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Nonspecific Resistance Against Infection with *Salmonella typhi* and *Salmonella typhimurium* Induced in Mice by Cord Factor (Trehalose-6,6'-Dimycolate) and Its Analogues

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Mice pretreated intraperitoneally with trehalose-6,6'-dimycolate (cord factor) were protected against an intraperitoneal challenge with *Salmonella typhi* strain Ty2 or *Salmonella typhimurium* strain SR 11. The nonspecific resistance to *S. typhi* and *S. typhimurium* was still detectable 7 and 14 days, respectively, after administration of cord factor. The effect of cord factor was local. Synthetic analogues of cord factor—trehalose-6,6'-dipalmitate and trehalose monopalmitate—also induced nonspecific resistance to the above virulent bacteria. The results are discussed.

Some biological activities displayed by cord factor (CF), a glycolipid extractable from mycobacteria (11), are similar to those of living BCG. Both, after intravenous (i.v.) injection into mice, induce lung granulomas composed of epithelioid cells, macrophages, and lymphocytes (2). Administration of cord factor (CF) or BCG into the footpads of mice causes formation of granulomas, marked hyperplasia of the lymphoid tissue in the paracortical zone, and accumulation of macrophages in the draining lymph nodes (3). Mice injected with both agents into the footpads react with increased antibody response to an unrelated antigen subsequently injected into the same site (7, 15). This increased antibody formation has been related to the granulomatous reaction induced in the draining lymph nodes (7). Mice with lung granulomas induced by CF were protected against i.v. challenge with the virulent H37Rv strain of *Mycobacterium tuberculosis*, a protection similar to that observed by others after i.v. injection of intact mycobacterial cells (2, 22, 23). It was observed that CF activates peritoneal macrophages of mice in vivo as judged by an increased acid phosphatase activity and increased phagocytic activity of *Listeria monocytogenes*. This activation was comparable to that induced by heat-killed or living BCG (E. Yarkoni and A. Bekierkunst, in preparation). Living BCG bacilli are able to stimulate the immunological response of the host, as evident in nonspecific resistance to heterologous bacterial injections as well as to grafted tumors in animals sensitized with BCG (4, 5, 8, 10, 13, 14, 17). This immunopotentiating activity is also evident in suppression and regression of intradermal tumors and prevention of their metastases in guinea pigs by injection of living BCG or oil emulsions of nonliving BCG cells or cell walls into the tumors (6, 9, 24-26). An important role in tumor regressive activity was related to CF (1, 6), which is apparently identical to P_3 (11, 15).

We now report the induction of nonspecific resistance against infection with *Salmonella bacilli* by CF and its synthetic analogues.

MATERIALS AND METHODS

Animals. Locally bred albino mice were used throughout.

Bacteria. *Salmonella typhi* strain Ty2, obtained from our stock, and *Salmonella typhimurium* strain SR 11, obtained from L. J. Berry (Department of Microbiology, University of Texas, Austin), were grown at 37°C in tryptic soy agar (Difco) for 16 to 18 h. The mean lethal dose of *S. typhi* strain Ty2 was 6×10^6 bacteria, and that of *S. typhimurium* strain SR 11 was less than 10 bacteria.

Preparation of emulsions and other materials. CF, its synthetic analogues, and their emulsions were prepared as described previously (2, 18, 19, 21). CF from *M. tuberculosis* strain Peurois and strain H37Ra, as well as the synthetic analogues trehalose-6,6'-dipalmitate and trehalose-6-monopalmitate were prepared in the Institut de Chimie des Substances Naturelles, Gif-sur-Yvette, France, and kindly supplied by E. Lederer.

Survival test. Mice pretreated intraperitoneally (i.p.) with CF or its analogues were challenged with a lethal dose of bacteria i.p. The percentage of the animals that survived for 4 or 30 days after the administration of *S. typhi* or *S. typhimurium*, respectively, served for the evaluation of nonspecific resistance.

Statistical evaluation of results. The nonparametric rank test of Wilcoxon was used (16).

RESULTS

Preliminary experiments showed that host resistance to *S. typhi* and *S. typhimurium* was increased by CF (Table 1). Seventy to 100% of the animals treated with CF 3 days before a challenge with *S. typhi* survived (experiments 1 and 2); 50% of such pretreated mice survived after challenge with *S. typhimurium* (experiment 3).

Nonspecific resistance with different amounts of CF. The above results raised the question of whether the protective effect was dose dependent. Groups of 10 mice were injected i.p. with CF in amounts ranging from 5 to 40 μg. After 3 days, these mice and the controls were inoculated i.p. with *S. typhi* (Table 2, experiment 1) or *S. typhimurium* (Table 2, experiment 2). The results indicate that 20 to 40 μg of CF was enough to induce a significant degree of resistance against both bacterial species.

Time course of the resistance effect induced by CF. Since CF was effective when given 3 days before a challenge with the bacteria, the effect of the time interval between the administration of CF and bacteria was investigated (Tables 3 and 4). When CF and the bacteria were injected at the same time, the survival time of the mice was not different from that of the controls. One- to 4-day intervals were needed to bring about established resistance. After 7 days the nonspecific resistance declined, and it disappeared after 10 days in mice challenged with *S. typhi* Ty2. In the case of *S. typhimurium*, the nonspecific resistance appeared after 3 days and lasted for at least 14 days.

Effect of route of administration of CF on nonspecific resistance. After an i.p. administration into mice, CF induced activation of macrophages as judged by an increased acid phosphatase activity and increased phagocytic activity. No such change was observed after i.v. administration of CF (Yarkoni and Bekierkunst, in preparation). It was of interest, therefore, to test whether the route of administration of CF is important in nonspecific resistance to *S. typhi*. CF was injected i.p. or i.v. into groups of 10 mice. After 3 days, the challenge dose was administered i.p. Table 5 demonstrates clearly that CF was effective only after i.p. administration.

Effect of analogues of CF on nonspecific

TABLE 1. *Nonspecific resistance of mice against Salmonella infections*

Expt no.	Material[a]	Dose (μg)	Survivors/ total	*P*	Bacterial challenge
1	Emulsion		1/10		
	Cord factor (H37Ra)	40	10/10	0.001	*S. typhi* Ty2
2	Emulsion		1/10		
	Cord factor (Peurois)	30	7/10	0.025	*S. typhi* Ty2
	Cord factor (Peurois)	60	7/10	0.025	
3	No treatment		0/10		
	Emulsion		0/10		*S. typhimurium* SR 11
	Cord factor (H37Ra)	40	5/10	0.05	

[a] Emulsion and cord factor were given i.p. 3 days before administration of 8 mean lethal doses of *S. typhi* Ty2 or 50 mean lethal doses of *S. typhimurium*. Final concentration of Bayol F in the emulsion was 0.4%; that of Tween 80 was 0.04%. The volume of the material injected was 0.2 ml.

TABLE 2. *Effect of pretreatment with different amounts of cord factor on the resistance of mice to Salmonella infections*

Expt no.	Material[a]	Dose (μg)	Survivors/ total	*P*	Bacterial challenge
1	Emulsion		1/10		
	Cord factor (H37Ra)	5	1/10	NS[b]	*S. typhi* Ty2
		10	4/10	NS	
		20	6/10	<0.05	
		40	8/10	<0.005	
2	Emulsion		0/10		
	Cord factor (H37Ra)	5	6/10	<0.025	*S. typhimurium* SR 11
		10	7/10	<0.005	
		20	7/10	<0.005	
		40	8/10	0.001	

[a] Experimental details are as in Table 1, except that 3 mean lethal doses of *S. typhi* were administered.
[b] NS, Not significant.

TABLE 3. *Time relationship between administration of cord factor and S. typhi infection*

Expt no.	Material	Dose (μg)	Time interval[a] (days)	Survivors/ total	P
1	Emulsion		0	0/10	
	Cord factor	40		0/10	
2	Emulsion		1	1/10	
	Cord factor	40		10/10	<0.001
3	Emulsion		3	1/10	
	Cord factor	40		10/10	<0.001
4	Emulsion		4	1/10	
	Cord factor	40		10/10	<0.001
5	Emulsion		7	1/10	
	Cord factor	40		5/10	0.05
6	Emulsion		10	1/10	
	Cord factor	40		0/10	
		60		0/10	
		80		2/10	

[a] Time interval between administration of cord factor (Peurois) and *S. typhi* inoculation (3 mean lethal doses). Final concentration of Bayol F in the emulsion was 0.4%; that of Tween 80 was 0.04%. The volume of the material injected was 0.2 ml.

TABLE 4. *Time relationship between administration of cord factor and S. typhimurium infection*

Expt no.	Material	Dose (μg)	Time interval[a] (days)	Survivors/ total	P
1	Emulsion		0	0/10	
	Cord factor	80		3/10	NS[b]
	Emulsion		1	0/10	
	Cord factor	80		3/10	NS
	Emulsion		3	0/10	
	Cord factor	80		8/10	0.001
2	Emulsion		7	2/10	
	Cord factor	100		9/10	<0.005
	Emulsion		10	1/10	
	Cord factor	100		6/10	<0.05
	Emulsion		14	2/10	
	Cord factor	100		7/9	0.01

[a] Time interval between administration of cord factor (H37Ra) and *S. typhimurium* inoculation (50 mean lethal doses). Final concentration of Bayol F in the emulsion was 1%; that of Tween 80 was 0.1%. The volume of the material injected was 0.2 ml.

[b] NS, Not significant.

TABLE 5. *Resistance of mice pretreated with cord factor i.v. or i.p.*

Material[a]	Dose (μg)	Route of injection	Survivors total	P
Emulsion		i.p.	1/10	
Emulsion		i.v.	1/10	
Cord factor	40	i.p.	7/10	<0.025
Cord factor	40	i.v.	0/10	NS[b]
	80	i.v.	1/10	NS
	160	i.v.	1/10	NS

[a] Emulsion and cord factor (Peurois) were given 4 days before administration of 3 mean lethal doses of *S. typhi*. Final concentration of Bayol F in the emulsion was 0.4%; that of Tween 80 was 0.04%. The volume of the material injected was 0.2 ml.

[b] NS, Not significant.

resistance. Earlier observation showed that not only CF but also its analogues increased the host resistance to Ehrlich ascites tumor cells (20). A study of the nonspecific resistance against *Salmonella* infections induced with analogues was therefore indicated. The analogues trehalose monopalmitate and trehalose-6,6'-dipalmitate were tested. Groups of 10 mice were pretreated i.p. with the analogues in oil-in-water emulsions and challenged 3 days later. Both were highly effective in induction of nonspecific resistance against *S. typhi* (up to 100% of mice survived) (Table 6, experiments 1 and 2); however, only trehalose-6,6'-dipalmitate brought about resistance against *S. typhimurium* (60% survivors), whereas trehalose monopalmitate was not effective (experiments 3 and 4).

DISCUSSION

It is clear that in mice, pretreatment with CF induces nonspecific resistance against *S. typhi* Ty2 and *S. typhimurium*. Another fact shown by this study is the local effect of CF. The latter injected i.v. has no inhibitory effect on the

TABLE 6. *Effect of pretreatment with analogues of cord factor on the resistance of mice to Salmonella infections*[a]

Expt no.	Material	Dose (μg)	Survivors/ total	P	Bacterial challenge
1	Emulsion[b]		1/10		*S. typhi*
	Cord factor (Peurois)	40	10/10	<0.001	(7 LD_{50})[c]
	Trehalose monopalmitate	80	10/10	<0.001	
	Trehalose-6,6'-dipalmitate	80	10/10	<0.001	
2	Emulsion[d]		1/10		*S. typhi*
	Trehalose monopalmitate	100	7/10	<0.025	(6 LD_{50})
	Trehalose-6,6'-dipalmitate	100	10/10	<0.001	
3	Emulsion[b]		0/10		*S. typhimurium*
	Trehalose-6,6'-dipalmitate	20	0/10	NS[e]	(50 LD_{50})
		40	0/10	NS	
		80	6/10		
4	Emulsion[f]		1/10		*S. typhimurium*
	Trehalose-6-,6'-dipalmitate	160	6/10	<0.05	(50 LD_{50})
	Trehalose monopalmitate	200	2/10	NS	
	Cord factor (H37Ra)	100	8/10	<0.005	

[a] Emulsion and cord factor were given i.p. 3 days before challenge. The volume of the material injected was 0.2 ml.

[b] Final concentrations of Bayol F and Tween 80 were 0.4 and 0.04%, respectively.

[c] LD_{50}, mean lethal dose.

[d] Final concentrations of Bayol F and Tween 80 were 0.5 and 0.05%, respectively.

[e] NS, Not significant.

[f] Final concentrations of Bayol F and Tween 80 were 2 and 0.2%, respectively.

growth of the bacteria injected i.p. To exert an inhibitory effect, both CF and the challenge must be administered into the same site. This effect is consistent with the local effect of CF, which had been shown already in tumor growth inhibition and in activation of peritoneal macrophages in vivo (4, 21; Yarkoni and Bekierkunst, in preparation).

The inhibition of growth of the virulent bacteria raises the question of what kind of host mechanism limits the growth of these intracellular bacteria. The explanation seems to be the inflammatory reaction induced at sites of inoculation of CF. However, not every kind of inflammatory reaction is sufficient to inhibit the growth of the bacilli. Administration of emulsion alone, for instance, is not enough to create conditions limiting the growth of the bacteria. Such a condition is created by CF, which displays a strong chemotactic activity for leukocytes, concentrating, at the site of its lodgment, large numbers of macrophages, which are subsequently activated (12). Such activated macrophages probably play an important role in increased resistance to virulent bacteria. CF has been shown to be effective in prevention (4, 21) and in immunotherapy of tumors in guinea pigs (1, 6) and humans (H. Cohen and A. Bekierkunst, in preparation), and in combination with nonliving BCG it was used for preparing a tumor vaccine (1). These activities of CF indicate that it may be useful as an adjunct in preparing effective vaccines against bacteria.

The results of the experiments in the present study with the CF analogues trehalose monopalmitate and trehalose-6,6'-dipalmitate indicate that the mycolic acid moiety is not essential for the activity observed; mycolic acid can be replaced by palmitic acid. Similar results were observed with these analogues in granulomagenic and tumor-suppressive activity in mice (20). It has recently been shown that trehalose-6,6'-dipalmitate and trehalose-6,6'-dibehenate can partially substitute for CF in its tumor-regressive activity in guinea pigs (A. Bekierkunst and E. Lederer, in preparation). Synthetic analogues of CF such as trehalose-6,6'-dipalmitate and trehalose-6,6'-dibehenate, as well as monoesters, open the way for possible use of these substances as adjuncts in preparation of specific active vaccines against tumors and other infectious agents.

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