Antibody Formation to Trehalose-6,6'-Dimycolate (Cord Factor) of *Mycobacterium tuberculosis*

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A complex of trehalose-6,6'-dimycolate (cord factor) and methylated bovine serum albumin was prepared, and its effect on the toxicity of cord factor in mice was studied. Either the active immunization with cord factor-methylated bovine serum albumin complex or the passive transfer of sera of rabbits vaccinated with the complex protected mice against the toxic action of cord factor. The antisera of mice and rabbits and gamma globulin fraction of rabbit antiserum demonstrated a precipitin reaction with an aqueous emulsion of cord factor. The reactivity of anticord factor was totally lost by treatment with 2-mercaptoethanol. The antigenic determinant group of cord factor was α -D-trehalose. Cord factor-anti-cord factor complex was devoid of activity to induce in vitro swelling and inhibition of respiratory and phosphorylative activity of mouse liver mitochondria. These results suggest that vaccination with cord factor-methylated bovine serum albumin complex induced the production of antibody to cord factor in mouse and rabbit serum which binds to cord factor and neutralizes its toxic activity either in vivo or in vitro.

Previous studies of the cellular biochemical lesion produced by trehalose-6,6'-dimycolate (cord factor), a toxic glycolipid of *Mycobacterium tuberculosis* (18), have shown that cord factor induced either in vivo (7, 12) or in vitro (10, 11) a disintegration of the mitochondrial membrane system and a functional damage of the membrane-associated respiration and phosphorylation in mouse liver. This metabolic lesion was assumed to be the biochemical basis of the toxicity of cord factor (11). In an attempt to confirm this assumption and to analyze further the role of cord factor in the pathogenesis of tuberculosis, a method for producing antibody to cord factor was devised by vaccinating animals with a cord factor-protein complex, since cord factor, a glycolipid of molecular weight of 2,900 ($C_{186}H_{366}O_{17}$), is not antigenic by itself (2).

This paper describes a method for preparing cord factor-methylated bovine serum albumin (MBSA) complex and its effect on the toxic property of cord factor. It is shown in this study that vaccination with cord factor-MBSA complex protected mice against the toxic effect of cord factor and that antibody to cord factor was produced in sera of mice and rabbits vaccinated with the complex. This antibody demonstrated a precipitin reaction with cord factor and neutralized its toxicity either in vivo or in vitro.

MATERIALS AND METHODS

Animals. Male albino mice of random-bred ddO stock (13) 4 to 8 weeks of age were used. They were fed a standard commercial diet (Oriental Pellet Diet MF, Oriental Yeast Industr. Co., Tokyo, Japan) and water ad lib. Rabbits weighing 2 to 3 kg were obtained from a commercial source and maintained on an Oriental Pellet Diet RC-5 and water.

Bacterial strain. An attenuated *M. bovis*, BCG (strain 1331), was supplied by the Department of Tuberculosis, National Institute of Health, Tokyo, Japan. A 7-day-old culture of BCG in Tween-albumin medium was used as reference vaccine.

Cord factor and related compounds. Cord factor was prepared by the method of Noll and Bloch (17) from live cultures of virulent human tubercle bacilli, strain H37Rv, grown for 4 weeks in Sauton synthetic liquid medium. The chemical and physicochemical specifications of the preparation used in the present study were given previously (13). Mycolic acid was prepared from the purified wax of H37Rv by the method of Asselineau and Lederer (1). Acetylated cord factor was prepared as previously described (8). Trehalose-6,6'-dicorynomycolate [the cord factor of *Corynebacterium diphtheriae* (20)] was prepared by T. Itoneda and generously supplied by E. Lederer, Institut de Chimie des Substances Naturelles, Gif-sur-Yvette, France. Methyl 6-mycoloyl α -D-glucopyranoside, 6,6'-dimycoloyl sucrose and 6,x-di-(3'-acetoxy,x-methoxymycoloyl)-N-acetyl-D-glucosamine were given by J. Asselineau, Laboratoire de Biochimie Structurale et Métabolique, Université Paul Sabatier,

Toulouse, France. The aqueous emulsion of these natural and semisynthetic lipids was prepared by a modification (11) of a previously reported procedure (6).

For injection into mice, cord factor was dissolved in Bayol F (Esso Standard Oil Co., New York, N.Y.), sterilized by heating at 100 C for 15 min, and injected intraperitoneally.

Preparation of ^{14}C -labeled cord factor. *M. tuberculosis* H37Rv was grown for 6 weeks in Sauton medium containing 0.3 μCi per ml of sodium acetate- $U\text{-}^{14}\text{C}$ (specific activity = 22.2 mCi/mmol). The bacilli were collected on a sintered glass filter and washed with distilled water until the filtrate was free of radioactivity. Cord factor was purified from wax C fraction by the method of Noll and Bloch (17). The radioactivity of ^{14}C -labeled cord factor was measured in an aqueous emulsion with Bray's reagent (3) in a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., La Grange, Ill.).

Preparation of MBSA. Bovine serum albumin powder fraction V (Armour Pharmaceutical Co., Kankakee, Ill.) was methylated by the method of Mandell and Hershey (16). The methylated product was repeatedly washed with methanol and ether and stored in a desiccator over KOH.

Preparation of cord factor-MBSA complex. An aqueous emulsion of cord factor (2 mg/ml) was added into a MBSA solution in distilled water to give a cord factor-MBSA ratio of 1:3 (w/w). A flocculent precipitate appeared after standing at 2 C overnight. This was allowed to sediment at the bottom of the tube by standing for 3 days at 2 C and then was precipitated by centrifuging at $10,000 \times g$ for 30 min in a refrigerated centrifuge. The packed sediment was taken in distilled water and stored at 2 C. The content of protein in the complex was determined by the Folin phenol reagent as described by Inoue and Nojima (5). Crystalline bovine serum albumin (BSA) was used as a standard. The content of cord factor was determined by using ^{14}C -labeled cord factor and measuring the radioactivity in the formed complex by liquid scintillation counting.

Vaccination. The aqueous suspension of cord factor-MBSA complex was emulsified with an equal volume of Freund's incomplete adjuvant (Difco) and injected subcutaneously into mice and rabbits. Vaccination of mice was started at 4 weeks of age, and the complex equivalent to 50 μg of cord factor was injected twice a week for 3 weeks. Rabbits were vaccinated with the complex equivalent to 1 mg of cord factor in the same manner. One to two weeks after the last vaccinating injection, the animals were subjected to a variety of experiments to test the effect of vaccination.

Live BCG vaccine was injected intravenously, and the effect of vaccination was tested 4 to 6 weeks after injection.

Preparation of serum from vaccinated animals. Mice were bled by decapitation and rabbits by cardiac puncture 10 to 14 days after the last vaccinating injection. After the blood was allowed to clot at 37 C for 1 hr and then at 2 C overnight, serum was collected by centrifugation at 2,000 rev/min for 1 hr and then

at $14,000 \times g$ for 1 hr in a Beckman model L-2 ultracentrifuge with a swing bucket-type SW25.1 rotor at 0 to 2 C. The pooled mouse and rabbit sera were inactivated by heating at 56 C for 30 min and stored frozen at -25 C. Sera from nonvaccinated and BCG-vaccinated animals were used as controls.

Preparation of gamma globulin fraction. Gamma globulin was prepared from antiserum by the method of Kekwick (14). A solution (10 mg/ml) in 0.2 M glycine-NaOH buffer, pH 8.05, was stored frozen at -25 C.

Serologic test. For the determination of precipitin titer, the aqueous emulsion of cord factor was added to a series of tubes containing increasing dilutions of serum in 0.2 M glycine-NaOH buffer, pH 8.05, containing 0.1% gelatine (GGB). The mixture was allowed to stand for 3 to 7 days at 2 C. For the quantitative precipitin test, various amounts of ^{14}C -labeled cord factor were added to a constant volume of undiluted antiserum. The mixture was kept at 2 C for 7 days with daily shaking. The formed precipitate was collected by centrifugation at 2,000 rev/min for 1 hr at 2 C. The precipitate was washed twice with ice-cold saline and suspended in 2 ml of a mixture of 50 volumes of 0.2 N NaOH + 4% Na_2CO_3 and 1 volume of 1% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ + 1% sodium tartrate. One milliliter of the suspension was incubated at 25 C for 30 min and extracted with 2 ml of ether to remove cord factor. The aqueous solution was heated for 5 min in a boiling-water bath, and the protein content in a sample was determined by using Folin phenol reagent (15) with BSA as a standard. Another 1 ml of the suspension of the washed precipitate was subjected to measurement of radioactivity by liquid scintillation counting with Bray's reagent (3) to determine the amount of cord factor bound to antibody.

The supernatant fluid was analyzed either for antibody excess by addition of cord factor or for antigen excess by radioactivity counting after the separation of precipitate.

For the precipitin inhibition test, varying amounts of sugars and mycolic acid were mixed with antiserum and incubated at 2 C overnight. To this mixture was added an appropriate amount of ^{14}C -labeled cord factor, and the mixture was allowed to stand for 3 days at 2 C. Radioactivity and protein content in the washed precipitate was measured as described above, and the per cent inhibition of the precipitin reaction between cord factor and antiserum was determined in terms of either radioactivity (antigen) or protein (antibody) content in the formed precipitate.

Reduction of antibodies with 2-mercaptoethanol. Either antiserum or the gamma globulin solution was mixed with an equal volume of 0.2 M 2-mercaptoethanol in 0.2 M glycine-NaOH buffer, pH 8.05, and incubated for 1 hr at 37 C. Antibodies treated without 2-mercaptoethanol served as a control.

Cord factor toxicity. The toxicity of cord factor was tested either by injecting mice intraperitoneally with 10 μg of cord factor at 2-day intervals and recording the loss of body weight and the number of deaths for 2 weeks or by injecting the graded amounts of cord factor intraperitoneally and estimating the 50% lethal

dose (LD₅₀) at 14 days by the method of Reed and Muench (19).

Mitochondrial swelling. The swelling of mouse liver mitochondria was measured as reported previously (10). Liver mitochondria prepared by a slight modification (11) of the Hogeboom procedure (4) were suspended in a test medium containing 0.154 M KCl + 0.02 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.4. The change of absorbancy at 520 nm was read every 5 min for 15 to 30 min after the addition of either cord factor or cord factor-anti-cord factor complex.

Measurement of mitochondrial oxidative phosphorylation. Phosphorylation coupled to the aerobic oxidation of either succinate or pyruvate+malate was measured as previously described (11).

Chemicals. Cytochrome *c* (type VI from horse heart), adenosine diphosphate (ADP; grade 1 from equine muscle), hexokinase (type III from yeast), and crystalline bovine serum albumin were products of Sigma Chemical Co., St. Louis, Mo.; bovine gamma globulin (fraction II) was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio; rotenone was purchased from K & K Laboratories, Plainview, N.Y.; sodium acetate-*U*-¹⁴C was the product of Daiichi Chemical Co., Tokyo, Japan.

RESULTS

Determination of binding of cord factor to MBSA. Table 1 summarizes the results of component analysis of cord factor-MBSA complex. As reported in a previous paper (6), cord factor never forms precipitate at 2°C when it is properly emulsified in water. The precipitate formed in the presence of MBSA was analyzed for the content of either protein or cord factor by using ¹⁴C-labeled cord factor. In a number of preparations, the ratio of cord factor to MBSA was found to be 10:1. When BSA was substituted for MBSA, no precipitate was formed even after a prolonged storage at 2°C.

Effect of vaccination with cord factor-MBSA complex on cord factor-toxicity. Repeated vaccination with cord factor-MBSA complex exhibited no apparent toxic effect in mice except that a certain degree of induration was produced by subcutaneous injection and a swelling of the foot by injection into the footpad. Figure 1 demonstrates that the vaccinated mice showed a marked resistance to the toxic action of the repeated injection of cord factor. The body weight of the vaccinated mice decreased after the first injection of cord factor but increased thereafter as that of control mice. The vaccinated mice survived the successive injection of cord factor, whereas all the nonvaccinated control mice gradually lost their body weight and died between 1 and 2 weeks.

Vaccination with cord factor-MBSA complex protected mice also from the toxic effect of a single large dose of cord factor. In a number of

TABLE 1. Protein content in cord factor-methylated bovine serum albumin (MBSA) complex

Prepn	Amt of cord factor ^a (μg)	Amt of MBSA ^b (μg)	Ratio of cord factor to MBSA ^c
1	250	24.5	10.5
2	250	25.0	10.0
3	500	52.0	9.6
4	500	48.5	10.3
5	1000	96.5	10.4

^a The content of cord factor was determined by the use of ¹⁴C-labeled cord factor (specific activity = 36,000 counts per min per mg).

^b MBSA was determined by Folin phenol reagent as described in the text.

^c Mean ratio = 10.2 ± 0.4.

FIG. 1. Effect of cord factor on mice vaccinated with cord factor-MBSA complex. Ten mice in each group were injected intraperitoneally with either 0.1 ml of Bayol F (Bayol F control) or 10 μg of cord factor per 0.1 ml of Bayol F (nonvaccinated control and cord factor-MBSA vaccinated) at 2-day intervals as indicated by arrows. Upper curve: mean body weight of mice, with standard deviation. Lower curve: survival of mice.

experiments, the LD₅₀ of cord factor ranged from 50 to 76 μ g in the nonvaccinated control mice and higher than 1 mg in the vaccinated mice. These results indicate that mice vaccinated with cord factor-MBSA complex acquire an enhanced resistance to the toxic action of cord factor.

In accord with the above results, the liver mitochondria prepared from mice vaccinated with cord factor-MBSA complex were not affected *in vivo* by cord factor (Table 2). The difference of the mitochondrial response to cord factor between vaccinated and nonvaccinated mice was evident when mice received repeated injections of 10 μ g of cord factor. Such repeated injection of cord factor caused a marked decline in both the protein content and the respiratory and phosphorylative activities in mitochondria in control mice, whereas the protein content and the above enzymatic processes in mitochondria were not affected in the vaccinated mice.

The protective potency of cord factor-MBSA complex against the toxicity of cord factor became manifest after repeated vaccination during at least 3 weeks.

The results summarized in Table 3 compare the protective effect of cord factor-MBSA complex against cord factor-toxicity with those of other relating preparations. It is shown that cord factor MBSA complex vaccinated with Freund's incomplete adjuvant by the subcutaneous route or into the footpad was most effective. Without adjuvant, it was completely ineffective even by the same routes. Intravenous vaccination without adjuvant was less effective. Either cord factor or MBSA alone was entirely ineffective. When mycolic acid or acetylated cord factor was substituted for cord factor and BSA for MBSA, neither the formation of a lipid-protein complex

nor the protective effect against cord factor toxicity was noted. Methyl 6-mycoloyl α -D-glucopyranoside and 6,6'-dimycoloyl sucrose formed a complex with MBSA, but vaccination with this complex did not protect mice against cord factor toxicity. As reported previously (9), live BCG vaccine was without effect on the toxicity of cord factor in mice.

Effect of antiserum on cord factor toxicity in mice. The above results led to a hypothesis that the vaccination of mice with cord factor-MBSA complex induced the production of antibody to cord factor in mouse serum, which neutralized the toxic action of cord factor *in vivo*. To obtain substantial evidence for this view, the effect of the transfer of sera of rabbits vaccinated with cord factor-MBSA complex on cord factor toxicity in mice was studied.

Figure 2 illustrates the effect of transfer of antiserum on the toxic manifestation of cord factor in mice. Mice which received daily injection of either normal or BCG-vaccinated rabbit sera, as well as those which received no serum injection, lost their body weight by repeated intraperitoneal injection of cord factor and died within 1 to 2 weeks. Mice injected with serum of rabbits vaccinated with cord factor-MBSA complex gained their body weight, as did those which received Bayol F only, and survived five injections of cord factor. Characteristic wasting symptoms due to the delayed toxicity of cord factor were not seen in mice injected with the sera of rabbits vaccinated with cord factor-MBSA complex.

Serologic data. The above results indicate that the serum of rabbits vaccinated with cord factor-MBSA complex neutralizes the toxicity of cord factor *in vivo*. This suggests that the serum

TABLE 2. *Effect of vaccination with cord factor-MBSA complex on in vivo action of cord factor on oxidative phosphorylation in mouse liver mitochondria*

Injection	No. of injections	Nonvaccinated			Cord factor-MBSA-vaccinated		
		Total protein (mg/g of liver)	Oxidation rate ^b	P/O	Total protein (mg/g of liver)	Oxidation rate ^b	P/O
Bayol F	1	39.2 $\pm$ 2.4 ^a	254 $\pm$ 13	1.68 $\pm$ 0.05	38.8 $\pm$ 2.0	242 $\pm$ 28	1.74 $\pm$ 0.08
Cord factor	1 ^c	28.4 $\pm$ 1.8 ^d	186 $\pm$ 16 ^d	1.51 $\pm$ 0.10 ^d	32.3 $\pm$ 4.1	208 $\pm$ 12	1.65 $\pm$ 0.03
Bayol F	5	36.7 $\pm$ 2.4	248 $\pm$ 23	1.77 $\pm$ 0.12	35.2 $\pm$ 3.6	228 $\pm$ 18	1.67 $\pm$ 0.11
Cord factor	5 ^e	25.7 $\pm$ 1.3 ^d	159 $\pm$ 24 ^d	0.96 $\pm$ 0.06 ^d	34.0 $\pm$ 1.5	217 $\pm$ 15	1.79 $\pm$ 0.04

^a Figures represent mean $\pm$ standard deviation of six mice in each group. Substrate: 10 mM succinate + 100 nM rotenone.

^b Nanoatoms of oxygen taken up per minute per milligram of protein.

^c Twenty-four hours after the intraperitoneal injection of 50 μ g of cord factor per 0.1 ml of Bayol F.

^d Variance ratio exceeds 1% level of significance.

^e In this experiment, mice received five successive intraperitoneal injections of 10 μ g of cord factor per 0.1 ml of Bayol F at 2-day intervals. Determination of protein content and assay of oxidative phosphorylation of mitochondria were carried out 24 hr after the last injection.

TABLE 3. *Protective effect of various vaccines against toxicity of cord factor*

Vaccine ^a	Adjuvant	Route of vaccination	Protection (no. of survivors/ no. tested at 14 days) ^b	
			Expt 1	Expt 2
Cord factor-MBSA	+	Subcutaneous	9/10	13/15
Cord factor-MBSA	+	Footpad	10/10	14/15
Cord factor-MBSA	—	Subcutaneous	1/10	1/15
Cord factor-MBSA	—	Footpad	0/10	ND ^c
Cord factor-MBSA	—	Intravenous	5/10	8/15
Cord factor only	+	Subcutaneous	0/10	ND
MBSA only	+	Subcutaneous	1/10	ND
Cord factor + BSA	+	Subcutaneous	2/10	2/15
Mycolic acid + MBSA	+	Subcutaneous	0/10	2/15
Acetylated cord factor + MBSA	+	Subcutaneous	0/10	ND
Methyl 6-mycoloyl α -D-glucopyranoside-MBSA	+	Subcutaneous	1/10	0/15
6,6'-Dimycoloyl sucrose-MBSA	+	Subcutaneous	0/10	ND
Live BCG	—	Intravenous	0/10	2/20
None	—		1/10	0/15

^a All vaccines except BCG were injected twice a week for 3 weeks with or without Freund's incomplete adjuvant by the designated route of vaccination. BCG (1.5×10^7 viable units) was vaccinated intravenously 4 weeks before the test of cord factor toxicity.

^b Cord factor toxicity was tested by injecting mice with 10 μ g of cord factor at 2-day intervals for 14 days.

^c Not done.

contains antibody to cord factor which may show a specific serologic reaction with cord factor. In a preliminary experiment to determine such a system, it was found that the antiserum of mice and rabbits demonstrates a precipitin reaction with the aqueous emulsion of cord factor. Table 4 shows that this precipitin reaction was seen only with the sera of rabbits vaccinated with cord factor-MBSA complex, whereas sera of either normal or BCG-vaccinated rabbits showed no reactivity with the cord factor emulsion. As shown in Table 5, anti-cord factor antibody was produced in all mice and rabbits vaccinated with cord factor-MBSA complex, whereas no antibody against cord factor was demonstrated in sera of normal and BCG-vaccinated mice and rabbits.

Representative results of quantitative precipitin reaction between cord factor and anti-cord factor rabbit serum (with $\times 320$ antibody titer) are shown in Fig. 3. Cord factor was completely precipitated in the region of antibody excess where the ratio of antibody to antigen was linear and ranged between 2 and 1. Beyond the equivalence zone, plots of the ratio of antibody to antigen yielded a less steep curve, declining from 1 to 0.5. The inhibition of the precipitate formation in the region of antigen excess was not marked.

Figure 4 gives the results of a precipitin reaction between cord factor and partially purified gamma globulin fraction of the antiserum. With constant

amount of cord factor and the increasing concentration of gamma globulin, the contents of both cord factor and protein in the formed precipitate increased and reached a maximum when all the added cord factor was precipitated with antibody. The plot of the ratio of antibody to antigen gave a linear curve and ranged between 0.8 and 0.5.

The precipitin reaction between cord factor and anti-cord factor antibody was completely abolished when either antiserum or the gamma globulin solution was treated with 2-mercaptoethanol, indicating that anti-cord factor produced under the present vaccination schedule is composed mainly of 19S IgM.

Antigenic specificity. In Table 6, the reactivity of the anti-cord factor serum toward several analogues of cord factor is shown. In addition to the cord factor of *M. tuberculosis* (trehalose-6,6'-dimycolate), trehalose-6,6'-dicorynomycolate of *C. diphtheriae* reacted with the antiserum, whereas neither mycolic acid, methyl 6-mycoloyl α -D-glucopyranoside, 6,6'-dimycoloyl sucrose, nor 6,x-di-(3'-acetoxy, x-methoxymycoloyl)-N-acetyl-D-glucosamine showed precipitin reaction with anti-cord factor serum. These results indicate that the antigenic determinant group of the cord factor molecule in the precipitin reaction is its sugar part, namely α -D-trehalose. To confirm this, precipitin inhibition studies using a number of sugar and mycolic acid were carried out. As shown

in Fig. 5, α -D-trehalose inhibited approximately 50% of the precipitin reaction between cord factor and anti-cord factor serum, whereas D-glucose, sucrose, maltose, and cellobiose were entirely

FIG. 2. Toxic action of cord factor on mice injected with serum of rabbits vaccinated with cord factor-MBSA complex. Ten mice in each group were injected intraperitoneally with either 0.1 ml of Bayol F only (Bayol F control) or 10 μ g of cord factor per 0.1 ml of Bayol F (all other groups) at 2-day intervals as indicated by arrows. Serum (0.2 ml) of nonvaccinated and BCG- or cord factor-MBSA-vaccinated rabbits were injected intraperitoneally everyday. Bayol F controls were not injected with serum. Upper curve: mean body weight of mice, with standard deviation. Lower curve: survival of mice.

FIG. 3. Quantitative precipitin reaction between cord factor and anti-cord factor serum. 14 C-labeled cord factor (specific activity = 15,200 counts per min per mg) emulsified in 0.5 ml of water was added to 0.5 ml of undiluted antiserum. The precipitate was collected after the mixture was allowed to stand at 2 C for 7 days. Antibody excess was detected by the addition of 100 μ g of cord factor to the supernatant fluid after the removal of the precipitate. In the region of antigen excess, free cord factor was demonstrated in the supernatant fluid, after the precipitate was removed, by radioactivity counting.

FIG. 4. Precipitin reaction between cord factor and gamma globulin fraction prepared from anti-cord factor serum. To 0.5 ml of the serial dilution of gamma globulin solution in 0.2 M glycine-NaOH buffer, pH 8.05, was added 0.5 mg of 14 C-labeled cord factor (specific activity = 15,200 counts per min per mg) per 0.25 ml; the mixture was incubated at 2 C for 3 days.

TABLE 4. Precipitin reaction between cord factor and serum of rabbit vaccinated with cord factor-MBSA complex

Serum	Reciprocal of serum dilution ^a									GGB
	1	2	10	20	40	80	160	320	640	
Cord factor-MBSA-vaccinated rabbit.....	3+ ^b	3+	3+	3+	2+	1+	1+	1+	+	—
BCG-vaccinated rabbit...	+	—	—	—	—	—	—	—	—	—
Nonvaccinated rabbit....	+	—	—	—	—	—	—	—	—	—

^a Cord factor (100 μ g) emulsified in 0.05 ml of water was added to the serial dilutions of serum (0.25 ml) in 0.2 M glycine-NaOH buffer, pH 8.05, containing 0.1% gelatine (GGB). The mixture was allowed to stand for 3 days at 2 C.

^b The intensity of the precipitin reaction was scored on an arbitrary scale from 3+ (complete precipitation with clear supernatant) to —.

TABLE 5. *Production of anti-cord factor antibody in mice and rabbits*

Animal	Vaccine	No. of animals with antibody/no. tested	Antibody titer by precipitation ^a
Mice	Cord factor-MBSA complex	9/9	1:320 (±) 1:80 1:160 1:80 1:160 1:40 1:160 1:40 1:80
Mice	Live BCG	0/10	No precipitate with undiluted sera
Mice	None	0/20	No precipitate with undiluted sera
Rabbits	Cord factor-MBSA complex	15/15	1:640 (±) 1:320 1:80 1:320 1:160 1:80 1:320 1:160 1:80 1:320 1:160 1:40 1:320 1:160 1:40
Rabbits	Live BCG	0/4	1:2 (±) 1:1 1:1 1:1
Rabbits	None	0/5	1:1 1:1 1:1 1:1 1:1

^a The last dilution of serum which gives 1+ or ± precipitin reaction.

TABLE 6. *Reactivity of anti-cord factor serum toward various cord factor analogues*

Lipid ^a	Reciprocal of antiserum dilution							
	2	10	20	40	80	160	320	GGB
Trehalose-6,6'-dimycolate.....	3+	3+	3+	2+	1+	1+	1+	—
Trehalose-6,6'-dicorynomycolate.....	3+	3+	3+	2+	1+	±	—	
Mycolic acid.....	—	—	—	—	—	—	—	
Sucrose-6,6'-dimycolate.....	±	—	—	—	—	—	—	
Methyl 6-mycoloyl α-D-glucopyranoside.....	—	—	—	—	—	—	—	
6, x-di-(3'-acetoxy, x-methoxy-mycoloyl)-N-acetyl-D-glucosamine.....	—	—	—	—	—	—	—	

^a An aqueous emulsion of 100 µg of each lipid in 0.05 ml of distilled water was added to the serial dilutions of the anti-cord factor serum (0.25 ml) in 0.2 M glycine-NaOH buffer, pH 8.05, containing 0.1% gelatine (GGB). The mixture was allowed to stand for 3 days at 2 C. Scale is as described in footnote b, Table 4.

ineffective as inhibitors of the precipitin reaction. Mycolic acid (not shown in Fig. 5) was inactive as an inhibitor in the range tested (100 to 1,000 µg/ml).

Hypersensitive state. All rabbits vaccinated with cord factor-MBSA complex showed a weak immediate skin hypersensitivity to MBSA but no delayed hypersensitive reaction to the purified-protein derivative test for tuberculosis, whereas the BCG-vaccinated rabbits were hypersensitized to the latter. The skin reactivity to cord factor in the cord factor-MBSA-vaccinated rabbits was difficult to demonstrate, since the test antigen (cord factor) is toxic and caused a necrotic lesion in the injection area. At least, the neutralization of this skin toxicity of cord factor was not seen

in the rabbits vaccinated with cord factor-MBSA complex.

Neutralization of cord factor toxicity by anti-cord factor in vitro. As shown previously (10), cord factor induced in vitro a swelling accompanied by partial disruption of mouse liver mitochondria. This structural damage of the mitochondrial membrane system was associated with the impairment of the metabolic activity of mitochondrial oxidative phosphorylation in both succinate and nicotinamide adenine dinucleotide pathways of the electron transport chain (11). It seemed of interest to determine whether the binding of anti-cord factor to cord factor neutralizes the action of the latter to induce the above structural and functional damage in mitochondria.

TABLE 7. *Effect of cord factor and cord factor-anti-cord factor complex on oxidative phosphorylation in mouse liver mitochondria*

Substrate	Cord factor concn ($\mu\text{g}/\text{mg}$ of protein) ^a	Cord factor		Cord factor-anti-cord factor complex	
		Oxidation rate ^b	P/O	Oxidation rate ^b	P/O
Succinate + rotenone	0	210	1.93	205	1.99
	25	200	1.58	208	1.95
	50	186	1.24	212	1.91
	100	172	1.03	206	2.01
Pyruvate + malate	0	72	2.96	68	3.01
	25	65	2.45	70	2.98
	50	42	2.03	70	3.02
	100	33	1.78	69	3.01

^a Mitochondria were preincubated for 15 min at 20 C with either cord factor or cord factor-anti-cord factor complex before addition to the assay medium.

^b Nanoatoms of oxygen taken up per minute per milligram of protein.

FIG. 5. *Inhibition of precipitin reaction between cord factor and anti-cord factor serum with α -D-trehalose. Symbols: —○—, α -D-trehalose (measured by radioactivity counting); —○—, α -D-trehalose (measured by protein determination); ●, sucrose; ○, glucose; □, maltose; Δ, cellobiose.*

As shown in Fig. 6, the cord factor-anti-cord factor complex did not induce the mitochondrial swelling, whereas cord factor ranging from 25 to 100 μg produced a dose-dependent swelling of mouse liver mitochondria. Since neither BSA nor bovine gamma globulin protected the cord factor-induced swelling of mitochondria, the above effect of anti-cord factor is not attributable to a nonspecific protective effect of protein.

Cord factor-anti-cord factor complex did not inhibit the mitochondrial respiration and accompanying phosphorylation in the presence of either succinate or pyruvate + malate as substrate (Table 7).

These results indicate that the action of cord factor to induce in vitro structural and functional damage in mouse liver mitochondria was completely lost by the binding to its specific antibody.

FIG. 6. *Effect of cord factor-anti-cord factor complex on mouse liver mitochondria. The test system contained in a final volume of 3.0 ml: 0.154 M KCl + 0.02 M Tris-hydrochloride (pH 7.4), 0.6 mg of mitochondrial protein, and either cord factor or cord factor-anti-cord factor complex emulsified in 0.25 M sucrose + 0.01 M Tris-hydrochloride (pH 7.4). Bovine serum albumin (BSA) and bovine gamma globulin (BGG) were added in a volume of 0.1 ml. Optical density reading was done at 20 C.*

DISCUSSION

Data presented herein demonstrate that anti-body to cord factor (trehalose-6,6'-dimycolate) is produced in sera of animals vaccinated with a complex of cord factor and MBSA and that the antibody precipitates cord factor and neutralizes its toxicity either in vivo or in vitro.

The use of MBSA to obtain a complex with

cord factor was based on recent work of Inoue and Nojima (5). These authors reported that potent anticardiolipin antibodies were produced in sera of rabbits vaccinated with a complex of cardiolipin antigen (cardiolipin, lecithin, and cholesterol) and MBSA. Since BSA becomes basic after methylation, it was assumed that cardiolipin, a strongly acidic phospholipid, and MBSA form a complex probably by ionic bonds and become an effective immunogen for producing cardiolipin antibodies (5). However, the nature of the bond between cord factor, a neutral glycolipid, and MBSA is unknown. It is at present assumed that cord factor binds to MBSA through hydrophobic bonds, since MBSA is more hydrophobic than BSA and binds even to a mixture of lecithin and cholesterol (5). There remains an alternative possibility that the hydroxyl groups in the cord factor molecule play a certain role in binding to MBSA through some physical grouping, since the totally acetylated cord factor did not form a complex with MBSA. Unexpectedly, mycolic acid, though acidic in nature, was found to be quite ineffective in forming a complex with MBSA. BSA, on the other hand, was entirely ineffective in producing a complex with cord factor. Taking the molecular weight of MBSA as 60,000 to 70,000 and that of cord factor as 2,900, the ratio of cord factor to MBSA, 10:1, indicates that an appreciable amount of cord factor was bound to MBSA as a haptenic glycolipid.

The precise mechanism of the serologic reaction between cord factor and anti-cord factor serum is not fully understood. The aqueous emulsion of cord factor is translucent and forms no precipitate when stored at 2°C for a prolonged time (6, 11). The precipitate formed between cord factor and anti-cord factor gave a packed sediment, by low-speed centrifugation, which could be repeatedly washed with cold saline without loss of bound protein. A typical quantitative precipitin curve thus was obtained with all antisera to cord factor, except that the ratio of antibody to antigen showed a biphasic curve and the content of antibody in the immune precipitate was extremely low at equivalence zone and even in the region of antibody excess. This is also true in the system with cord factor and partially purified gamma globulin from antiserum, indicating that a large amount of cord factor is bound to antibody to form the immune precipitate. Thus, the precipitin reaction between cord factor and anti-cord factor seems to be a greatly modified one. Further study of the nature of this immune reaction and the characterization of the antibody to cord factor is in process.

Precipitin inhibition studies clearly indicate that the antigenic determinant group of cord

factor is α -D-trehalose. This seems of interest since there are several indications that the hydroxyl groups of trehalose play an important role in the toxic property of cord factor (8, 10-12) and in the formation of an antigenic complex with MBSA. The fact that the sugar moiety defined the antigenic specificity of the haptenic glycolipids in the complex with MBSA gives a reasonable explanation for the ineffectiveness of the complex of MBSA with either methyl 6-mycoloyl α -D-glucopyranoside or 6,6'-dimycoloyl sucrose as the protective vaccine against cord factor toxicity.

It should be noted that the protein content and activity of oxidative phosphorylation in liver mitochondria of the cord factor-MBSA-vaccinated mice were not affected *in vivo* by the injection of cord factor and that cord factor lost its activity to induce *in vitro* mitochondrial swelling and impairment of respiratory and phosphorylative activity in mouse liver mitochondria when it was bound to anti-cord factor. These findings are consistent with an earlier assumption (11) that the structural and functional damage in host cell mitochondria is the biochemical basis of the toxic action of cord factor.

The possibility that antibodies to cord factor are produced in animal sera by vaccination with live BCG and participate in the immunogenic activity of the latter seems to be excluded since, as shown previously (9), the BCG-vaccinated mice were as susceptible to the toxic action of cord factor as nonvaccinated control mice. It is also shown in the present study that the serum of BCG-vaccinated rabbits neither protected mice against cord factor toxicity nor showed any reactivity to cord factor. Thus, it may be concluded that vaccination with live BCG vaccine does not produce antibodies to cord factor in animal sera and that the mechanism involved in the immunogenic activity of live BCG vaccine and of cord factor-MBSA complex is essentially different.

Whether cord factor plays a certain role in the pathogenesis of tuberculosis has long been a question. To obtain a substantial answer to this question, study of the effect of immunization with cord factor-MBSA complex or the injection of anti-cord factor serum on experimental tuberculosis in mice is in process.

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