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Nonspecific Immunostimulant Activities of Synthetic Trehalose-6,6'-Diesters (Lower Homologs of Cord Factor)

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Mycobacterial cord factors (6,6'-diesters of trehalose with mycolic acids ranging from C_{80} to C_{90}) have been shown to protect mice effectively against infection with *Klebsiella pneumoniae* or with *Listeria monocytogenes*. Our present findings indicate that the low-molecular-weight cord factor of *Corynebacterium diphtheriae* (with corynomycolic acids ranging from C_{28} to C_{36}) is equally active. Moreover, its synthetic analog (with synthetic C_{32} mycolic acid) has the same activity. Two lower synthetic 6,6'-diesters of trehalose with C_{22} acids, which are described here for the first time, as well as dipalmitate and a dioleate of sucrose, were found inactive. The synthetic C_{76} trehalose diesters, which are capable of enhancing nonspecific resistance to infection, increase the immune response in mice, even when injected in metabolizable oil. They induce in the injected paws an inflammatory process weaker and more transient than the natural cord factor.

The immunostimulant properties of cord factor (6,6'-dimycolate of trehalose; CF), a glycolipid produced by mycobacteria, and particularly its marked antitumor activity have been recently reviewed by several authors (1a, 5, 11, 14, 15). CF also seems to contribute to the stimulation of nonspecific immunity of mice to bacterial infections since rigorously purified preparations are able to restore the full activity of delipidated mycobacterial cell walls suspended in saline (17) and, moreover, since by itself CF increases the resistance of mice against heterologous infectious challenges (17, 28). The protective effect of the mycobacterial glycolipid was demonstrated when administered not only in Bayol but also in a metabolizable oil or even as a suspension in water (17). CF has also been shown to enhance the susceptibility of mice to endotoxin (22), to be arthritogenic (3), and to enhance various immune responses (6, 12, 19), although its adjuvant activity in guinea pigs depends on the experimental conditions (1, 4, 12, 19).

Lower homologs of mycobacterial CF with shorter-chain mycolic acids have been isolated from other microorganisms such as nocardiae and corynebacteria (see 1a, 11, 14, 15). Such compounds, particularly those in which the primary hydroxyl groups of trehalose are esterified with synthetic C_{32} and C_{44} mycolic acids, have been synthesized (8, 23, 25). Recently (J. Polonsky, E. Soler, and J. Varenne, Carbohydr. Res., in press), one of the first published syntheses (8), consisting of the condensation of trehalose-6,6'-ditosylate with a salt of mycolic acid, has been improved. Condensation of the 6,6'-ditosylate of trehalose with each of the two racemic (*threo* and *erythro*) diastereoisomers of synthetic C_{32} mycolic acids gave two C_{76} trehalose diesters (*7* and *8*). The same reaction with the two C_{44} synthetic mycolic acid diastereoisomers gave two C_{100} trehalose diesters (*10* and *11*) (Polonsky et al., in press).

The present study compares the in vivo effect of natural and synthetic lower homologs of mycobacterial CF against two bacterial infections (*Klebsiella pneumoniae* and *Listeria monocytogenes*) with that of purified mycobacterial CF, which has been shown previously to protect mice very effectively (17). The influence of synthetic preparations on several host responses (hepatosplenomegaly, enhancement of susceptibility to endotoxin, adjuvant activity, and arthritogenicity) was also evaluated in comparison to natural CF. The results show that the natural dicorynomycolates of trehalose obtained from *Corynebacterium diphtheriae* and their synthetic isomers have the same degree of activity as mycobacterial CF in protecting mice against unrelated bacterial infections. The synthetic preparations were also shown to be adjuvant active in mice even when administered in a metabolizable oil emulsion.

MATERIALS AND METHODS

Mice. Five- to six-week-old (C57Bl/6 × AKR)F_1 hybrids raised at the Pasteur Institute, Paris, were

used in most of the experiments. Adjuvanticity tests and adjuvant arthritis assays were performed with female mice of Swiss common stock.

Trehalose diesters. All the trehalose diesters used in this study are listed in Table 1. Mycobacterial CF preparations from *Mycobacterium tuberculosis* Peurois and *M. bovis* AN5, as well as corynebacterial CF preparations from *C. diphtheriae,* were produced at the Extraction Laboratory of the Institut de Chimie des Substances Naturelles at Gif-sur-Yvette under the supervision of A. Escaut.

CF *1* from strain Peurois had mp 43 to 45°C, optical rotation $[\alpha]_D = +32°$ ($CHCl_3$), and CF *3* from strain AN5 had mp 43 to 44°C, $[\alpha]_D = +31.6°$ ($CHCl_3$). These preparations were homogenous on thin-layer chromatography and gave correct elemental analysis.

Peracetylated CF *2*. CF *1* (350 mg) was acetylated with acetic anhydride-pyridine at room temperature overnight. After the usual work-up, the product (390 mg) was dissolved in ether and precipitated by addition of methanol to yield the peracetylated CF *2* (364 mg) as an amorphous white powder, homogenous on thin-layer chromatography (benzene-ether, 4:1). Its infrared spectrum is devoid of hydroxyl absorption and has mp 34 to 37°C, $[\alpha]_D = +42.3°$ ($c = 0.94$; $CHCl_3$) (found: C 75.63, H 11.95%; $C_{202}H_{382}O_{25}$ requires: C 75.60, H 11.91%).

Semisynthetic CF *4*. Mycolic acid was obtained by alkaline hydrolysis of the CF of *M. bovis* AN5. It had mp 55 to 56°C, $[\alpha]_D = +7.6°$ ($c = 1.05$; $CHCl_3$). Condensation of the potassium salt of this mycolic acid with trehalose-6,6′-ditosylate by the procedure described by Polonsky et al. (in press) gave the semisynthetic CF *4*, mp 42 to 44°C, $[\alpha]_D = +31.2°$ ($CHCl_3$).

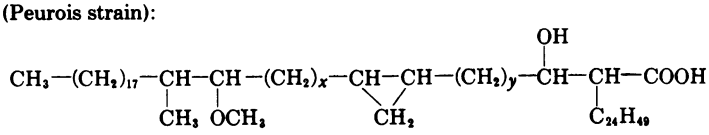
CF preparations *5* and *6* from *C. diphtheriae*. The lipid fraction from *C. diphtheriae* that was insoluble in boiling acetone was used as the starting material. Repeated chromatography of these lipids on Silica Gel 60, 70 to 230 mesh (Merck), afforded several fractions by elution with chloroform containing increasing concentrations of methanol. The glycolipid was found in fractions 4 to 8, which were eluted with chloroform-methanol (94:6). Dissolution of fractions 5 and 7 in ether and reprecipitation by the addition of 4 volumes of acetone gave the CF preparations *5* and *6* as colorless waxes, homogenous on thin-layer chromatography (chloroform-benzene-methanol, 45:40:15).

Synthetic CF analogs, *7, 8, 9, 10,* and *11*. The β-hydroxy acids, 2-tetra-decyl-3-hydroxyoctadecanoic acid and 2-eicosyl-3-hydroxytetracosanoic acid, were prepared from palmitic and behenic acid, respectively. The separation of their two *erythro* and *threo* diastereoisomers, called α and β, were performed by chromatography of the methyl esters on silica gel (18). Condensation of the potassium salt of each C_{32} hydroxy acid and of each C_{44} hydroxy acid with trehalose-6,6′-ditosylate as described by Polonsky et al. (in press) gave the CF *7, 8, 10,* and *11*, respectively. CF *9* was

TABLE 1. *Chemical properties of trehalose diesters*

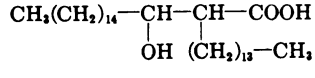
Trehalose diester	Acids esterifying the 6,6′ position
1 Natural CF (Peurois strain)	Mycolic acid $C_{85\pm5}$[a]
2 Peracetylated natural CF	3-Acetoxymycolic acid $C_{87\pm5}$
3 Natural CF (AN5 strain)	Mycolic acid $C_{85\pm5}$
4 Semisynthetic CF	Mycolic acid obtained by hydrolysis of *3*, $C_{85\pm5}$
5 Natural CF (*C. diphtheriae*) fraction 5	Corynomycolic[b] $C_{32}H_{64}O_3$ and corynomycolenic acid $C_{32}H_{62}O_3$
6 Natural CF (*C. diphtheriae*) fraction 7	*idem*, contaminated with lower homologs
7 Synthetic CF (C_{76})	2-Tetradecyl-3-hydroxyoctadecanoic acid (*erythro*), $C_{32}H_{64}O_3$
8 Synthetic CF (C_{76})	2-Tetradecyl-3-hydroxyoctadecanoic acid (*threo*), $C_{32}H_{64}O_3$
9 Synthetic CF (C_{76})	2-Tetradecyl-3-hydroxyoctadecanoic acid (*erythro* and *threo*), $C_{32}H_{64}O_3$
10 Synthetic CF (C_{100})	2-Eicosyl-3-hydroxytetracosanoic acid (*erythro*), $C_{44}H_{88}O_3$
11 Synthetic CF (C_{100})	2-Eicosyl-3-hydroxytetracosanoic acid (*threo*), $C_{44}H_{88}O_3$
12 Dibourgeanate of trehalose	Bourgeanic acid $C_{22}H_{42}O_5$[c]
13 Dibehenate of trehalose	Behenic acid $C_{22}H_{44}O_2$
14 Dipalmitate of trehalose	Palmitic acid $C_{16}H_{32}O_2$

[a] Mycolic acid (Peurois strain):

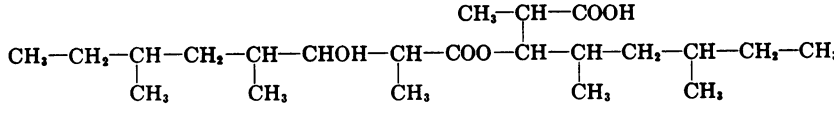


where $x = 7$ to 19, $y = 18$ to 6, maximum $x + y = 37(C_{90}H_{178}O_4)$, minimum $x + y = 25(C_{78}H_{154}O_4)$.

[b] Corynomycolic acid (*C. diphtheriae*) $C_{32}H_{64}O_3$:



[c] Bourgeanic acid $C_{22}H_{42}O_5$:



similarly prepared by using the diastereoisomeric *threo* and *erythro* mixture of the C_{32} hydroxy acids.

6,6′-di-*O*-bourgeanyl-α,α-trehalose, *12*. The potassium salt of bourgeanic acid (7) (308 mg) and 6,6′-di-*O*-tosyl-α,α-trehalose (300 mg) in dimethyl formamide (6 ml) were heated with stirring at 130°C. The evolution of the reaction is monitored by thin-layer chromatography (chloroform-methanol, 9:1). After 4 h the reaction product (364 mg) was isolated and chromatographed on silica gel (Polonsky et al., in press). Elution with chloroform-methanol, 8:2, afforded the CF *12* as a wax, $[\alpha]_D = +69.2°$ ($c = 1.01$; $CHCl_3$) (found: C 62.21, H 9.45%; $C_{56}H_{102}O_{19}$ requires: C 62.33, H 9.20%).

6,6′-di-*O*-behenyl-α,α-trehalose, *13*. Methyl behenate was transesterified with trehalose in dry dimethyl formamide by the procedure described in (27). The reaction product was isolated and chromatographed on silica gel (Polonsky et al., in press). Elution with chloroform-methanol, 94:6, followed by recrystallization in acetone gives the diester *13* as a white powder, homogenous on thin-layer chromatography (chloroform-benzene-methanol, 45:40:15), mp 106 to 112°C, $[\alpha]_D = +67°$; (chloroform) (found: C 67.98, H 10.58, O 21.83%; $C_{56}H_{106}O_{13}$ requires: C 68.15, H 10.75, O 21.10%; mass spectrum of the hexaacetate: 1,238 [M^+], *m/e* 611 [oxonium ion]).

6,6′-di-*O*-palmityl-α,α-trehalose, *14*. Transesterification of methyl palmitate by trehalose as described in (27) gives the diester *14*, mp 155 to 156°C, $[\alpha]_D = +67.5°$ ($c = 1.22$; $CHCl_3$) (14, 15) (found: C 64.24, H 10.05%; $C_{44}H_{82}O_{13}$ requires: 64.55, H 10.05%).

Dioleate of sucrose, *15*. A commercial preparation of dioleate of sucrose (Sucrose-Chemical Division, Gramercy, La.) was purified by chromatography on silica gel. Recrystallization from acetone gives a powder, homogenous on thin-layer chromatography (chloroform-benzene-methanol, 45:40:15) (found: C 65.35, H 10.02%; $C_{48}H_{86}O_{13}$ requires: C 66.2%, H 9.9%).

All the preparations were dissolved in mineral oil (Bayol F, Serva) or peanut oil.

Infectious challenge. Mice were challenged by the intravenous route with a virulent strain of *K. pneumoniae* or *L. monocytogenes* (17). They were treated by the same route 14 days previously with CF preparations dissolved in Bayol F and emulsified in saline by sonic treatment (10% oil emulsion). Cumulative results of several comparable experiments using groups of eight mice were expressed as the percentage of survivors 2 weeks after infection. The *P* values were obtained by the adjusted chi-square test (21).

Endotoxin sensitivity and evaluation of hepatosplenomegaly. Endotoxin sensitivity and hepatosplenomegaly were evaluated in mice 14 days after treatment with preparations injected under the same conditions as described above. The 50% lethal dose of *Salmonella enteritidis* endotoxin (phenol-water extract) was calculated as described previously (17) after intravenous injection of serial fivefold dilutions (from 1 to 125 μg) to groups of eight mice, in three identical experiments.

Antibody estimation. A water-in-peanut oil emulsion containing a purified preparation of bovine serum albumin (fraction V, Miles Laboratories) with or without various preparations of CF was injected into the mouse footpads as described previously (2). Some controls were immunized with bovine serum albumin alone administered in saline. Thirty days later a recall of 100 μg of antigen dissolved in saline was administered alone by the same route to all groups. At days 14 and 34, sera were pooled for each group of eight mice, and at day 36 they were collected separately. Antibody titers were expressed as the highest dilution of serum agglutinating bovine serum albumin-coated formalinized sheep erythrocytes (9). The *P* values were calculated by Student's *t* test.

Induction of inflammatory reaction. Preparations dissolved in Bayol F were injected into the mouse footpads in a volume of 0.05 ml. Control groups received Bayol only. Severity of lesions was estimated as described previously (3).

RESULTS

Effect of natural CF from *M. tuberculosis* or *C. diphtheriae* and of the synthetic C_{76} CF *7* on the resistance of mice against bacterial infections. The activity of CF preparation *5* from *C. diphtheriae* was compared with that of its synthetic isomer *7* and that of CF from *M. bovis* AN5 *3*. Two weeks before challenge, either with *K. pneumoniae* or with *L. monocytogenes*, mice received 150 μg of either preparation. Controls were treated with the emulsion containing 10% Bayol.

Cumulative results of two identical experiments are shown in Fig. 1. Mice were protected effectively against both types of infection, and there were no marked differences among the effects of the three preparations. Survivors were protected indefinitely, since no deaths occurred after 10 days.

Comparative effect of lower synthetic homologs of CF on the resistance of mice against bacterial infections. As in the former assays, all preparations previously dissolved in Bayol were administered by intravenous route in the oil-in-water emulsion. Results in Table 2 are given as the number of survivors 2 weeks after the infectious challenge by *K. pneumoniae* or by *L. monocytogenes*.

In these experiments CF from *M. tuberculosis* human strain Peurois *1* and bovine strain AN5 *3* were used as positive controls (Table 2). Peracetylated Peurois CF *2* has only low protective activity, in agreement with previous reports on weak toxicity of peracetylated CF under certain experimental conditions (1a, 14, 15), although, as will be seen later, toxicity is not necessarily required for protection. These results show, however, that free hydroxyl groups seem to be necessary for biological activity. The semisynthetic CF *4* was less active on *K. pneumoniae*, but just as active on *L. monocytogenes* as the natural CF *3*. Two different chromatographic fractions of natural CF from *C. diphtheriae* *5*

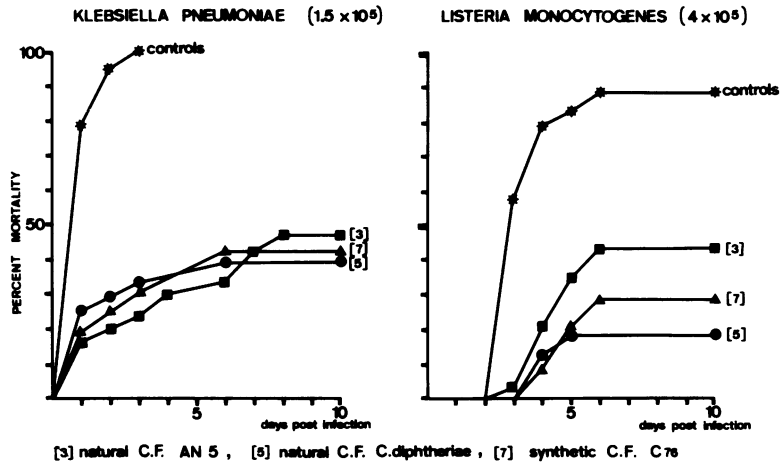


FIG. 1. *Protective effect of natural trehalose diesters and a synthetic analog of C. diphtheriae CF against infection in mice.*

and *6* were both strongly active, particularly against *Listeria* infection.

The activity of synthetic trehalose esters is also reported in Table 2. The C_{32} and C_{44} β-hydroxy acids esterifying trehalose in the synthetic CF analogs *7* to *11* were obtained by sodium borohydride reduction of the corresponding β-ketoesters. Chromatography on silica gel allows the separation of the two diastereoisomers formed. The more polar β-isomer of the C_{32} hydroxy acid has been previously shown (18) to be the racemate of corynomycolic acid of *C. diphtheriae* (20), which has been proven to be the *threo* diastereoisomer (24).

Both synthetic CF analogs *7* and *8*, with the *erythro* and the *threo* C_{32} isomer, and the CF analog *9*, prepared by using the diastereoisomeric mixture of the C_{32} hydroxy acids, were fully active (Table 2).

An unexpected result was obtained with the C_{100} diesters of trehalose, *10* and *11*, which had only slight activity. This might be due to their relative insolubility in the oily vehicle used for injection.

A synthetic dibourgeanate of trehalose *12* was weakly active in the *Listeria* test, whereas the dibehenate *13* and the dipalmitate *14* were entirely inactive. A mixture of sucrose dioleates prepared by chromatography of an industrial preparation was also inactive.

Influence of CF preparations on spleen and liver weight. Mice were treated with three compounds, *3*, *5*, and *7*, which had been shown to be active against infectious challenge under the same conditions. BCG was used as controls with a suspension of killed cells in the 10% mineral oil emulsion, as in the case of CF. Controls received the emulsion alone. All mice were sacrificed 14 days later.

A strong enlargement of the spleen and the liver was observed, as usual, in the group of mice treated with killed BCG cells. Whereas the natural CF of *C. diphtheriae 5* had a nonsignificant effect, the natural CF from AN5 strain *3* and the synthetic C_{76} diester *7* produced marked increase in organ weight (Table 3).

Influence of CF preparations on sensitivity to endotoxin. Resistance to the lethal effect of endotoxin was evaluated under conditions identical to the assays described above. After treatment with BCG in the oily emulsion, mice became highly susceptible to the lethal effect of endotoxin (Table 4), similar to previous reports on studies during which BCG was administered in saline (10). Although the effect was less marked than in BCG-treated mice, the natural CF of AN5 strain *3* significantly decreased resistance to endotoxin, since the 50% lethal dose of the untreated controls was about 320 μg. The synthetic C_{76} diester *7* also increased their susceptibility. However, the natural CF of *C. diphtheriae* was ineffective since, as in the control group, 23 out of 24 mice survived doses up to 125 μg, which was the highest dose administered.

Adjuvant activity of natural CF preparations and synthetic trehalose esters. Preparations were dissolved in peanut oil and administered in mice as a water-in-oil emulsion. The immunoadjuvant effect of synthetic trehalose esters was evaluated by comparison with two natural CF preparations, from Peurois strain *1* and from AN5 strain *3*, using bovine serum albumin as antigen. Controls were immunized with the antigen alone, either in saline or in the oily vehicle.

Natural CF from Peurois strain *1* was found

TABLE 2. *Protective activity of various natural or synthetic CF preparations against K. pneumoniae or L. monocytogenes infection in mice*

Treatment[a]	Survival after intravenous challenge[b]	
	K. pneumoniae	*L. monocytogenes*
Control	0/48	4/32 (12.5)
1 Natural CF (Peurois)	9/24 (37.5)[c]	21/32 (65.6)[c]
2 Peracetylated natural CF (Peurois)	6/24 (25)[d]	NT[e]
3 Natural CF (AN5)	24/48 (50)[c]	18/32 (56.3)[c]
4 Semisynthetic CF (AN5)	11/48 (23)[c]	11/16 (68.8)[c]
5 Natural CF (*C. diphtheriae*)	30/48 (62.5)[c]	26/32 (81.2)[c]
6 Natural CF (*C. diphtheriae*)	21/48 (43.7)[c]	30/32 (93.8)[c]
7 Synthetic CF C_{76} (*erythro* acid)	29/48 (60.4)[c]	23/32 (71.9)[c]
8 Synthetic CF C_{76} (*threo* acid)	21/48 (43.8)[c]	32/32 (100)[c]
9 Synthetic CF C_{76} (*erythro* + *threo* acids)	15/24 (62.5)[c]	24/32 (75)[c]
10 Synthetic CF C_{100} (*erythro* acid)	9/48 (18.8)[c]	3/16 (18.8)
11 Synthetic CF C_{100} (*threo* acid)	9/48 (18.8)[c]	2/16 (12.5)
12 Dibourgeanate of trehalose	6/24 (25)[d]	8/16 (50)
13 Dibehenate of trehalose	3/24 (12.5)	0/16
14 Dipalmitate of trehalose	0/24	NT[e]
15 Dioleate of sucrose	2/24 (8.3)	0/16

[a] Each preparation (150 μg) was given intravenously in 10% mineral oil emulsion at day −14.

[b] Number of survivors/total number tested (percentage) 2 weeks after infection with 1.5×10^5 *Klebsiella* or 4×10^5 *Listeria*.

[c] $P < 0.01$ = level of significance as compared with controls.

[d] $P < 0.05$ = level of significance as compared with controls.

[e] NT, Not tested.

to be adjuvant active, even at a dose of 10 μg (Table 5). Potentiation of the immune response by the CF of the bovine AN5 strain *3* or by the synthetic C_{76} diester *7* was also significant. However, the synthetic C_{100} diester *10* was inactive (Table 5).

Examination of the injected paws showed that granuloma formation was much less important with the active synthetic compound *7* than in groups treated with the natural product *3*. No granuloma occurred after administration of the inactive synthetic preparation *10*.

Inflammatory reaction induced by natural CF preparations and synthetic trehalose esters. It has been previously observed that Swiss mice are even more susceptible than rats to the local inflammation produced by natural CF preparations injected into their footpads (3).

In the following experiment, inflammatory activity of adjuvant active or inactive synthetic preparations was evaluated in comparison with natural CF *3* from the AN5 strain. The data obtained after administration in mineral oil in one posterior footpad are reported in Table 6. They show that the inactive synthetic trehalose ester *10* had no inflammatory action in contrast to natural CF *3*, which induced a very severe reaction. The active synthetic compound *7* induced an inflammatory but weaker and more transient process than the natural CF. These results agree with those of the adjuvant experiments reported above.

DISCUSSION

In previous experiments with mice in vivo, it was shown that several synthetic analogs of CF

TABLE 3. *Influence of CF preparations on hepatosplenomegaly in mice*

Treatment[a]	Wt[b]		
	Body (g)	Liver (mg)	Spleen (mg)
Control	19.6 ± 1.8	1,117 ± 145	120 ± 60
Killed BCG	19.6 ± 2	1,931 ± 162[c]	377 ± 63[c]
3 Natural CF (AN5)	20 ± 2.4	1,521 ± 392[d]	267 ± 104[c]
5 Natural CF (*C. diphtheriae*)	21.2 ± 1.7	1,237 ± 204	158 ± 68
7 Synthetic CF C_{76} (*erythro* acid)	22.4 ± 1.6	1,486 ± 117[c]	286 ± 85[c]

[a] Each preparation (150 μg) was given intravenously in 10% mineral oil emulsion to groups of eight mice at day −14.

[b] Given as arithmetic mean ± standard deviation.

[c] $P < 0.01$.

[d] $P < 0.05$.

TABLE 4. *Hyperreactivity to endotoxin in mice pretreated with natural or synthetic CF*

Treatment[a]	Endotoxin LD_{50}[b] (μg)
Untreated controls	320
Killed BCG	1
3 Natural CF (AN5)	21
5 Natural CF (*C. diphtheriae*)	>125
7 Synthetic CF C_{76} (*erythro* acid)	62

[a] Each preparation (150 μg) was given intravenously in 10% mineral oil emulsion at day −14.

[b] LD_{50} (50% lethal dose) as calculated after intravenous injection of serial fivefold dilutions of endotoxin (from 1 to 125 μg) to groups of eight mice.

TABLE 5. *Antibody response of mice to BSA administered in water-in-peanut oil emulsion with natural or synthetic CF preparations*

Treatment	Primary antibody response	Secondary antibody response	
	Day 14	Day 34	Day 36
Control (saline)	<3	<3	<3
Control (water-in-peanut oil emulsion)	12	25	500
1 Natural CF (Peurois)			
10 μg	6	800	1,600[a]
30 μg	50	400	1,900[b]
100 μg	50	200	1,300[a]
Control (saline)	<3	<3	10
Control (water-in-peanut oil emulsion)	6	6	100
3 Natural CF (AN5), 100 μg	50	200	600[c]
7 Synthetic CF C_{76} (*erythro* acid), 100 μg	50	200	700[a]
10 Synthetic CF C_{100} (*erythro* acid), 100 μg	<3	6	70

[a] $P < 0.01$.
[b] $P < 0.02$.
[c] $P < 0.05$.
[a, b, c] Levels of significance as calculated by Student's *t* test, comparison between experimental groups with their water-in-peanut oil control.

TABLE 6. *Inflammation induced by natural or synthetic CF preparations administered in Bayol to Swiss mice*

Treatment[a]	Severity of lesions[b]		
	Day 7	Day 14	Day 21
Control (Bayol)	–	–	–
3 Natural CF (AN5)	++++	+++++ necrosis	+++++ necrosis
7 Synthetic CF C_{76} (*erythro* acid)	++++	+++	++
10 Synthetic CF C_{100} (*erythro* acid)	+	±	–

[a] 100 μg of each preparation was injected into one posterior footpad to groups of seven mice in 0.05 ml of Bayol.
[b] Severity of lesions was estimated by comparing injected and noninjected paws.

could elicit various biological responses such as granuloma formation and tumor rejection. Thus, trehalose 6,6′-dipalmitate, sucrose 6,6′-dimycolate, 6-mycolate of methyl α or β D-glucopyranose, and even trehalose monopalmitate induce granuloma in the lungs of mice similar to those caused by CF and inhibit the growth of Ehrlich ascites tumor as does CF, but generally larger doses of the analogs are necessary (1a, 29). Other carbohydrate esters, such as sucrose myristate, have also a marked activity against Ehrlich ascites carcinoma in mice (16). Saito et al. (19) report that a trehalose diester from *Arthrobacter paraffineus* containing C_{32} to C_{36} corynomycolic acids showed "no apparent difference in toxicity or in adjuvant effect" in comparison to "cord factor from Aoyama B." Quite recently, Toubiana et al. (26) have found a synthetic 6,6′-trehalose diester of 2-eicosyl-3-hydroxytetracosanoic acid (in fact, a mixture of the compounds *10* and *11* described here) to have about 60% of the antitumor activity of mycobacterial CF (P_3) in regressing line-10 tumors in strain 2 guinea pigs when emulsified with oil droplets and endotoxins from O-antigen-deficient Re mutants of *Salmonella typhimurium*.

We have already reported that, in contrast to what was observed in tumor systems (1a, 29), the dipalmitate, as well as glycerol mycolate, is unable to enhance nonspecific immunity against bacterial challenges by *K. pneumoniae* or *L. monocytogenes* (17). The data reported here show, however, that, like natural CF from mycobacteria (6,6′-diesters of trehalose with mycolic acids ranging from C_{80} to C_{90}, *1* and *3*), a lower homolog isolated from *C. diphtheriae* (with two C_{32} mycolic acids, *5* and *6*) is able to increase effectively nonspecific resistance of mice to infectious challenge. Moreover, synthetic isomers of the latter natural trehalose ester (C_{76}-diesters, *7* and *8*, as well as *9*, which is a mixture of these two) have the same activity and can definitely protect animals infected with either *K. pneumoniae* or *L. monocytogenes* (Fig. 1). This is important because the tedious separation of the *threo* and *erythro* isomers of racemic corynomycolic acid is thus not necessary.

The two synthetic C_{100} diesters, *10* and *11*, protect mice against *Klebsiella* to a much lesser extent than the C_{76} diester and showed no activity against *Listeria*. Lower synthetic esters of trehalose, the dibourgeanate *12*, the dibehenate *13*, and the dipalmitate *14* showed little or no activity. A mixture of sucrose dioleates was also inactive. In a previous work differences were shown with mycobacterial CF suspended in water or dissolved in oil (17). Attempts to obtain a stable suspension in water with lower homologs of CF were unsuccessful. Therefore, the relative insolubility of certain preparations could be responsible for their low activity in certain experiments. The time interval between treatment and challenge could also be critical. Therefore, studies are being pursued to check whether these differences of activity depend on the time of injection and/or the nature of the vehicle.

CF has been reported to increase nonspecific immunity, to produce inflammation and lymphoid hyperplasia, to enhance susceptibility to endotoxin, and to be adjuvant active in mice.

Are these various biological responses necessarily interrelated? The capacity of various mycobacterial preparations to enhance susceptibility to endotoxin appeared in a previous study to be correlated to their ability to enlarge lymphoid organs and to their anti-infectious activity (17). In contrast, Yarkoni et al. (30) found no sensitization to endotoxin in CF-treated mice. These latter experiments were performed, however, under different conditions and with a different strain of mice. Although increased susceptibility to endotoxin and its relationship with splenomegaly (10, 13, 17) was confirmed in our present study, when certain CF preparations were used, the capacity to enhance reactivity to endotoxin was not correlated to the anti-infectious activity in natural trehalose ester from *C. diphtheriae*. The absence of such a side effect increases the interest of this well-defined molecule.

CF in incomplete Freund adjuvant has little if any adjuvant effect in guinea pigs (1, 19), but was shown to induce lasting delayed hypersensitivity when administered with protein antigen in an oil-in-water emulsion (4, 12). Highly purified CF in a water-in-oil emulsion enhances both the antibody production in mice and the development of delayed hypersensitivity in rats (6, 19). In these experiments it was administered in mineral oil (6, 12, 19). The findings reported here show that CF, like whole mycobacterial cells (2), increases the immune response when injected in peanut oil. They show also that the activity of natural CF could be matched by that of a synthetic analog, *7*, which is less toxic, as shown by the reduced inflammation (Table 6) and by the less important granuloma in the injected paws (adjuvant experiments). Therefore, the various biological activities of CF are not necessarily interrelated.

Our findings show that a synthetic C_{76} trehalose diester, a close analog of the natural *C. diphtheriae* CF, is capable of enhancing nonspecific resistance to infection. Moreover, this analog can stimulate the immune response in mice even when administered in metabolizable oil and, therefore, elicit less side effects than those observed usually. Such well-defined synthetic preparations are easily available for large-scale testing of their immunostimulant properties.

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