Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 737–740, February 1991
Biochemistry

Cord factor (α,α-trehalose 6,6′-dimycolate) inhibits fusion between phospholipid vesicles

(trehalose/membrane fusion/liposomes/tuberculosis/nocardiosis)

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Communicated by John D. Baldeschwieler, October 15, 1990

ABSTRACT **The persistence of numerous pathogenic bacteria important in disease states, such as tuberculosis, in humans and domestic animals has been ascribed to an inhibition of fusion between the phagosomal vesicles containing the bacteria and lysosomes in the host cells [Elsbach, P. & Weiss, J. (1988) *Biochim. Biophys. Acta* 974, 29–52; Thoen, C. O. (1988) *J. Am. Vet. Med. Assoc.* 193, 1045–1048]. In tuberculosis this effect has been indirectly attributed to the production of cord factor (α,α-trehalose 6,6′-dimycolate). We show here that cord factor is extraordinarily effective at inhibiting Ca^{2+}-induced fusion between phospholipid vesicles and suggest a mechanism by which cord factor confers this effect. These findings are likely to be important in our understanding of the pathogenesis and treatment of many diseases of bacterial etiology.**

Foreign bacteria are normally identified as such and phagocytized by macrophages in higher animals, including humans (1–3, 41). In macrophages the phagocytic vesicle containing the bacteria (the phagosome) fuses with lysosomes, ultimately resulting in degradation of the bacteria. However, in numerous diseases, phagosomal–lysosomal fusion is inhibited in some way, resulting in persistence of the bacteria within the host cell. For example, the bacteria *Mycobacterium tuberculosis* and *Nocardia asteroides*, the etiological agents for tuberculosis and nocardiosis, respectively, elicit normal cellular responses to foreign bacteria such as release of superoxide and phagocytosis by macrophages (4, 5), but the bacteria-containing phagosome does not fuse with either primary or secondary lysosomes (4–7). The bacteria are thus free to continue to divide and to invade other host cells. Although the means of persistence of the bacteria are therefore clear, the mechanism by which they evade phagosomal–lysosomal fusion has not been elucidated, despite the importance of this phenomenon in human welfare. In this report we provide data that suggest such a mechanism.

Cord factor (CF), or α,α-trehalose 6,6′-dimycolate, is a cell wall glycolipid of *Mycobacterium*, *Nocardia*, *Rhodococcus*, and *Corynebacterium*. The term cord factor was coined by Noll *et al.* (8) to describe the glycolipid obtained after a gentle petroleum ether extract of *Mycobacterium tuberculosis*, which prevented formation of bacterial cords, or filaments. Observations of the effects of CF and related analogues have provided the basis for suggesting that this molecule plays a role in the persistence of the bacteria in the host cell (5–7). The CF molecule consists of the sugar trehalose esterified to two mycolic acid residues (Fig. 1), which range in length from 20 to 80 carbons, depending on the bacterial species. Three decades of research on CF have uncovered a myriad of biological activities in the pathogenesis of mycobacterial and nocardial diseases, including high toxicity in mice (9, 10), antitumor activity (11), immunomodulation (12, 13), and granulomagenic activity (14). Indirect evidence has been provided that CF might be responsible for inhibiting fusion between adjacent membranes *in vivo* (6). This finding is particularly appealing in view of the work of Goodrich and Baldeschwieler (15, 16) and Hoekstra and coworkers (17, 18), where carbohydrates anchored to the membrane by a hydrophobic group have been shown to confer an inhibition of fusion in model membrane systems. Goodrich and Baldeschwieler (15, 16) reported that galactose anchored to cholesterol prevents fusion damage to liposomes during freezing and drying. Using the glycosphingolipid globoside GL-4, Hoekstra and coworkers (17, 18) have observed that a reduction in the initial rate of fusion is related to the size of the headgroup of the globoside. Furthermore, trehalose, a sugar found at high concentrations in many organisms that naturally survive freezing or dehydration (19, 20), strongly inhibits fusion between membranes during freezing (21, 22) or dehydration (19, 20). This remarkable effect, which is thought to account partly for the survival of extreme dehydration by many organisms, requires direct interaction between the trehalose and the membranes, probably involving hydrogen bonding between hydroxyl groups on the sugar and the polar head group of the phospholipids (19, 20). In the present study we show, using a model liposome system under fusogenic conditions, that CF is more effective than free trehalose in inhibiting fusion and suggest a mechanism for this effect that may help to elucidate the role of CF under conditions thought to exist *in vivo*. We wish to point out at the outset, however, that in order for CF to inhibit phagosomal–lysosomal fusion *in vivo* it must be found in the phagosomal membrane. As yet, there is no evidence that this molecule is transferred from the bacterial cell, where it is synthesized, to the phagosomal membrane. Thus, the data presented here establish only that CF can strongly inhibit fusion between bilayers but do not demonstrate that this is the mechanism *in vivo*.

MATERIALS AND METHODS

Reagents. All analytical grade organic solvents were glass distilled and stored in glass containers. Palmitoyloleoylphosphatidic acid (Pam,Ole-PA) and egg phosphatidylethanolamine (PE) were obtained from Avanti Polar Lipids and checked for purity by TLC. Fluorescent probes 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) and *p*-xylenebispyridinium bromide (DPX) were procured from Molecular Probes. High purity α,α-trehalose dihydrate was obtained from Pfanstiehl Laboratories.

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Abbreviations: PE, egg phosphatidylethanolamine; Pam,Ole-PA, palmitoyloleoylphosphatidic acid; LUVs, large unilamellar vesicles; ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid; DPX, *p*-xylenebispyridinium bromide; CF, cord factor.

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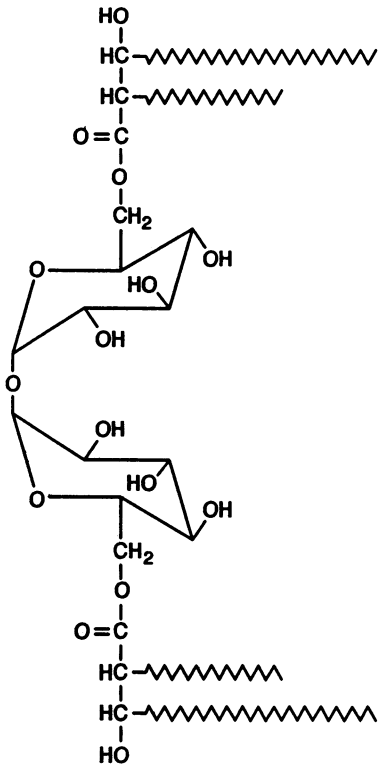

FIG. 1. CF, or α,α-trehalose 6,6'-dimycolate, derived from *N. asteroides* strain GUH-2. This strain produces CF with mycolic acid residues ranging from C_{48} to C_{54} in length and 0–4 degrees of unsaturation.

Purification of Cord Factor. *N. asteroides* CF was purified from *N. asteroides* strain GUH-2. *N. asteroides* was grown to late logarithmic phase (24 hr) on brain heart infusion medium containing 1% glycerol at 37°C. Formalin-killed bacteria were collected by filtration, rinsed of medium with Dulbecco's phosphate-buffered saline, and freeze-dried (lyophilized) prior to extraction of the cell wall. The cell wall was extracted three times with chloroform/methanol, 1:2 (vol/vol). The chloroform and methanol were removed from cell wall extract by flash evaporation; the extract was redissolved in chloroform. The chloroform-soluble fraction was purified essentially as described by Silva *et al.* (23). Purity of CF was determined by mass spectroscopy (Hewlett–Packard) and infrared spectroscopy (Perkin–Elmer model 1750). Free mycolic acids were isolated by alkaline hydrolysis of CF and purified by silica gel TLC with *n*-hexane/diethyl ether/acetone/acetic acid, 70:30:11:1 (vol/vol) as the mobile phase.

Liposomes. Large unilamellar vesicles (LUVs) of Pam,-Ole-PA and PE [1:1 molar ratio (Pam,Ole-PA/PE)] with and without CF as indicated were prepared by modification of the ether vaporization method of Deamer (24). Briefly, an ether emulsion of lipid was injected into aqueous buffer containing fluorescent probe at 70°C, and the resulting liposomes were then extruded at 500 psi (1 psi = 6.89 kPa) of N_2 through two 0.2-μm Nuclepore filters maintained at 70°C (25). LUVs were prepared to contain either 25 mM ANTS, 40 mM NaCl, or 90 mM DPX in 10 mM *N*-tris(hydroxymethyl)methyl-2-aminosulfonic acid (Tes) buffer (26). External, untrapped buffer and probe were replaced by 10 mM Tes/100 mM NaCl (pH 7.4 at 25°C) by centrifugation across Bio-Rad P-6 100- to 200-mesh resin (27).

Fusion Assay. Ca^{2+}-induced fusion was measured by the mixing of aqueous internal contents of two populations of liposomes. One population was prepared to contain a fluorescent probe (ANTS); the other was prepared to contain a fluorescent quencher (DPX). Upon fusion of these two liposomes, a decrease in fluorescence is seen by collision-dependent quenching. Leakage of either probe from the liposome does not result in fluorescence quenching (28). Cuvettes were prepared to contain lipid at 0.1 mg/ml in 10 mM Tes/100 mM NaCl. Total fusion (0% fluorescence) was determined by preparation of LUVs containing both fluorescent probe and quench probe. Maximum fluorescence for each sample was determined prior to addition of $CaCl_2$. Calcium-induced fusion was allowed to take place in a cuvette at 25°C with periodic mixing. Final fluorescence was measured 5 min after the addition of $CaCl_2$. Independent measurements of these LUVs indicate that no further quenching of fluorescent probe occurs after a 5-min incubation.

Freeze-Fracture Electron Microscopy. Control and calcium-fused vesicles were quenched from room temperature in liquid propane and maintained in liquid nitrogen. A Balzers BAF 400D freeze-fracture apparatus was used to obtain standard knife-fracture replicas (at −100°C) by vaporization of platinum at 45°. Replicas were examined and photographed on a Philips 410 electron microscope.

Photon Correlation Spectroscopy. Quasi-elastic light scattering was performed using a Langley–Ford model 1096 correlator run in the autocorrelation mode. The light source was the 488-nm line of an argon laser. Scattered light was collected at 90° by using a Hamamatsu R585 photomultiplier tube. Data were analyzed by cumulative fit. Liposomes were incubated with 10 mM Ca^{2+} at 25°C for 5 min prior to sampling. Average frequency (Γ) was determined by using cumulative data collected eight times for 120 sec each.

RESULTS AND DISCUSSION

To investigate the effects of CF on fusion between membranes, we have used as a model system artificial lipid vesicles (liposomes) prepared with and without CF incorporated into the bilayer. Separate studies (to be described elsewhere) suggest that CF is incorporated into the phospholipid bilayers, as one might expect from consideration of the structure of the CF molecule. Since membrane fusion in cells is known to be influenced strongly by Ca^{2+} (29–32), a lipid mixture was chosen that easily undergoes fusion upon addition of this cation. We are well aware that this lipid mixture does not mimic the composition of biological membranes *in vitro*, but suggest that it nevertheless provides a useful model. Results from three independent techniques show that CF strongly inhibits Ca^{2+}-induced fusion in this model.

Freeze Fracture. The results from freeze-fracture studies (Fig. 2) provide visual evidence of the stabilizing effect of CF with respect to fusion. We found that liposomes made either with or without CF could be kept for hours at room temperature without evidence of fusion. When 10 mM Ca^{2+} was added to vesicles that had been prepared without CF, massive fusion resulted, leading to formation of vesicles ≈10 times the diameter of the original vesicles (Fig. 2*B*). By contrast, when Ca^{2+} was added to vesicles containing 0.1 mol of CF per mol of phospholipid, only slight amounts of fusion were evident in the freeze-fracture images (Fig. 2*D*).

Quasi-Elastic Light Scattering. Data on the size of the vesicles obtained by quasi-elastic light scattering are consistent with the freeze-fracture data (Table 1). The average diameter of the unilamellar vesicles as calculated from the average frequency (Γ) increased 15-fold in the presence of 10 mM Ca^{2+}, whereas those prepared with CF increased in average diameter less than 4-fold under the same conditions.

Fluorescence Quenching. The effects of CF on the inhibition of Ca^{2+}-induced fusion were quantified most extensively by using fluorescence quenching to monitor fusion (28). In this particular assay, two water-soluble fluorescent probes were

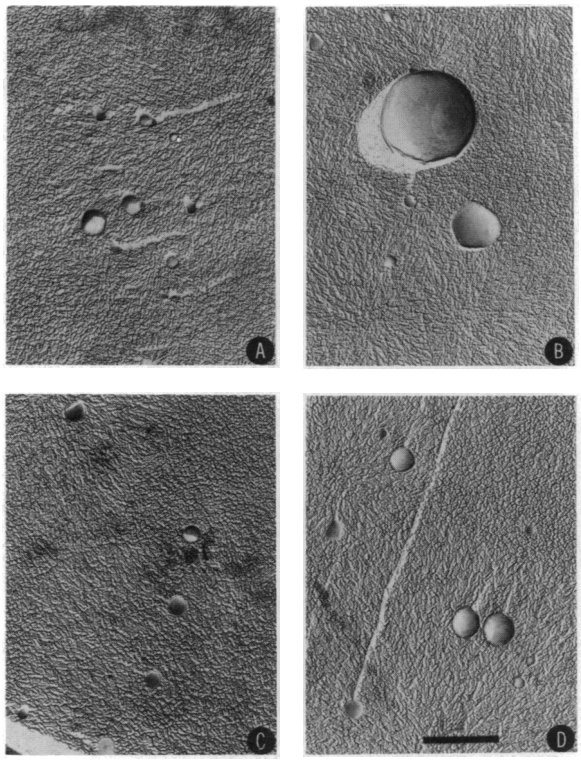

FIG. 2. Freeze-fracture micrographs of Pam,Ole-PA/PE LUVs in the absence of Ca^{2+} (*A*), Pam,Ole-PA/PE LUVs in the presence of 10 mM Ca^{2+} (*B*), Pam,Ole-PA/PE LUVs containing 0.1 mol of CF per mol of phospholipid (*C*), and Pam,Ole-PA/PE LUVs containing 0.1 mol of CF per mol of phospholipid in the presence of 10 mM Ca^{2+} (*D*). (Bar = 500 nm.)

trapped separately in the aqueous interior of two populations of vesicles during their preparation (see *Materials and Methods*). The two populations of vesicles were mixed at equal concentrations immediately before the experiment. One of the two probes (the donor) is quenched by the other (the acceptor), provided that both probes reside in the same vesicle. As a result, fluorescence decreases as fusion increases (Fig. 3). Since the probability of fusion of a vesicle containing the donor probe with one containing the acceptor probe was the same as that for fusion with one containing the donor, a decrease in fluorescence of 50% means that every vesicle has, on average, undergone one fusion event. Therefore, these data indicate a conservative estimate of the actual fusion. The resulting data show an inhibition of fusion in the vesicles made with CF (Fig. 3).

Effects of Components of CF on Fusion. The component of CF that is responsible for inhibiting fusion was investigated in the following experiments.

(*i*) *Effects of mycolic acids.* The mycolic acids were de-esterified from CF and purified. Liposomes were then prepared with the addition of 0.2 mol of mycolic acids per mol of phospholipid, an amount equivalent to that added when the vesicles were made with the intact CF. Since free fatty acids are known to be fusogenic (33–36), we expected these

Table 1. Quasi-elastic light scattering of Pam,Ole-PA/PE LUVs with or without CF in the presence and absence of Ca^{2+}

CF	Ca^{2+}	Γ, sec^{-1}	D, $cm^2 \cdot sec^{-1}$	Diameter, Å
−	−	2922 ± 77	4.9×10^{-8}	1,000
−	+	185 ± 173	3.2×10^{-9}	15,200
+	−	2757 ± 115	4.7×10^{-8}	1,020
+	+	726 ± 198	1.2×10^{-8}	4,030

D, diffusion coefficient.

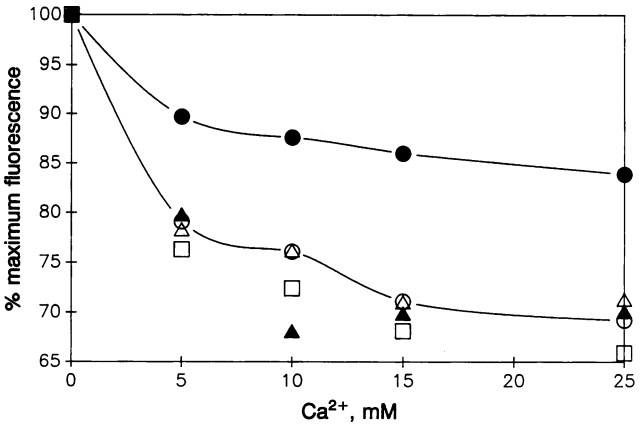

FIG. 3. Calcium-induced fusion of Pam,Ole-PA/PE LUVs (○) as a control and Pam,Ole-PA/PE LUVs containing 0.1 mol of CF per mol of phospholipid (●). These data indicate that fusion is inhibited by the presence of CF in the bilayer. The effects of free mycolic acids (△), trehalose (▲), and both free mycolic acids and trehalose (□) on Ca^{2+}-induced fusion of LUVs are shown. These data indicate that there is no effect of the trehalose or mycolic acid moieties of CF at equivalent concentrations to that in vesicles containing CF.

mycolic acids to have similar effects, if they affected fusion at all. The results showed that addition of these hydrocarbons to the vesicles had no effect (Fig. 3), possibly because the effects of Ca^{2+} on fusion are so pronounced that a further stimulus towards fusion by the mycolic acids could not be detected by this assay. The conclusion can be drawn, however, that the mycolic acids clearly do not inhibit fusion.

(*ii*) *Effects of trehalose.* Trehalose was added to vesicles that had been made without CF at 1.25 mM, a concentration equivalent to that in the vesicles containing CF. Addition of this amount of trehalose had no effect on Ca^{2+}-induced fusion (Fig. 3), and increasing the free trehalose concentration to 200 mM had no further effect on the inhibition of fusion (data not shown).

(*iii*) *Synergism between mycolic acids and trehalose.* We tested the possibility that the free mycolic acids and trehalose might have a synergistic effect in inhibiting fusion. The results (Fig. 3) showed that this was not the case. We concluded from these experiments that the intact CF was required to inhibit fusion.

Mechanism for Inhibition of Fusion by CF: An Hypothesis. These data clearly show that CF can inhibit Ca^{2+}-induced fusion between phospholipid bilayers *in vitro*, a finding that is consistent with the suggestion that it may play a similar role in inhibiting fusion between phagosomes and lysosomes *in vivo*. On the basis of these and other observations (cf. ref. 19), we suggest the following mechanism for this effect. Since CF consists of trehalose attached to a hydrophobic component, this molecule would be expected to show a hydrophobic interaction with phospholipid bilayers, thus immobilizing the trehalose at the aqueous interface between the hydrophobic interior of the bilayer and the surrounding bulk water. With the trehalose immobilized at the surface of the bilayer, one would expect CF to be particularly effective at inhibiting fusion between phospholipid bilayers, where we suggest it could affect fusion in at least two ways: it could increase the hydration force that is known to be an important primary barrier to fusion (37–40); alternatively, it could act as a steric block to fusion. We believe that the results reported here will have important implications for our understanding of the pathogenic mechanism in diseases such as tuberculosis and nocardiosis, and they may suggest possible approaches to clinical treatment by the eventual elucidation of the mechanism of inhibition of fusion between phagosomal and lyso-

somal membranes *in vivo*. It is conceivable that a greater understanding of this mechanism could lead to drug therapies that would reduce the length of the therapy and increase the eradication of these and related diseases in many Third World countries in which they are endemic.

We thank Dr. J. Vesenka for assistance with collection of the quasi-elastic light scattering data and Drs. T. J. Anchordoguy and E. Z. Drobnis for critical review of this manuscript. This work was supported in part by Grants DMB 85-18194 from the National Science Foundation to J.H.C., 2RO1 AI20900 from the National Institutes of Health to B.L.B., and CNPq, FAPESP, FINEP, and BIO/Universidade de São Paulo to T.I.

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