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Adjuvant Effect of Cord Factor, a Mycobacterial Lipid

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Cord factor was a good adjuvant in mice and rats but exerted little if any adjuvant effect in guinea pigs. In rats, cord factor not only enhanced antibody production but also induced delayed hypersensitivity. Wax D was a weak adjuvant in mice. Cord factor caused a marked inflammatory swelling at the sites of injection in mice, but it caused no such swelling in rats and guinea pigs.

Mycobacterium tuberculosis acts as an immunological adjuvant that enhances the production of humoral antibody and induces delayed hypersensitivity. Its lipid fraction, designated wax D, has been widely regarded as an active principle responsible for its adjuvant activity, based, for the most part, on experiments in guinea pigs (16). Recently, cord factor, a toxic trehalose mycolate, but not wax D was reported to enhance antibody production in mice (3). However, in seeking to confirm what would appear to be a paradoxical observation, we considered that at least two points required clarification. The first is whether unsuspected impurities in the cord factor preparation used might contribute to its adjuvant activity, since cord factor preparations have from time to time been demonstrated to be contaminated with considerable amounts of wax D or other components (1). The second point is whether adjuvant activity of a given mycobacterial component may be exerted in some animal species but not in others. In the present study we investigated primarily these two points. For this purpose, two highly purified trehalose mycolate samples were used; one was cord factor purified by diethylaminoethyl (DEAE)-cellulose column chromatography (6), and the other was P3 prepared by pressure-accelerated chromatography (1).

MATERIALS AND METHODS

Trehalose mycolates. The following samples were used. (i) A cord factor fraction prepared by the method of Noll and Bloch (11) from live cultures of *M. tuberculosis* H37Rv grown 4 weeks in Sauton synthetic medium was further purified on DEAE-cellulose as described (6). This product is referred to as cord factor. (ii) P3 was isolated from *M. tuberculosis* Aoyama B as described previously (1). *Arthrobacter paraffineus* strain KY 4303 was also extracted by similar techniques (13), and the purity of the glycolipid obtained was examined by the same technique (1). The fatty acids of this glycolipid from *Arthrobacter* were corynomycolic acid (C_{32}-C_{36}), which forms the ester linkages with trehalose. Details of the structure of this glycolipid will be published elsewhere.

Sulfolipid. SL-I, the principal sulfatide of H37Rv, was isolated and purified as described previously (5). SL-III, a minor sulfatide recovered by extensive rechromatography of polar fractions recovered during preparation of SL-I, is principally 2-palmitoyl-3,6-bishydroxyphthioceranoyl-trehalose-2'-sulfate (M. Goren, O. Brokl, and B. C. Das, in preparation).

Wax D. Wax D was isolated by the method of Noll and Bloch (11) and purified by DEAE-cellulose chromatography (6).

Acetylated wax D and ADb. Acetylated wax D and its subfraction, AD6, were prepared as described previously (15).

Animals. Eight-week-old CF1 female mice, Hartley female guinea pigs weighing 300 g, and 8-week-old female Sprague-Dawley rats were used. Experimental groups consisted of six to eight mice, five rats or five guinea pigs.

Immunization. In most experiments, Freund-type water-in-oil emulsion containing saline, Drakeol, and Arlacel A (Freund incomplete adjuvant) was employed for immunization, mycobacterial lipids being dissolved in the oil phase. Either 0.025, 0.05, or 0.1 ml of the inocula was injected into the left hind footpad in mice, rats, or guinea pigs, respectively.

Measurement of immune reactions. Antibody titers against the protein antigen were determined by passive hemagglutination, using either tannic acid- (4) or carbodiimide- (7) treated erythrocytes.

Measurement of the thickness of mouse footpads. This was measured microscopically with a 20-fold magnification.

RESULTS

Effect of cord factor on antibody production and on injection sites and lymph nodes in

mice. Experiments were repeatedly performed, the sort and amount of the antigens and the amount of cord factor as well as the date of blood sampling being changed. Representative data are shown in Table 1 and Fig. 1. When 1 μg or more of cord factor was incorporated in the oil phase of the sensitizing antigen emulsion, the level of antibody titer was elevated generally four to eight times that of control groups. Since equal volumes of blood from two to three mice were pooled in these experiments, the number of samples was too small for statistical analysis. Some of these experiments were performed, however, under the same conditions, and the data were collected for statistical analysis. There was a statistically significant difference between the control and cord factor groups (Table 2). Roughly, the effect of 10 μg of cord factor corresponded to that of 50 to 100 μg of tubercle bacilli and was stronger than 100 μg of wax D. Cord factor at doses of more than 1 μg also caused a marked swelling at the site of injection (footpad) (Fig. 2). The swelling was striking even 1 day after injection and lasted for the entire experimental period (28 days), peaks appearing around 10 to 14 days after injection. The aqueous suspension of cord factor also caused a significant swelling, but the degree of swelling was much lower than that produced when the lipid was incorporated in the oil phase of the emulsion.

The use of P3 isolated from either *M. tuberculosis* strain Aoyama B or *Arthrobacter* produced results similar to those of cord factor with regard to the enhancement of antibody production and footpad swelling (Fig. 3).

When cord factor (10 μg) was injected in the form of an aqueous suspension along with sheep erythrocytes, no enhancement of antibody production was observed. However, if the antigen was injected into the site where the cord factor suspension had been injected 5 days previously, a moderate enhancement of antibody production was observed (data not shown).

When cord factor incorporated in a water-in-oil emulsion with or without sheep erythrocytes was injected into the hind footpads of mice, a significant increase in the weight of popliteal lymph nodes was observed (Fig.4). Interestingly, a greater increase in weight was observed in inguinal lymph nodes in the groups injected with cord factor under the same conditions, which was further strengthened by the addition of the antigen (Fig. 4). No such swelling of inguinal lymph nodes was observed when cord factor was injected as an aqueous suspension, though the suspension caused a significant swelling of popliteal lymph nodes. Very similar results were obtained when the number of cells of lymph nodes instead of weight was measured.

Effect of sulfolipids on antibody production

TABLE 1. *Effect of cord factor incorporated in a water-in-oil emulsion on antibody production in mice (1)*

No. of mice/ group	Antigen	Test substance[a]	Mean antibody titer (log_2)[b]		
			(day 21)	(day 28)	
8	Egg albumin[c] (250 μg)	IFA	7	10	
		CF (20 μg)	9	14	
		AD6 (100 μg)	8	12	
			(day 17)	(day 24)	
6	Hamster erythrocytes[d]	IFA	3.0 ± 1.5	5.5 ± 0.7	
		CF (10 μg)	7.0 ± 0.5	7.8 ± 0.4	
		TB (100 μg)	4.0 ± 1.3	6.5 ± 0.4	
			(day 9)	(day 14)	(day 21)
6	Sheep erythrocytes	IFA	3.5 ± 0.7	4.8 ± 0.4	6.0 ± 0.4
		CF (10 μg)	6.0 ± 0	5.8 ± 0.4	8.8 ± 0.4
		TB (50 μg)	5.5 ± 0.7	7.0 ± 0	8.0 ± 0
			(day 5)	(day 10)	(day 17)
6	Sheep erythrocytes	IFA	2.0 ± 0	3.0 ± 1.4	4.5 ± 0.7
		CF (10 μg)	4.5 ± 0.7	7.0 ± 1.4	8.3 ± 1.1
		Wax D (100 μg)	2.5 ± 0.7	5.5 ± 0.7	7.0 ± 0

[a] IFA, Freund incomplete adjuvant; CF, cord factor; TB, tubercle bacilli; AD6, acetylated wax D subfraction.

[b] Numbers in parentheses indicate days after immunization.

[c] Footpad test was performed on day 21 after immunization.

[d] Footpad test and secondary immunization were performed on days 10 and 17, respectively.

and injecton sites in mice. One hundred micrograms of sulfolipids I and III caused no enhancement of antibody production. Sulfolipid III at a dose of 100 μg caused a transient slight swelling at the site of injection. However, 10 μg of sulfolipid III or 10 to 100 μg of sulfolipid I caused no swelling. Kato and Goren (8) observed a synergistic action between cord factor and sulfolipid in relation to toxicity in mice. No synergistic action, however, was observed between cord factor (0.1 μg) and sulfolipid III (100 μg) with regard to enhancement of antibody production and footpad swelling (data not shown).

Effect of mycobacterial lipids on immune responses in guinea pigs. As shown in Table 3, 500 μg of cord factor incorporated in a water-in-oil emulsion containing 1 mg of egg albumin as antigen induced no delayed hypersensitivity to the antigen in guinea pigs as measured by corneal as well as skin reactions, whereas the same amounts of wax D and acetylated wax D induced a high level of delayed hypersensitivity. The same tendency was observed concerning antibody production. In contrast to the reaction in mice, no inflammatory swelling occurred at the sites of injection in guinea pigs.

Effect of mycobacterial lipids on immune responses in rats. Rats were sensitized with 100 μg of egg albumin incorporated in a water-in-oil emulsion with or without mycobacterial lipids. A delayed skin reaction was elicited 15 days after immunization in the groups in which wax D (200 and 500 μg) or cord factor (40 and 250 μg) had been injected together with the antigen (Fig. 5). Significant increase in antibody formation was also observed in both groups injected with cord factor and wax D. A

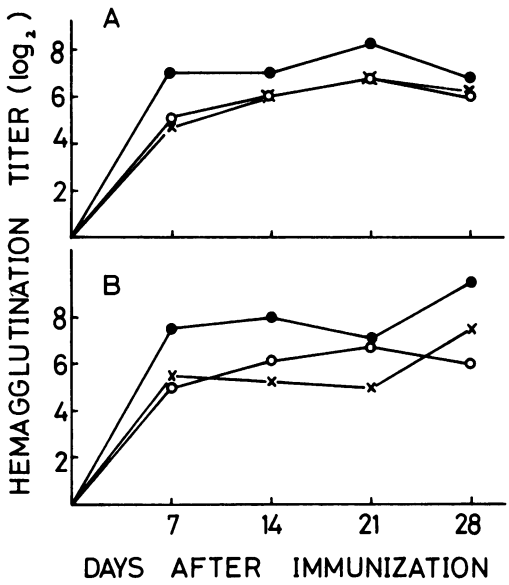

FIG. 1. *Effect of cord factor and wax D on antibody production of mice. Sheep erythrocytes were incorporated in Freund incomplete adjuvant containing no addition (○), cord factor (●), or wax D (×). One microgram of cord factor and wax D was used for experiment A and 5 μg of those materials was used for B. Each point represents the mean of eight mice.*

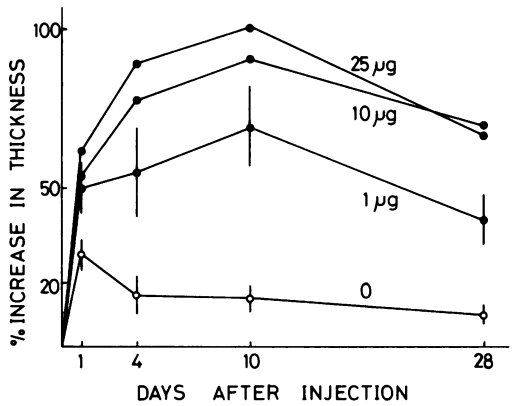

FIG. 2. *Effect of cord factor on footpad swelling of mice. Various amounts of cord factor were incorporated in Freund incomplete adjuvant and injected into the left hind footpads of mice. Each point indicates the mean of six mice, and vertical lines indicate standard deviation.*

TABLE 2. *Effect of cord factor incorporated in a water-in-oil emulsion on antibody production in mice (2)*[a]

No. of mice/ group	Antigen	Test substance	Mean antibody titer (log_2)[b] (Day 10)	(Day 15)	(Day 21)	(Day 23)
15	Egg albumin (200 μg)	IFA				6.0 ± 0
15		CF (10 μg)				8.5 ± 0.6 ($P < 0.001$)
18	Sheep erythrocytes	IFA	3.2 ± 1.0	4.6 ± 0.5	5.2 ± 0.7	
18		CF (10 μg)	6.4 ± 0.8 ($P < 0.001$)	6.7 ± 1.3 ($P < 0.01$)	7.3 ± 1.5 ($P < 0.025$)	

[a] IFA, Freund incomplete adjuvant; CF, cord factor.
[b] Numbers in parentheses indicate days after immunization.

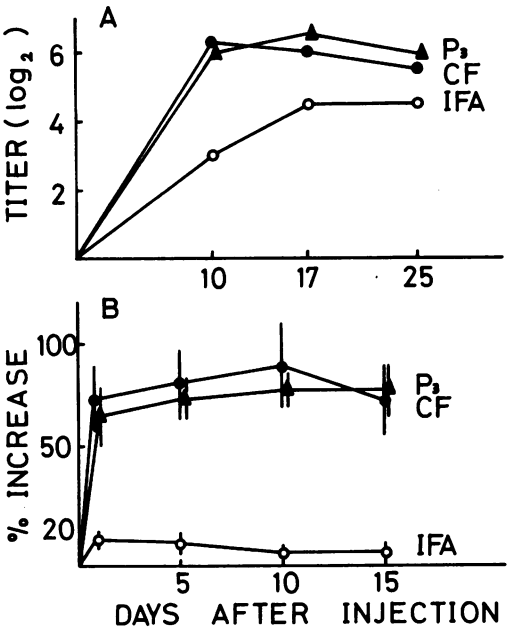

FIG. 3. *Effect of P3 and cord factor (CF) on antibody production (A) and footpad swelling (B) of mice. Six mice for each group were injected with sheep erythrocytes incorporated in Freund incomplete adjuvant (IFA) containing no addition, 10 μg of cord factor, or P3.*

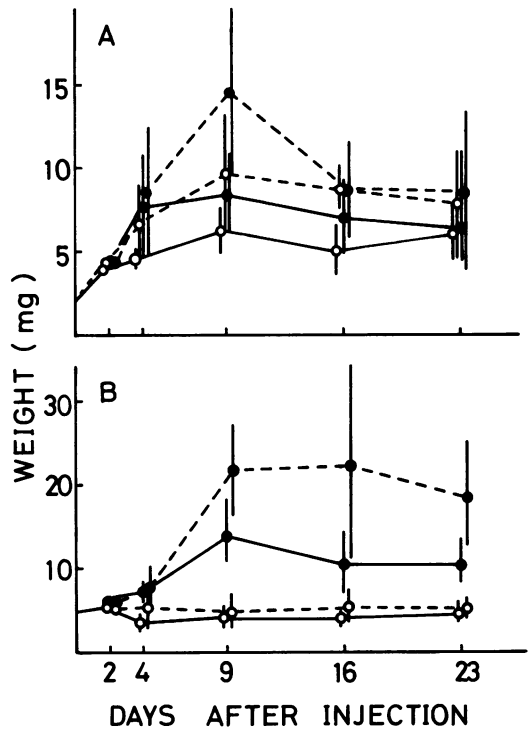

FIG. 4. *Effect of cord factor and antigen (sheep erythrocytes) on the weight of draining popliteal (A) and inguinal (B) lymph nodes of mice. Eight micrograms of cord factor (—●—) or 10^8 sheep erythrocytes (- -○- -) or both (- -●- -) were incorporated in Freund incomplete adjuvant and injected into the left hind footpad. Control mice were injected with Freund incomplete adjuvant alone (—○—). Each point represents six mice.*

slight inflammatory swelling occurred in the sites of injection only when 250 μg of cord factor was injected.

DISCUSSION

Recently, Bekierkunst et al. reported that cord factor can exert an adjuvant effect in mice (3). However, the possibility that some contaminant(s) in cord factor preparations might contribute to its adjuvant effect has not been excluded. We considered that this was important for two reasons. First, Azuma et al. showed clearly that various cord factor samples that were examined contained considerable amounts of various contaminants designated as P1, P2, and polar top materials as revealed by pressure-accelerated chromatography (1). Second, though Bekierkunst and Yarkoni reported that cord factor produced an epithelioid granuloma in the lungs of mice (2), Meyer et al. found that the granuloma was produced in lungs of mice when P3 was injected along with cell wall skeleton (a delipidated tubercle bacilli cell wall), whereas granuloma formation was slight when P3 alone was injected (9). Also, a synergistic action was noticed between cord factor and sulfolipid for toxicity in mice (8) and between P3 and cell wall skeleton for regression of tumors (10), for production of lung granuloma (9), and for protection from aerosol challenge with H37Rv in mice (12).

These findings suggest that a purified sample must be used to ascertain whether the adjuvant activity of cord factor in mice is exerted only by cord factor or trehalose mycolate itself or is attributable either to some contaminants such as wax D or to the synergistic action of trehalose mycolate and some other unidentified contaminant(s). Therefore, in the present study we used two highly purified trehalose mycolate preparations: P3, which showed one band in the pressure-accelerated chromatography (1), and a highly purified (DEAE-cellulose) cord factor that gave only one spot in thin-layer chromatography (6).

Data obtained with these purified samples showed that cord factor or trehalose mycolate

TABLE 3. *Effect of mycobacterial components incorporated in a water-in-oil emulsion on immune response of guinea pig against egg albumin*

Components tested[a]	Enhancement of antibody production[b]	Delayed skin reaction[c]	Corneal reaction[d]
Wax D (500 μg)	+++	+	++
AD (500 μg)	+++	+	++
AD6			
(500 μg)	+++	+	++
(50 μg)	+	+	−
Cord factor			
(500 μg)	±	−	−
(50; μg)	±	−	−

[a] AD, Acetylated wax D; AD6; acetylated wax D subfraction.

[b] Evaluated by the difference in the level of antibody titer between experimental and control groups. +++, Highly significant ($P < 0.005$); ++, significant ($P < 0.05$); ±, only slight, but statistically not significant ($P < 0.05$).

[c] Evaluated by the difference in the size of the induration measured at 48 h between experimental and control groups. +, Statistically significant ($P < 0.05$); −, not significant.

[d] Evaluated by the degree of the corneal opacity examined at 48 h. ++, Opacity of moderate intensity by our arbitrary grading; −, no opacity.

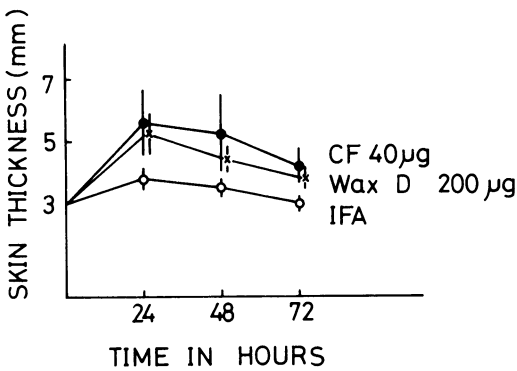

FIG. 5. *Effect of cord factor (CF) and wax D on skin reaction in rats. Five rats for each group were immunized with 100 μg of egg albumin in Freund incomplete adjuvant (IFA) containing no addition, cord factor, or wax D. Two weeks later, they were injected with 50 μg of egg albumin intradermally in the flank, and doubled skin thickness was measured for induration.*

itself can in fact exert an adjuvant effect in mice in terms of enhancement of antibody production, confirming the data of Bekierkunst et al. (3). The fact that 1 μg of cord factor enhanced antibody production but 5 μg of wax D did not agree with the view that cord factor itself acts as the adjuvant (Fig. 1). Enhancement of antibody production by cord factor was repeatedly confirmed with different antigens (sheep erythrocytes, hamster erythrocytes, and egg albumin) and different mouse strains (CF1 and C57BL). On a weight basis, cord factor appeared to exert a stronger adjuvant effect than tubercle baccilli in mice. No apparent differences were observed in toxicity or in adjuvant effect between cord factor from Aoyama B possessing mycolic acid and that of *Arthrobacter* possessing corynomycolic acid (C_{32-36}).

Since cord factor showed a remarkable inflammatory capacity in mice and this capacity appeared to correlate with its adjuvant activity, it is tempting to speculate that the tissue-damaging mechanism might operate in the adjuvant activity of cord factor in mice. However, cord factor showed neither general toxicity nor local inflammatory activity in rats, in which the lipid nevertheless behaved as a good adjuvant (R. Saito, A. Tanaka, K. Sugiyama, and M. Kato, Am. Rev. Respir. Dis., in press). The mechanism for its adjuvant effect remains to be resolved.

In the study of Bekierkunst et al., 5 to 10 μg of wax D did not act as adjuvant (3), and in our study also, 5 μg of wax D did not enhance antibody production in mice. However, 100 μg of wax D acted as adjuvant. This wax D preparation caused no footpad swelling in mice on injection and gave no extractable material on repeated extractions by hot acetone, suggesting that it is almost free from cord factor. Wax D is possibly a weak adjuvant in mice. Acetylated wax D and its subfraction, AD6, were also weak adjuvants in mice.

In contrast to mice, guinea pigs reacted to the mycobacterial lipids very differently. Five hundred micrograms of cord factor showed little adjuvant effect, whereas the same amounts of wax D and acetylated wax D exerted a strong adjuvant effect both in antibody production and in induction of delayed hypersensitivity. This confirms previous observations (14, 16).

Also, rats gave results different from those of mice and guinea pigs. Cord factor acted as a good adjuvant in this species, not only stimulating antibody production but also inducing delayed hypersensitivity. However, in contrast to mice, little inflammatory swelling was observable in the site of injection. General toxicity was also not observable except the effusion of ascites after the intraperitoneal injection of a large amount (1,350 μg) of cord factor dissolved in oil. Wax D exerted about the same degree of adjuvant activity as cord factor in rats. It is interesting that cord factor is not active as adjuvant in inducing delayed hypersensitivity in one animal species (guinea pigs) but is active in another (rats).

Tubercle bacilli as a whole act as a good adjuvant in many animal species. However, the present data indicate that at the level of extracted bacillary components, different animal species react differently to the separate components; a component active as adjuvant in one species is not always active in another. It is suggested that this should be taken into consideration when the adjuvant-active mycobacterial components are applied to humans.

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