and (v) antitumor activities *in vivo*.[5] Its structure has been established to be trehalose-6,6′-dimycolate (TDM) with high molecular weight α-branched β-hydroxyfatty acid moieties.[6] TDM and its analogs have been synthesized.[7]

Ribi *et al.*[8] reported that in line 10 hepatoma system, a combination of BCG cell-wall skeleton and P_3 (TDM) was immunotherapeutically more active than either of the two fractions alone, and later Toubiana *et al.*[9] showed that trehalose-6,6′-diester of 3-hydroxy-2-icosyltetracosanoic acid (44 carbon atoms) was also as active as TDM in this system. Yarkoni *et al.*[10] have shown that two synthetic analogs of TDM with shorter-chain (32 carbon atoms) fatty acids were much less toxic, but had the same antitumor activities as natural TDM in the line 10 hepatoma system when it was combined with endotoxin. Parant *et al.*[11] have also reported that the natural trehalose dicorynomycolates and their synthetic isomers have the same degree of activity as TDM in protecting mice against unrelated bacterial infections.

In this study, we have synthesized trehalose-6,6′-diesters of fatty acids, such as linear C_{30}-acid (L30), α-branched C_{30}-acid (B30), α-branched β-hydroxy C_{32}-acid (BH32), α-branched β-hydroxy C_{48}-acid (BH48), β-acyloxy C_{28}-acid (D28), and mycolic acid. We also prepared bis-*N*-acyl derivatives of 6,6′-diamino-6,6′-dideoxy-α,α-trehalose (pseudo cord factor) using BH32 and mycolic acid by the method of Liav *et al.*[12] with some modifications. The effects of the synthetic analogs on biological activities, such as lethal toxicity to mice, mouse peritoneal

macrophage activation, and protective activity against Sendai virus infection in mice were examined in comparison with those of TDM. The relationships between the structures and biological activities of TDM analogs are discussed.

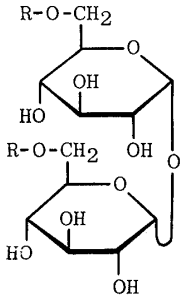
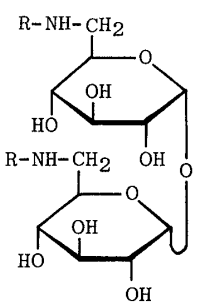
Materials and Methods

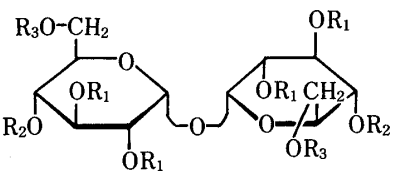
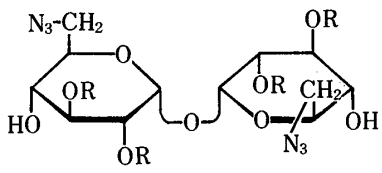
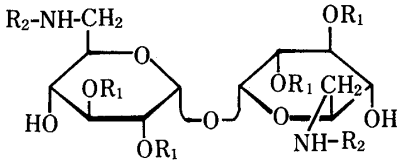
Trehalose Dimycolate (TDM) and Its Synthetic Analogs——TDM from *Mycobacterium tuberculosis* strain Peurois was kindly supplied by Dr. E. Lederer, Laboratoire de Biochimie, C.N.R.S., Gif-sur-Yvette, and Institut de Biochimie, Université de Paris-Sud, Centre d'Orsay, France. TDM and its analogs used in this study (Table I) were synthesized according to the procedure illustrated in Chart 1.

General Methods——Melting points were determined with a Yamato micro melting-point apparatus and are uncorrected. Specific rotations were determined with a Union PM-101 polarimeter, and infrared (IR) spectra were recorded with a Shimadzu IR-27G spectrophotometer. The field desorption-mass spectra (FD-MS) were recorded on a JEOL JMS-OISG-2 spectrometer. Preparative chromatography was performed on silica gel (Merck Co., 200 mesh) with the solvent systems specified. All evaporations were conducted *in vacuo*.

2,3,2′,3′-Tretra-*O*-benzyl-6,6′-bis-*O*-(2-tetradecylhexadecanoyl)-α,α-trehalose (2)——A mixture of the potassium salt of 2-tetradecylhexadecanoic acid[13b] (500 mg), 2,3,2′,3′-tetra-*O*-benzyl-6,6′-di-*O*-tosyl-α,α-trehalose[14] (500 mg) and 18-crown-6 (150 mg) in toluene (5 ml) was stirred for 5 h at 90°C. The mixture was evaporated to a syrup, which

Table I. Chemical Structures of TDM Analogs

	Acyl group (R)	Abbreviation	Compd. No.
	–mycoloyl	TDM (Aoyama B)	**11**
		TD (BH48)	**9**
		TD (BH32)	**10**
		TD (B30)	**3**
		TD (L30)	**8**
		TD (D28)	**13**
	–mycoloyl	TDNM (Aoyama B)	**17**
		TDN (α-BH32) TDN (β-BH32)	**19**

	R_1	R_2	R_3
1:	$PhCH_2$	H	Ts
2:	$PhCH_2$	H	B30
3:	H	H	B30
4:	H	H	Ts
5:	$SiMe_3$	$SiMe_3$	Ts
6:	$SiMe_3$	$SiMe_3$	L30
7:	$SiMe_3$	$SiMe_3$	BH48 ($SiMe_3$)[a]
8:	H	H	L30
9:	H	H	BH48
10:	H	H	BH32
11:	H	H	mycoloyl
12:	$PhCH_2$	$PhCH_2$	H
13:	H	H	D28

14: $R = PhCH_2$

	R_1	R_2
15:	$PhCH_2$	H
16:	$PhCH_2$	mycoloyl
17:	H	mycoloyl
18:	$PhCH_2$	BH32
19:	H	BH32

a) Trimethylsilylated BH48.

Chart 1

was applied to a column of silica gel. Elution with hexane–AcOEt (10 : 1) gave pure **2** as a syrup; yield, 660 mg (85%). $[\alpha]_D^{25}$ +50° (c = 1.09, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 1735, 1465. *Anal.* Calcd for $C_{100}H_{162}O_{13}$: C, 76.39; H, 10.38. Found: C, 76.29; H, 10.34.

6,6′-Bis-*O*-(2-tetradecylhexadecanoyl)-α,α-trehalose [3; TD(B30)]——Compound **2** (520 mg) was dissolved in EtOH (15 ml), and hydrogenolyzed in the presence of palladium black catalyst for 4 h. The catalyst was filtered off, and the filtrate was evaporated to a syrup, which was applied to a column of silica gel. Elution with $CHCl_3$–MeOH (20 : 1) removed minor products. Continued elution with the same solvent system, followed by $CHCl_3$–MeOH (10 : 1), yielded pure **3** as a wax; yield 330 mg (82%). $[\alpha]_D^{25}$ +47° (c = 0.43, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3300, 1740, 1465, 720. FD-MS *m/e*: 1212 ($M+1^+$), 1194 ($1212-H_2O$), 906, 598 (oxonium ion, $C_{29}H_{59}COOC_6H_{10}O_4^+$). *Anal.* Calcd for $C_{72}H_{138}O_{13}$: C, 71.36; H, 11.48. Found: C, 70.96; H, 11.56.

6,6′-Di-*O*-tosyl-α,α-trehalose (4)——Compound **1** (0.50 g) in MeOH (15 ml) was hydrogenolyzed in the presence of palladium black catalyst for 3 h. After removal of the catalyst and evaporation of the solvent, the residue was crystallized from EtOH–Et_2O, to give **4** as needles; yield, 250 mg (78%). mp 133—136 °C, $[\alpha]_D^{25}$ +115° (c = 0.246, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3370, 1355, 1175. *Anal.* Calcd for $C_{26}H_{30}O_{15}S_2$: C, 47.99; H, 5.27. Found: C, 47.90; H, 5.14.

6,6′-Di-*O*-tosyl-2,3,4,2′,3′,4′-hexakis-*O*-trimethylsilyl-α,α-trehalose (5)——Chlorotrimethylsilane (0.5 ml) and 1,1,1,3,3,3-hexamethyldisilazane (1 ml) were added to a solution of **4** (0.30 g) in pyridine (3 ml), and the mixture was kept for 50 min at room temperature. Ice was then added and the mixture was extracted with $CHCl_3$. The extract was successively washed with 2 M HCl, saturated $NaHCO_3$ and water, then dried (Na_2SO_4) and evaporated to give a crystalline mass **5**; yield, 0.51 g (quantitative). mp 153–156 °C. $[\alpha]_D^{25}$ +119° (c = 0.087, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 1250, 840. *Anal.* Calcd for $C_{44}H_{82}O_{15}S_2Si_6$: C, 48.76; H, 7.63. Found: C, 48.71; H, 7.71.

6,6′-Di-*O*-triacontanoyl-2,3,4,2′,3′,4′-hexakis-*O*-trimethylsilyl-α,α-trehalose (6)——A mixture of the potassium salt of triacontanoic acid (390 mg), **5** (417 mg) and 18-crown-6 (120 mg) in toluene was stirred for 24 h at 120 °C. After evaporation of the solvent, the residue was dissolved in pyridine (5 ml); 1,1,1,3,3,3-hexamethyldisilazane (1 ml) and chlorotrimethylsilane (0.5 ml) were added to the solution, and the mixture was kept for 30 min at room temperature. The solvent was evaporated off, and the residue was triturated in hexane. The insoluble material was filtered off, and the filtrate was evaporated to a syrup, which was chromatographed on silica gel. Elution with hexane–AcOEt (30 : 1) and recrystallization from Et_2O–EtOH gave **6**; yield, 444 mg (70%). mp 68.5—71.5 °C, $[\alpha]_D^{25}$ +57° (c = 1.04, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 1740, 1465, 720. *Anal.* Calcd for $C_{90}H_{186}O_{13}Si_6$: C, 65.71; H, 11.40. Found: C, 65.85; H, 11.45.

6,6′-Bis-*O*-(2-docosyl-3-trimethylsilyloxyhexacosanoyl)-2,3,4,2′,3′,4′-hexakis-*O*-trimethylsilyl)-α,α-trehalose (7)——Similarly, **7** was obtained from **5** and the potassium salt of 2-docosyl-3-hydroxyhexacosanoic acid[13] in 83% yield. mp 32.0—32.5 °C, $[\alpha]_D^{25}$ +40° (c = 0.39, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 1740, 1470, 720. *Anal.* Calcd for $C_{132}H_{274}O_{15}Si_8$: C, 68.15; H, 11.87. Found: C, 68.35; H, 12.24.

6,6′-Di-*O*-triacontanoyl-α,α-trehalose [8; TD(L30)]——A solution of **6** (350 mg) in a mixture of Et_2O (20 ml), EtOH (10 ml), AcOH (5 ml) and H_2O (2 ml) was kept for 8 h at 45 °C. The precipitate was collected by filtration, washed with EtOH, and recrystallized from EtOH to give **8**; yield 209 mg (81%), mp 143—147 °C. IR ν_{max}^{KBr} cm^{-1}: 3370, 1730, 1465, 720. FD-MS *m/e*: 1212 ($M+1^+$), 908, 906, 761 ($1212-C_{30}H_{59}O_2$), 598 (oxonium ion,

$C_{30}H_{59}O_2C_6H_{10}O_4^+$). *Anal.* Calcd for $C_{72}H_{138}O_{13}\cdot 2H_2O$: C, 69.30; H, 11.47. Found: C, 69.63; H, 11.30.

6,6′-Bis-*O*-(2-docosyl-3-hydroxyhexacosanoyl)-α,α-trehalose [9; TD(BH48)]——A solution of **7** (209 mg) in a mixture of $CHCl_3$ (8 ml), MeOH (2 ml), AcOH (8 ml) and H_2O (2 ml) was kept for 10 h at 45—50 °C. After evaporation of the solvent, the residue was chromatographed on silica gel. Elution with $CHCl_3$–MeOH (40 : 1) removed minor by-products. Elution with $CHCl_3$–MeOH (20 : 1) gave pure **9**; yield, 116 mg (74%), mp 75 °C sinter 142 °C, $[\alpha]_D^{25}$ +40° ($c=0.55$, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3370, 1725, 1470, 720. *Anal.* Calcd for $C_{108}H_{210}O_{15}\cdot H_2O$: C, 73.42; H, 12.09. Found: C, 73.14; H, 12.15.

6,6′-Bis-*O*-(3-hydroxy-2-tetradecyloctadecanoyl)-α,α-trehalose [10; TD(BH32)]——Esterification of **5** (600 mg) with the potassium salt of 3-hydroxy-2-tetradecyloctadecanoic acid[13] was acomplished in a manner similar to that described above for **6**, but at 80—100 °C instead of 120 °C. After evaporation of the solvent, the residue was dissolved in 80% aqueous AcOH (20 ml) and kept for 4.5 h at 45 °C. The solvent was evaporated off, and the residue was chromatographed on silica gel. Elution with $CHCl_3$–MeOH (40 : 1), followed by $CHCl_3$–MeOH (20 : 1), removed minor by-products. Elution with $CHCl_3$–MeOH (10 : 1) afforded pure **10** as a wax; yield 340 mg (57%). $[\alpha]_D^{25}$ +53.9° ($c=0.701$, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3370, 1725, 1470, 720. FD-MS *m/e*: 1300 ($M+1^+$), 1282 ($1300-H_2O$), 1060 ($1300-C_{15}H_{31}CHO$), 1042 ($1060-H_2O$), 805 ($1300-C_{32}H_{68}O_3$), 641 (oxonium ion, $C_{32}H_{63}O_3C_6H_{10}O_4^+$). *Anal.* Calcd for $C_{76}H_{146}O_{15}$: C, 70.22; H, 11.32. Found: C, 69.80; H, 11.22.

6,6′-Di-*O*-mycoloyl-α,α-trehalose [11; TDM (Aoyama B)]——Mycolic acid was obtained from the cell wall by alkaline hydrolysis of *Mycobacterium tuberculosis* strain Aoyama B. The average molecular weight of mycolic acid was calculated as $C_{80}H_{158}O_{3.5}$.[15] Compound **11** was obtained from **5** and the potassium salt of mycolic acid by a procedure similar to the described for **10**; yield, 41%, mp 43—44 °C, $[\alpha]_D^{25}$ +39° ($c=0.695$, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3370, 1725, 1470, 720. *Anal.* Calcd for $C_{172}H_{334}O_{16}$: C, 77.71; H, 12.66. Found: C, 77.79; H, 12.47.

6,6′-Bis-*O*-(3-tetradecanoyloxytetradecanoyl)-α,α-trehalose [13; TD(D28)]——Dicyclohexylcarbodiimide (DCC) (330 mg) was added to a mixture of 2,3,4,2′,3′,4′-hexa-*O*-benzyl-α,α-trehalose[16] (500 mg) and 3-hydroxytetradecanoic acid (151 mg) in *N*,*N*-dimethylformamide (DMF) (2 ml) and pyridine (0.6 ml), with ice-cooling, and the mixture was stirred for 3 h at 4 °C. After evaporation of the solvent, the residue was extracted with $CHCl_3$. The extract was successively washed with 2 M HCl and water, dried (Na_2SO_4) and evaporated to give a syrupy product that was purified by chromatography on silica gel. Elution with $CHCl_3$–MeOH (400 : 1) gave the corresponding 6,6′-bis-*O*-(3-hydroxytetradecanoyl) derivative (340 mg). This compound (170 mg) was dissolved in pyridine (1 ml) and 1,2-dichloroethane (1 ml), then dimethylaminopyridine (31 mg) was added to the solution. Next, tetradecanoyl chloride was added at 0 °C, and the mixture was stirred for 30 min at 0 °C then for an additional 20 h at room temperature. After completion of the reaction, a small amount of methanol was added to the mixture, and the solvent was evaporated off. The residue was extracted with $CHCl_3$, and the extract was washed, then dried (Na_2SO_4) and evaporated to give a syrup that was applied to a column of silica gel. Elution with $CHCl_3$ gave the corresponding 6,6′-bis-*O*-(3-tetradecanoyloxytetradecanoyl) derivative (100 mg). This compound (100 mg) was dissolved in a mixture of EtOH (4 ml), AcOEt (2 ml) and AcOH (2 ml), and hydrogenolyzed in the presence of 10% palladium-on-carbon catalyst (50 mg), for 2 d. The catalyst was filtered off and washed with EtOH, and the combined filtrate and washing were evaporated, giving a residue that was chromatographed on silica gel. Elution with $CHCl_3$–MeOH (20 : 1) gave **13**; yield, 48 mg (14%), mp 109—114 °C, $[\alpha]_D^{25}$ +37° ($c=0.3$, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3400, 1740, 1465, 720. FD-MS *m/e*: 1238 ($M+Na^+$), 1254 ($M+K^+$), 1215 (M^+), 681. *Anal.* Calcd for $C_{68}H_{126}O_{17}\cdot 2H_2O$: C, 65.24; H, 10.46. Found: C, 65.23; H, 10.32.

6,6′-Diazido-2,3,2′,3′-tetra-*O*-benzyl-6,6′-dideoxy-α,α-trehalose (14)——A solution of **1** (700 mg) in DMF (5 ml) was treated with NaN_3 (505 mg), and the mixture was stirred for 17 h at 100 °C, then evaporated. The residue was extracted with $CHCl_3$. The extract was washed, dried (Na_2SO_4), and evaporated to give a syrup that was applied to a column of silica gel. Elution with $CHCl_3$–MeOH (400 : 1) afforded pure **14**; yield 520 mg (quantitative). $[\alpha]_D^{25}$ +99.2° ($c=1.0$, $CHCl_3$). IR ν_{max}^{film} cm^{-1}: 2070. *Anal.* Calcd for $C_{40}H_{44}N_6O_9\cdot 1/2H_2O$: C, 63.06; H, 5.95; N, 11.03. Found: C, 63.16; H, 5.92; N, 11.15.

6,6′-Diamino-2,3,2′,3′-tetra-*O*-benzyl-6,6′-dideoxy-α,α-trehalose (15)——Compound **14** (740 mg) was dissolved in EtOH (15 ml), and hydrogenolyzed in the presence of 10% palladium-on-carbon catalyst (350 mg) for 2 h. The catalyst was filtered off and washed with MeOH, and the combined filtrate and washing were evaporated to give an amorphous residue (520 mg), 75%) which was homogeneous on thin layer chromatography (TLC). This product was used without further purification. The physicochemical properties of this compound were not determined. The structure of **15** was confirmed by conversion of **15** into the 6,6′-di-*N*-acetyl-4,4′-di-*O*-acetyl derivative; mp 195.5—197.0 °C, $[\alpha]_D^{25}$ +76.0° ($c=0.3$, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3120, 1740, 1650, 1530, 1230. *Anal.* Calcd for $C_{48}H_{56}N_2O_{13}$: C, 66.35; H, 6.50; N, 3.22. Found: C, 65.95; H, 6.46; N, 2.97.

6,6′-Dideoxy-2,3,2′,3′-tetra-*O*-benzyl-6,6′-bismycoloylamino-α,α-trehalose (16)——*N*-(Mycoloyloxy)succinimide[17] (2.2 g) dissolved in 1,4-dioxane (20 ml) and 4 drops of Et_3N were added to a solution of **15** (465 mg) in DMF (10 ml). The mixture was stirred for 8 h at room temperature, and evaporated to a syrup that was purified by chromatography on silica gel. Elution with $CHCl_3$ gave the pure product; yield 1.25 g (62%). Recrystallization from EtOH gave white crystals mp 127.5—128.5 °C, $[\alpha]_D^{25}$ +21.5° ($c=0.3$, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3500, 3300, 1620, 1560, 1465, 720. *Anal.* Calcd for $C_{200}H_{360}N_2O_{14}$: C, 79.62; H, 12.03; N, 0.93. Found: C, 80.05; H, 12.19; N, 0.83.

6,6′-Dideoxy-6,6′-bismycoloylamino-α,α-trehalose [17; TDNM (Aoyama B)]——Compound **16** (550 mg) was dissolved in CH_2Cl_2 (6 ml), and hydrogenolyzed in the presence of palladium black catalyst for 12 h. The reaction mixture was worked up as usual, and the crude product was purified by column chromatography on silica gel. The pure product was eluted with $CHCl_3$–MeOH (20 : 1) and isolated as a syrup in 70% yield (340 mg). It was crystallized from Et_2O–MeOH to give an analytically pure sample; mp 60°C sinter 81.5°C, $[\alpha]_D^{25}$ +37.8° ($c=0.3$, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3400. *Anal.* Calcd for $C_{172}H_{336}N_2O_{14}$: C, 77.76; H, 12.75; N, 1.05. Found: C, 77.22; H, 12.77; N, 1.04.

6,6′-Dideoxy-2,3,2′,3′-tetra-*O*-benzyl-6,6′-bis(3-hydroxy-2-tetradecyloctadecanoylamino)-α,α-trehalose (18)——*N*-(3-Hydroxy-2-tetradecyloctadecanoyloxy)succinimide was prepared from the α- or β-isomer[13a] of 3-hydroxy-2-tetradecyloctadecanoic acid by the same procedure as described previously.[17] Compound **18** was obtained in the same manner as described for the preparation of **16**. It was crystallized from MeOH to give an analytically pure sample. α-Isomer: mp 118—129 °C, $[\alpha]_D^{25}$ +34° ($c=0.2$, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3350, 1640, 1540, 1465. *Anal.* Calcd for $C_{104}H_{172}N_2O_{13}\cdot CH_3OH$: C, 74.60; H, 10.49; N, 1.66. Found: C, 74.34; H, 10.42; N, 1.66. β-Isomer: mp 131—134 °C, $[\alpha]_D^{25}$ +36.8° ($c=1.0$, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3500, 3300, 1620, 1560, 1460. *Anal.* Calcd for $C_{104}H_{172}N_2O_{13}$: C, 75.32; H, 10.45; N, 1.69. Found: C, 74.94; H. 10.42; N, 1.68.

6.6′-Dideoxy-6,6′-bis(3-hydroxy-2-tetradecyloctadecanoylamino)-α,α-trehalose [19; TDN(α-BH32), TDN(β-BH32)]——Compound **18** was dissolved in EtOH and CH_2Cl_2, and hydrogenolyzed in the presence of palladium-on-carbon catalyst as usual. The product was purified by column chromatography on silica gel with $CHCl_3$–MeOH (5 : 1). α-Isomer: mp 189—193 °C, $[\alpha]_D^{25}$ +39.4° ($c=0.2$, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3400, 720. FD-MS *m/e*: 1298 ($M+1^+$), 1057 ($M^+ - C_{15}H_{31}CHO$), 641 (oxonium ion, $C_{32}H_{63}O_2NHC_6H_{10}O_4^+$). *Anal.* Calcd for $C_{76}H_{148}N_2O_{13}$: C, 70.33; H, 11.49; N, 2.16. Found: C, 70.02; H, 11.64; N, 2.06. β-Isomer; mp 182—184 °C, $[\alpha]_D^{25}$ +40.9° ($c=0.3$, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3350, 720. FD-MS *m/e*: 1298 ($M+1^+$), 1057 ($M^+ - C_{15}H_{31}CHO$), 641 (oxonium ion, $C_{32}H_{63}O_2NHC_6H_{10}O_4^+$). *Anal.* Calcd for $C_{76}H_{148}N_2O_{13}\cdot H_2O$: C, 69.36; H, 11.49; N, 2.12. Found: C, 69.51; H, 11.40; N, 2.11.

Animals——Seven- to nine-week-old female C57BL/6 mice and five-week-old male BALB/c mice were obtained from Shizuoka Agricultural Cooperative for Experimental Animals, Hamamatsu, Japan.

Toxicity Test——Oil-in-water emulsions were prepared according to the procedures described earlier.[1c] TDM and its analogs (3 or 8 mg) were ground with mineral oil (Drakeol 6-VR, 0.18 g) in a glass homogenizing vessel with a Teflon pestle. Saline (1.82 ml) containing 1.1% Tween 80 was added to the vessel, and the mixture was ground for 3 min. The emulsions containing TDM and its analogs were injected intravenously into seven- to eight-week-old female C57BL/6 mice in groups of 10 each, in a volume of 0.1 ml. The final concentrations of emulsion components were: TDM and its analogs, 150 or 400 μg/0.1 ml; mineral oil (Drakeol 6VR), 9 mg/0.1 ml (9%); Tween 80, 1%. The mice were observed for 30 d, and body weight and survival were recorded.

Preparation of Aqueous Suspensions of TDM and Its Analogs——TDM and its analogs were suspended in water, at a concentration of 1 mg/lml, according to the method of Kato.[18] The suspensions were sterilized for 30 min at 60 °C in a water bath, and diluted in saline before use.

Culture Medium——Hank's balanced salt solution (HBSS) (Nissui Seiyaku Co., Ltd., Tokyo, Japan) was supplemented with 100 units of penicillin per milliliter and 100 μg of streptomycin per milliliter. RPMI 1640 medium (Nissui Seiyaku Co., Ltd., Tokyo, Japan) was supplemented with 10% heat-inactivated fetal calf serum (FCS) (lot R 781615; GIBCO Laboratories, Grand Island, N. Y., U.S.A.), 2 mM L-glutamine, 100 units of penicillin per milliliter, and 100 μg of streptomycin per milliliter (RPMI-FCS).

Macrophages——Aqueous suspensions of TDM and its analogs or oil-in-water emulsions of them, containing 1% squalane and 0.5% HCO-60, were injected intraperitoneally into mice in groups of 5 each, in a volume of 0.5 ml. Various days (usually 7 or 9 d) later, the mice were killed and the peritoneal exudate cells were obtained by washing out the peritoneal cavity with 10 ml of HBSS containing 10 units of heparin per milliliter. The cells were resuspended in RPMI-FCS, and the suspensions were plated in 96-well micro tissue culture plate (Corning Cell Wells 25860, Corning, New York 14831) to result in uniform densities of adherent cells, according to the procedure described previously.[19] The cells were cultured for 2 h at 37 °C and then washed with RPMI-FCS to remove nonadherent cells.

Assay for Cytolytic Activity of Macrophages——FBL-3 leukemia cells (FBL-3), Lewis lung carcinoma (3LL) and lymphoma cells (EL-4) were maintained in *in vitro* cultures. Cytolysis of target cells was quantitated as previously described.[19] In brief, FBL-3, 3LL or EL-4 cells (5×10^5) in RPMI-FCS were incubated for 1 h at 37 °C with $Na_2{}^{51}CrO_4$ (Japan Radio Isotope Association, Tokyo, Japan) and washed three times with HBSS. ^{51}Cr-Labeled target cells (5×10^3) were cultured with peritoneal macrophages (2.5×10^5) for 18—20 h at 37 °C. The radioactivity in the culture supernatant was determined by γ-counting to estimate target cytolysis by means of the formula:

$$\%\,\text{cytolysis} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100$$

Maximum release of ^{51}Cr was determined by freezing and thawing of labeled target cells three times. The spontaneous release was measured as the radioactivity released from labeled cells in the absence of macrophages.

Protection against Sendai Virus Infection——The Sendai strain of parainfluenza type I virus was purchased from

Flow Laboratories, Inc., Rockville, Md, U.S.A. This virus was passed for 10 generations in suckling C3H/He mice before being stored. The mouse lungs of the 10th passage were homogenized in phosphate-buffered saline (PBS), and the supernatant fluid was dispersed in ampoules in 1 ml lots, frozen and stored as stock virus suspension at −70 °C until used. A 0.030 ml aliquot of the stock suspension of this virus was poured into the nasal cavity of BALB/c mice in groups of 8 to 9 each, under light Ketamin (Ketalar-50, Sankyo Co., Ltd., Tokyo, Japan) anesthesia. The infectious inoculum was $10^{1.7}$ TCID50 of virus in a volume of 0.03 ml of the stock suspension when determined in LLCMK2 cells. TDM and its analogs were suspended in water at a concentration of 1 mg/ml. N^{α}-(*N*-Acetylmuramyl-L-alanyl-D-isoglutaminyl)-N^{ε}-octadecanoyllysine (MDP-Lys-L18)[20] (as a positive control) was dissolved in PBS at a concentration of 0.5 mg/ml. A 0.020 ml aliquot of solution was poured into the nasal cavity under light Ketalar-50 anesthesia at 1 d before infection. The protective effect of TDM and its analogs was represented as per cent survivors recorded 21 d after infection.

Results and Discussion

Synthesis of TDM and Its Analogs

Introduction of acyl groups into the trehalose moiety was performed by essentially the same method as that described by Polonsky *et al.*[7c,d] Treatment of 2,3,2′,3′-tetra-*O*-benzyl-6,6′-di-*O*-tosyl-α,α-trehalose (**1**)[14] with the potassium salt of 2-tetradecylhexadecanoic acid[13b] in toluene in the presence of 18-crown-6 at 90 °C, followed by hydrogenolytic removal of the benzyl groups, yielded 6, 6′-bis-*O*-(2-tetradecylhexadecanoyl)-α,α-trehalose (**3**) in good yield. However, 6,6′-di-*O*-triacontanoyl-α,α-trehalose (**8**) could not be obtained by the same procedure as described above. Catalytic hydrogenolysis of 2,3,2′,3′-tetra-*O*-benzyl-6,6′-di-*O*-triacontanoyl-α,α-trehalose was found to be troublesome because of the low solubility of **8** in the reaction solvent.

After hydrogenolytic deprotection of **1**, the resulting compound was converted into 6,6′-di-*O*-tosyl-2,3,4,2′,3′,4′-hexakis-*O*-trimethylsilyl-α,α-trehalose (**5**), which was also a useful compound for introduction of acyl groups into the trehalose moiety. Compounds **8**, **9**, **10** and **11** were obtained from **5** and the potassium salt of the corresponding fatty acid. The exchange reaction of the ditosylate (**5**) with the potassium salt of each fatty acid proceeded smoothly, but partial desilylation occurred under the conditions used. Compounds **6** and **7** were obtained in good yields after trimethylsilylation of the reaction mixture. In all cases, the trimethylsilyl groups were removed under mild acidic conditions.

Compound **12**[16] serves as an ideal starting material for synthesis of 6,6′-bis-*O*-(3-tetradecanoyloxytetradecanoyl)-α,α-trehalose (**13**). Treatment of **12** with 3-hydroxytetradecanoic acid in DMF and pyridine in the presence of DCC gave the corresponding 6,6′-bis-*O*-(3-hydroxytetradecanoyl) derivative, which was treated with tetradecanoyl chloride in pyridine and CH_2Cl_2, affording the 6,6′-bis-*O*-(3-tetradecanoyloxytetradecanoyl) derivative. Catalytic hydrogenolysis of this compound gave **13**.

Bis-*N*-acyl derivatives of 6,6′-diamino-6,6′-dideoxy-α,α-trehalose (**17**, **19**) were synthesized by the method of Liav and Goren[16] with some modifications. Treatment of **1** with sodium azide in DMF gave the corresponding 6,6′-diazido derivative **14** in good yield. Catalytic hydrogenation of **14** in the presence of 10% palladium-on-carbon gave the 6,6′-diamino derivative **15**. The structure was confirmed by conversion to the acetylated derivative. Treatment of **15** in DMF and 1,4-dioxane with *N*-(mycoloyloxy)-succinimide[17] and hydrogenolytic deprotection gave **17**. Compound **19** was also synthesized by essentially the same procedure as described for **17**.

Toxicity in Mice

Bloch[1a] reported that multiple intraperitoneal injection of cord factor (TDM) in paraffin oil at 2- to 3-day intervals killed mice. A loss of body weight of the animals was observed after the injections. It was shown that a single intravenous injection of 5 to 100 μg of cord factor (TDM) emulsified by grinding in oil also causes loss of body weight and death in mice.[21] It

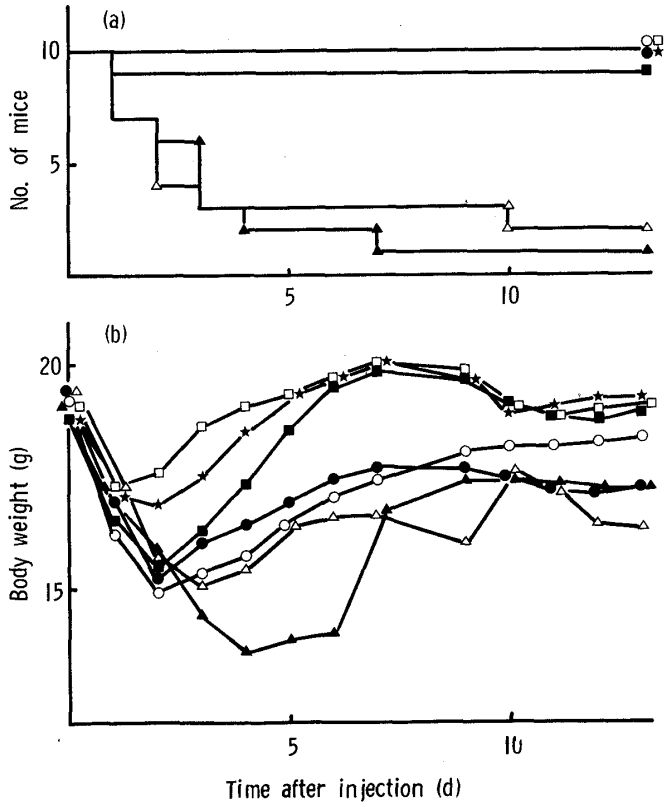


Fig. 1. The Number of Surviving Mice (a) and Average Weights of Surviving Mice (b) after Administration of TDM and Its Analogs

▲, TDM (Peurois); △, TDM (Aoyama B); □, TD (BH48); ○, TD (BH32); ●, TD (B30); ■, TD (L30); ☆, control. Oil-in-water emulsions containing 150 μg of TDM or an analog were prepared by grinding and were injected intravenously into C57BL/6 mice in a volume of 0.1 ml.

TABLE II. Toxicity of TDM and Its Analogs in Mice

Treatment	Number of death/number treated	
	150 μg/mouse	400 μg/mouse
TDM (Peurois)	9/10	—
TDM (Aoyama B)	36/40	10/10
TD (BH48)	0/10	—
TD (BH32)	0/10	0/10
TD (B30)	0/10	—
TD (L30)	1/10	—
TD (D28)	0/10	—
TDNM (Aoyama B)	3/20	4/10
TDN (α-BH32)	—	0/10
TDN (β-BH32)	—	0/10
Control (emulsion alone)	0/40	0/10

Oil-in-water emulsions of TDM and its synthetic analogs or emulsions alone (control) were prepared by grinding and were administered intravenously into C57BL/6 mice in a volume of 0.1 ml.

has been reported that the toxicity of TDM emulsions depends on the mineral oil concentration and the size distribution of the oil droplets.[1b,c]

In this toxicity study we used 150 μg of TDM in emulsions containing 9% oil prepared by grinding according to the method of Yarkoni and Rapp.[1c] Figure 1 shows that both natural TDM (Peurois) and semisynthetic TDM (Aoyama B) were lethal; 8 or 9 out of 10 treated mice died. The other synthetic analogs, TD(L30), TD(B30), TD(BH32) and TD(BH48) showed no lethal toxicity to mice at a dose of 150 μg. However, loss of body weight was observed after administration of any emulsion, even in the control group (oil emulsions alone). In the case of TD(L30) and TD(BH48), the body weight of the mice recovered to that

Table III. Effect of Administration Dose of TDM on Peritoneal Macrophage Activation in Mice

Treatment[a]	Timing (d)	Dose (μg)	% cytolysis (3LL)[b]
TDM (Peurois)	−7	200	32.3±1.3
	−7	100	21.4±2.3
	−7	50	28.2±1.9
	−7	10	20.7±1.3
Control (water)	−7	—	1.2±1.4

a) C57BL/6 mice were injected intraperitoneally with various doses of TDM suspended in water at 7 d before harvesting of macrophages. *b*) Each value is the mean ± standard error of 6 wells in each group. 3LL were used as target cells. All adjuvant groups were significantly different from the control group by Student's *t*-test ($p<0.001$).

Table IV. Effect of Timing of TDM Administration on Peritoneal Macrophage Activation in Mice

Treatment[a]	Timing (d)	Dose (μg)	% cytolysis (FBL-3)[b]
TDM (Aoyama B)	−21	50	25.7±2.8
	−14	50	26.0±2.1
	−7	50	27.5±3.2
Control (resident)	—	—	−5.4±1.4
Pyran copolymer	−3	500	48.3±2.0

a) C57BL/6 mice were injected intraperitoneally with TDM suspended in saline at 7, 14 and 21 d before harvesting of macrophages. *b*) See Table III for footnote *b*). FBL-3 were used as target cells.

of the control group at 7 d after administration. The fact that the treatment with TD(L30) emulsions killed 1 of 10 treated mice was not clearly explained, but the death was probably not due to the toxicity of TD(L30) but to an accident, because the body weight curve of the mice injected with TD(L30) was identical with that of the control mice later than 6 d after the injection (Fig. 1). As shown in Table II, TD(D28), which has unique fatty acids[22] contained in the lipid A moiety of gram-negative bacterial lipopolysaccharide, showed no toxicity in mice. TDNM was less toxic than TDM even when administered to mice at a dose of 400 μg per mouse. No lethal toxicity was observed in the mice injected with a maximum of 400 μg of TD(BH32), TDN(α-BH32) or TDN(β-BH32) (Table II). It seemed that the ester linkage on the sugar residue and the long chain fatty acid (especially mycolic acid having 80 carbon atoms) moiety played an important role in the manifestation of toxicity to mice. The biochemical mechanism of the toxicity of TDM and structural requirements of acyl-sugar derivatives for toxicity were investigated by Kato.[23] It was reported that TDM induced a marked swelling and fragmentation of mitochondrial membranes and functional damage of membrane-associated electron transport and coupling of respiration and phosphorylation specifically between coenzyme Q-cytochrome b complex and cytochrome c. That mechanism seems not to be sufficient to account for the death of the mice. Further detailed study on the toxicity of TDM is required.

Effect of TDM and Its Synthetic Analogs on Peritoneal Macrophage Activation in Mice

Lepoivre *et al.*[3c] reported the cytostatic activity of TDM-induced mouse peritoneal macrophages against syngeneic mastocytoma cells (P815). In this study, the cytolytic activity of mouse peritoneal macrophages induced by natural TDM (Peurois) in aqueous suspension was observed in the dose range of 10 to 200 μg per mouse (Table III). TDM was found to be

TABLE V. Effect of TDM and Its Analogs on Peritoneal Macrophage Activation in Mice

Treatment[a]	Timing (d)	Dose (μg)	% cytolysis[b]	
Expt. 1; saline suspension			FBL-3	3LL
TD (L30)	−7	50	12.8±1.2[c]	29.1±2.4[c]
TD (B30)	−7	50	10.9±1.7[d]	23.1±2.0[c]
TD (BH32)	−7	50	12.8±2.1[c]	29.1±2.9[c]
TD (BH48)	−7	50	17.8±1.3[c]	36.7±2.3[c]
TDM (Aoyama B)	−7	50	11.3±1.4[c]	30.4±2.0[c]
TDM (Peurois)	−7	50	10.3±1.7[d]	23.4±2.4[c]
Control (saline)	−7	—	4.3±1.6	1.8±2.7
Pyran copolymer	−3	500	16.3±2.1[c]	44.5±3.2[c]
Expt. 2; oil-in-water emulsion			3LL	EL-4
TD (L30)	−9	50	8.2±1.3	13.0±2.6
TD (B30)	−9	50	38.5±2.9[c]	33.6±2.0[c]
TD (BH32)	−9	50	20.9±3.6[c]	24.8±2.3[c]
TD (BH48)	−9	50	26.9±1.8[c]	24.5±1.7[c]
TDM (Aoyama B)	−9	50	19.4±1.8[c]	29.0±1.3[c]
TDM (Peurois)	−9	50	13.7±1.9[c]	23.6±2.6[c]
Control (emulsion alone)	−9	50	2.3±1.6	10.8±2.1
Pyran copolymer	−9	50	47.1±2.0	—

a) C57BL/6 mice were injected intraperitoneally with saline suspensions or oil-in-water emulsions of TDM and its analogs 7 or 9 d before harvesting of macrophages. *b*) Each value is the mean ± standard error of 6 wells in each group. 3LL, FBL-3 and EL-4 were used as target cells. *c*) Significant difference from the control by Student's *t*-test ($p<0.005$). *d*) Significant difference from the control ($p<0.02$).

active, even at a dose of 10 μg per mouse. In the following experiments, the assay of TDM and its analogs for induction of cytolytic macrophages was carried out at a dose of 50 μg per mouse. Peritoneal macrophages obtained 21, 14 and 7 d after intraperitoneal injection of TDM were shown to have potent cytolytic activity against FBL-3 leukemia cells (Table IV). Pyran copolymer was used as a positive control. Table V shows the effect of various TDM analogs on the activation of macrophages. The activity of all the synthetic analogs in Expt. 1 was comparable to that of natural TDM, or rather TD(BH48) was slightly more effective than natural TDM. The fact that even TD(L30), which is a normal chain fatty acid ester, has potent macrophage-activating ability indicates that the mycolic acid structure (α-branched β-hydroxy acid) is not essential for the activation of macrophages when the trehalose diester is administered to mice as a saline suspension. The cytolytic activity of peritoneal macrophages induced by the TDM analogs in oil-in-water emulsions was also investigated. We used oil-in-water emulsions because such an emulsion of TDM was reported to induce accumulation of macrophages and to cause formation of granulomas in the lungs of mice after intravenous injection.[1b,c] The intensity of the granulomatous reaction depended on the concentration of vehicle oil in the emulsion and on the size distribution of the oil droplets. Therefore, the oil probably plays an important role in macrophage activation by TDM. Macrophage-activating ability of the TDM analogs in Expt. 2 was similar to that in Expt. 1, except for the case of TD(L30) (Table V). The reason why TD(L30) was not effective when administered to mice as oil-in-water emulsion is not clear. However, it may be assumed that TD(L30) was hardly soluble in oil and that the low affinity of TD(L30) in oil-in-water emulsion accounted for the low activity of TD(L30).

TABLE VI. Effect of Pseudo Cord Factor on Peritoneal Macrophage Activation in Mice

Treatment[a]	Timing (d)	Dose (μg)	% cytolysis[b]	
			3LL	FBL-3
Expt. 1; saline suspension				
TDNM (Aoyama B)	−7	50	39.9±1.9	58.8±1.7
TDM	−7	50	36.2±4.1	55.7±2.9
Control (saline)	−7	—	−0.1±1.9	3.9±1.3
Pyran copolymer	−3	500	57.5±2.5	64.3±3.4
Expt. 2; saline suspension				
TDN (α-BH32)	−7	40	45.0±2.6	63.0±5.0
TDN (β-BH32)	−7	50	53.8±2.9	61.9±5.3
TD (BH32)	−7	50	17.3±1.8	30.6±2.5
TDNM (Aoyama B)	−7	50	64.1±2.1	68.9±5.7
TDM (Aoyama B)	−7	50	45.6±2.9	53.4±4.0
Control (saline)	−7	—	−1.6±1.2	2.1±1.8
Pyran copolymer	−3	500	60.7±3.8	27.5±3.5
Expt. 3; oil-in-water emulsion				
TDNM (Aoyama B)	−9	50	41.5±3.4	60.7±3.1
Control (emulsion alone)	−9	—	−0.4±1.7	5.6±1.6
Pyran copolymer	−3	500	51.3±3.4	55.5±5.3

a) See Table V for footnote *a*). *b*) See Table V for footnote *b*). 3LL and FBL-3 were used as target cells. All adjuvant groups were significantly different from the control group by Student's *t*-test ($p<0.001$).

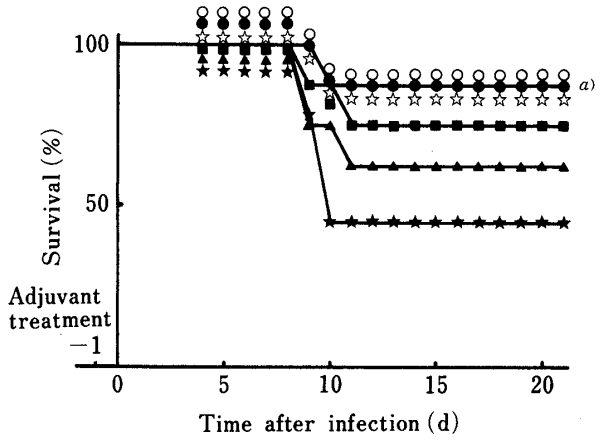


Fig. 2. Protective Activity of TDM and Its Analogs against Infection with Sendai Virus in Mice

○, TDM (Aoyama B); ●, TD (BH32); ■, TD (B30); ▲, TD (L30); ☆, MDP-Lys-L18; ★, control. TDM and its analogs (20 μg/0.02 ml/mouse) and MDP-Lys-L18 (10 μg/0.02 ml/mouse) were administered into the nasal cavity of BALB/c mice in groups of 8 to 9, at 1 d before infection. Adjuvant-treated groups were compared to the control group and *p* values were calculated for survival time by using the Mann-Whitney U-test. *a*) $p<0.05$.

TDNM (Aoyama B) was as effective as TDM in macrophage activation, whether administered to mice as a saline suspension or an oil-in-water emulsion (Table VI). TDN(α-BH32) and TDN(β-BH32) were slightly more effective than TD(BH32) in macrophage activation (Table VI, Expt. 2). The macrophage-activating ability of TDN(α-BH32) was similar to that of TDN(β-BH32). The amido linkage could substitute for the ester linkage of TDM in inducing cytolytic macrophages against tumor cells. Since TDNM was less toxic than TDM, bis-*N*-acyl derivatives of 6,6′-diamino-6,6′-dideoxy-α,α-trehalose with high macrophage activating ability were regarded as good adjuvants. TD(D28) did not induce cytolytic macrophages (data not shown).

The mechanism of the macrophage activation has not yet been clarified. Reisser *et al.*[24] reported that *in vitro* treatment of rat peritoneal macrophages with TDM did not induce

tumoricidal activity, and they considered that the activation process by TDM was not due to a direct effect on macrophages. We also failed to make mouse peritoneal macrophages cytolytic against tumor cells by *in vitro* TDM treatment (data not shown). The details are now under investigation.

Protective Activity of TDM and Its Analogs against the Infection of Sendai Virus in Mice

Natural trehalose dicorynomycolates obtained from *Corynebacterium diphtheriae* and their synthetic isomers have been shown to protect mice as effectively as mycobacterial TDM against infection with *Klebsiella pneumoniae* or with *Listeria monocytogenes*.[9] In the present study, the effect of TDM and its analogs against infection with Sendai virus was examined in mice. The results are shown in Fig. 2. TDM (Aoyama B) and TD(BH32) effectively stimulated nonspecific host resistance against Sendai virus infection. Intranasal administration of TDM or TD (BH32) gave an 88% survival rate, and these groups were significantly different from the control group in survival time ($p < 0.05$). TD(B30) and TD(L30) seemed to be less active in protecting mice against the infection.

Ribi *et al.*[25] reported that BCG cell-wall skeleton required addition of P_3 (TDM) to produce regression of transplanted line 10 hepatocarcinoma in strain 2 guinea pigs. It was shown that an endotoxin preparation from an O-antigen-deficient (Re) mutant of *Salmonella typhimurium* or synthetic muramyl dipeptides (MDP) combined with TDM in oil-in-water emulsions produced strong regression after intralesional injection in strain 2 guinea pigs bearing established line 10 dermal tumors.[5] In our experiment, TDM (Aoyama B) showed a very potent synergistic effect on line 10 tumor-regressive activity of quinonyl-MDP[26] (unpublished data). However, no synergistic effect of TDM on the activation of mouse peritoneal macrophages by quinonyl-MDP-66 was observed (data not shown). The mechanisms of the synergistic effect of TDM on tumor regression by BCG-CWS, endotoxin preparation, or MDP require detailed study.

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