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Mycobacteria Glycolipids as Potential Pathogenicity Effectors: Alteration of Model and Natural Membranes

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ABSTRACT: Four mycobacterial wall glycolipids were tested for their effects on phospholipidic liposome organization and passive permeability and on oxidative phosphorylation of isolated mitochondria. From fluorescence polarization of diphenylhexatriene performed on liposomes it was concluded that the two trehalose derivatives (dimycoloyltrehalose and polyphthienoyltrehalose) rigidified the fluid state of liposomes, the triglycosyl phenolphthiocerol slightly fluidized the gel state, while the peptidoglycolipid ("apolar" mycoside C) just shifted the phase transition temperature upward. Dimycoloyltrehalose was without effect on liposome passive permeability, as estimated from dicarboxyfluorescein leak rates, and polyphthienoyltrehalose and triglycosyl phenolphthiocerol slightly decreased leaks, while mycoside C dramatically increased leaks. Activity of these lipids on mitochondrial oxidative phosphorylation was examined. The two trehalose derivatives have been tested previously: both had the same type of inhibitory activity, dimycoloyltrehalose being the most active. Triglycosyl phenolphthiocerol was inactive. Mycoside C was very active, with effects resembling those of classical uncouplers: this suggested that its activity on mitochondria was related to its effect on permeability. All these membrane alterations were called nonspecific because it is likely that they result from nonspecific lipid-lipid interactions, and not from recognition between specific molecular structures. Such nonspecific interactions could be at the origin of some of the effects of mycobacteria glycolipids on cells of the immune system observed in the last few years.

Mycobacteria wall glycolipids are potential effectors of pathogenicity. For instance, it was shown that two mycolic acid derivatives, dimycoloyltrehalose (Cord factor) and mycoloyl diarabinoside, were active against mitochondrial oxidative phosphorylation (Kato, 1970; Rouanet & Lanêelle, 1983); a species-specific glycolipid, the triglycosyl phenolphthiocerol from *Mycobacterium leprae*, could have specific or nonspecific effects on lymphocytes (Mehra et al., 1984; Brett et al., 1984); a polar mycoside C of *Mycobacterium avium* (serovar 4) was also shown to be active on lymphocytes (Brownback & Barrow, 1988).

Lipids can represent 60% of mycobacteria walls, and some glycolipids are profusely produced in infected organs (Draper & Rees, 1973; Hunter & Brennan, 1981). As mycobacteria are causative agents of long-lasting diseases (tuberculosis, leprosy), and as pathogenic mycobacteria are intracellular parasites, glycolipids have enough time to diffuse and act. It is likely that membranes of host cells are the first target of these glycolipids, and as mycobacteria glycolipids have structures greatly differing from that of animal glycolipids, it is possible that they disturb the host membrane structure and function. However, very few studies have been devoted to effects of mycobacteria lipids on membranes, and they were performed with crude solvent extracts (Stewart-Tull et al., 1978; Roozemond et al., 1985).

We have looked for effects on model and natural membranes of four glycolipids (Figure 1): dimycoloyltrehalose (Cord factor, present in most mycobacteria), the diglycosyl peptidolipid (mycoside C) from *Mycobacterium smegmatis* (Daffê et al., 1983), whose structure is representative of the most abundant mycosides C present in *M. avium*, and two new glycolipids isolated from *Mycobacterium tuberculosis* (strain Canetti), namely, a triglycosyl phenophthiocerol (Daffê et al., 1987) and a polyphthienoyltrehalose (Minnikin et al., 1985; Daffê et al., 1988).

Materials and Methods

Mycobacteria Lipids. Polyphthienoyltrehalose and triglycosyl phenolphthiocerol, from *M. tuberculosis* (strain Canetti), were generous gifts of Drs. M. Daffê, C. Lacave, and M. A. Lanêelle. Mycoside C and dimycoloyltrehalose (Cord factor) were isolated from *M. smegmatis* (ATCC 607). Crude dimycoloyltrehalose was obtained in the methanol-precipitated fraction of lipid extracts, and mycoside C, in the supernatant. Both fractions were further purified by adsorption chromatography on silicic acid or on Florisil. The purity of the compounds was checked by thin-layer chromatography.

Molecular weights of the natural compounds were calculated from published formulae of the major compounds and from the relative percentages of their homologues as determined by mass spectrometry or gas-liquid chromatography: dimycoloyltrehalose (Cord factor), 2700 Da; polyphthienoyltrehalose, 1950 Da; triglycosyl phenolphthiocerol, 1820 Da; mycoside C, 1260 Da.

Miscellaneous Compounds. Dimyristoyl-*sn*-glycerophosphocholine, bovine brain phosphatidylserine (acyl residues 18:0, 54%; 18:1, 41%; higher homologues, 5%), and egg phosphatidylcholine and phosphatidic acid (acyl residues 16:0,

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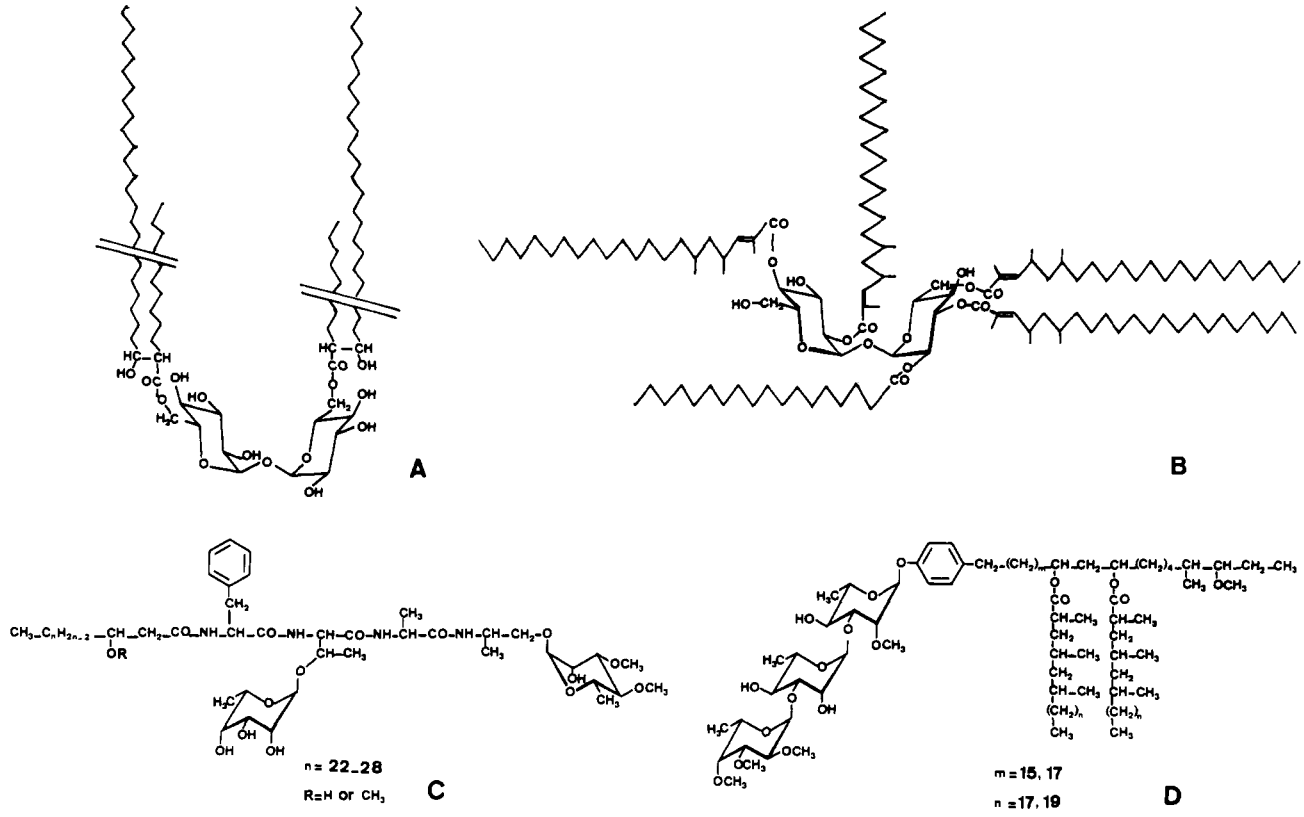

FIGURE 1: Formulas of the tested mycobacterial wall glycolipids: (A) dimycoloyltrehalose (Cord factor); (B) polyphthienoyltrehalose (Daffé et al., 1988); (C) glycopeptidolipid (mycoside C) (Daffé et al., 1983); (D) triglycosyl phenolphthiocerol (Daffé et al., 1987).

38%; 16:1, 5%; 18:0, 11%; 18:1, 31%; 18:2, 15%) were purchased from Sigma. Their purity was checked by thin-layer chromatography prior to use. Diphenylhexatriene was purchased from Sigma. Dicarboxyfluorescein was purchased from Eastman and purified according to Weinstein et al. (1983).

Liposomes and Glycolipid Suspensions. Liposomes used for fluorescence polarization assays contained dimyristoyl-*sn*-glycerophosphocholine, various amounts of the tested glycolipids, and 2% (molar ratio) of phosphatidic acid. In order to use liposomes with well-defined composition, the following procedure was used: chloroform solutions of lipids were mixed, and then solvent was evaporated; mixed lipids were dissolved in methylene chloride, and then the mixture was injected into buffer containing the fluorescent probe (5 mM sodium phosphate, 45 mM NaCl, 4×10^{-6} M diphenylhexatriene, pH 7.2) and maintained at 50 °C. Nitrogen was then bubbled through the suspension for 45 min at 50 °C to eliminate methylene chloride. There was no detectable effect of residual solvent since liposomes prepared with pure dimyristoyl-*sn*-glycerophosphocholine under these conditions had exactly the same fluorescence polarization characteristics as liposomes prepared without solvent from the same dry lipid. Fluorescence anisotropy ($I_{\parallel} - I_{\perp}/I_{\parallel} + 2I_{\perp}$) was measured on a fully automatic, temperature-programmed apparatus built in the laboratory by one of us (J.-F.T.).

Liposomes used for passive permeability were prepared with egg phosphatidylcholine, phosphatidylserine, and cholesterol (molar ratios 2/1/1) and used within 2 h. In a typical experiment, 4 μmol of total lipids was dispersed by sonication in 0.2 mL of buffer (5 mM sodium phosphate, pH 7.2) containing 120 mM carboxyfluorescein. Alternative 1-min periods of sonication and of cooling were used, until a clear solution was obtained. Liposomes were separated from this initial solution on Sephadex G-25 (1 × 10 cm column) and were maintained at 4 °C before use. This procedure was adopted, after testing several other published methods, since in our hands it gave the most stable liposomes and the most reproducible results.

Leaks of carboxyfluorescein were followed on a Jobin et Yvon JY3D spectrofluorimeter. Fluorescence increase was followed first for 10 min without any addition (control), and then the lipid suspension was added (assay, see Figure 3). Results were expressed as the ratio of the assay to the control slopes.

Glycolipid suspensions (0.4–0.8 mM) were prepared by injection into buffer (5 mM sodium phosphate, pH 7.2) of concentrated solutions of the glycolipids plus 2% (molar ratio) of phosphatidic acid. The solvents used were either hexamethylphosphoramide (it can be used for all lipids) or methylene chloride (mycoside C, triglycosyl phenolphthiocerol). For assays on mitochondria, suspensions contained 0.2 M sucrose. Suspensions up to 2 mg/mL were stable for several weeks.

In all experiments performed with these glycolipid suspensions, controls were done by adding to the cuvette a volume of buffer prepared in the same conditions as for the glycolipid suspension, but without glycolipid, in order to check that there was no influence of the solvent used to dissolve glycolipids.

Assays on Mitochondria. Rat liver mitochondria were isolated and tested according to routine laboratory conditions (Durand et al., 1979; Rouanet & Lanéelle, 1983). All preparations used had a respiratory control ratio (active to controlled respiration) at least of 3, in the control experiment. Glycolipids were tested either with a 10-min preincubation (at 25 °C, final volume 0.2 mL) with mitochondria (triglycosyl phenolphthiocerol) or by direct addition of the glycolipid suspension into the assay cuvette (mycoside C).

Results and Discussion

Effects on Phospholipid Bilayer Organization. Effects of glycolipids on membrane organization were tested by following

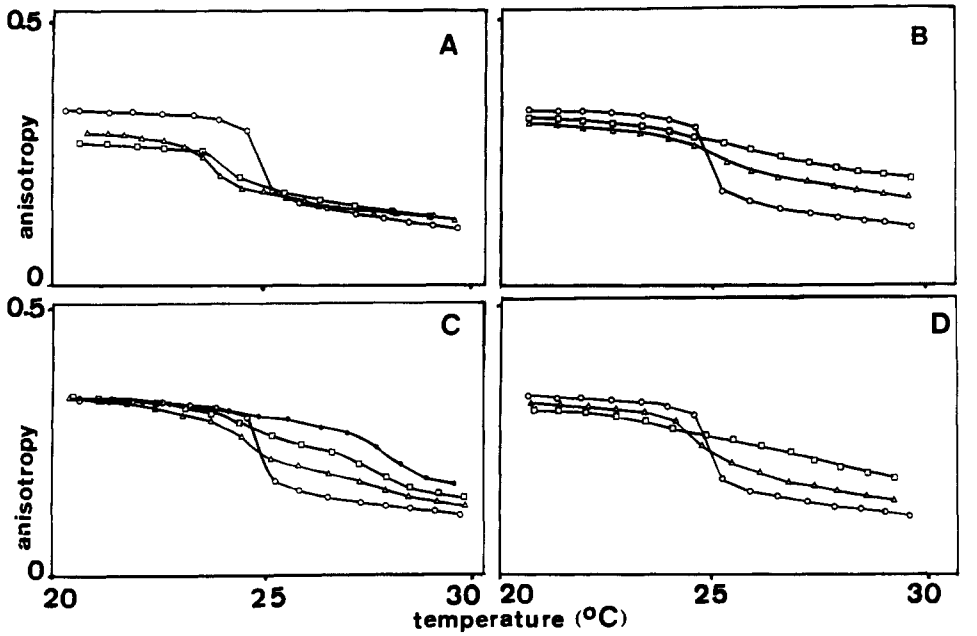

FIGURE 2: Glycolipid effects on the liposome phase transition critical temperature. Fluorescence anisotropy of diphenylhexatriene embedded in liposomes of dimyristoyl-*sn*-glycerophosphocholine containing various molar ratios of glycolipids (expressed below as mol %). (Empty circles) Pure phospholipid. (A) Triglycosyl phenolphthiocerol: (triangles) 1.8%; (squares) 3.6–5.5%. (B) Dimycoloyltrehalose (Cord factor): (triangles) 3.8%; (squares) 6.3%. (C) Glycopeptidolipid (mycoside C): (triangles) 2.7%; (squares) 8.1%; (full circles) 16.1%. (D) Polyphthienoyltrehalose: (triangles) 3.3%; (squares) 17%.

the fluorescence anisotropy of diphenylhexatriene embedded in liposomes of dimyristoyl-*sn*-glycerophosphocholine containing known amounts of glycolipids. The temperature dependence of the fluorescence anisotropy of the glycolipid–phospholipid liposomes is presented in Figure 2. It appeared that their respective effects on the "gel phase" (below the transition temperature) or on the "fluid phase" (above the transition temperature) were not the same for the four glycolipids.

Triglycosyl phenolphthiocerol (Figure 2A) decreased the fluorescence anisotropy of the gel phase, i.e., disorganized it slightly, but was without effect on the fluid phase. This resulted in a discrete lowering of the phase transition temperature. This agreed with the presence of branched fatty acids, known to introduce some disorder in ordered phases. As natural membranes are considered to be in a fluid state, it was not expected that this lipid could disturb them much.

The two trehalose derivatives (Figure 2B,D) had exactly the same effect, but dimycoloyltrehalose (Cord factor) (Figure 2B) acted at lower molar ratios than polyphthienoyltrehalose (Figure 2D). They did not disturb the gel phase, but both induced an increase of fluorescence anisotropy in the fluid state, i.e., some "rigidifcation" of the phase, with a progressive disappearance of the phase transition.

Mycoside C (Figure 2C) did not produce a detectable effect in the gel state, but widened and shifted upward the phase transition temperature, by about 3 °C for the highest mycoside C content tested (16% molar ratio), resulting in an apparent rigidification of the fluid state at temperatures slightly above the phase transition temperature.

Effects on Liposome Passive Permeability. The effects of glycolipids on membranes can be tested by addition of glycolipid suspensions to membrane preparations, since it has been shown that lipids are able to diffuse from their suspensions and to penetrate model or natural membranes (Lanêelle & Tocanne, 1980); moreover, dimycoloyltrehalose (Cord factor) and analogues were repeatedly tested on isolated mitochondria by this procedure [e.g., by Asselineau and Kato (1973), Durand et al. (1979), and Gillois et al. (1984)].

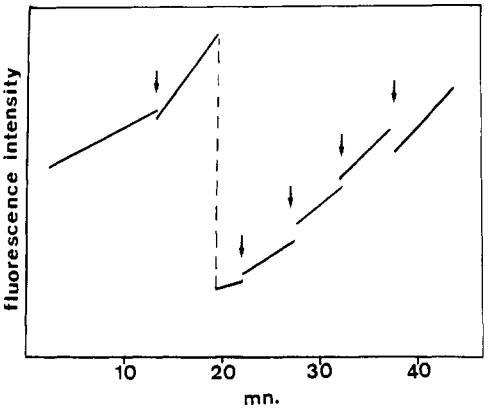

FIGURE 3: Liposome passive permeability to carboxyfluorescein. It was estimated by following the fluorescence intensity increase due to dilution in the medium of carboxyfluorescein that leaked out through the membrane of the liposomes. This figure shows a recorder trace of fluorescence intensity, before and after successive additions of the glycopeptidolipid (mycoside C): (arrows) mycoside C addition; (dotted line) scale reduction; (ordinates) fluorescence intensity (arbitrary units). Liposomes were prepared by sonication of a mixture of egg phosphatidylcholine, phosphatidylserine, and cholesterol (molar ratios 2/1/1).

The leakage of liposome-entrapped carboxyfluorescein was followed, before and after addition of glycolipid suspensions. This method allowed us to compare reliably the relative efficiency of glycolipids by comparing the ratios of the fluorescence intensity increase per minute in the presence of the glycolipid to the increase in the absence of the glycolipid (ratio, slope assay/slope control, S_a/S_c). A typical recording, resulting from an experiment performed with mycoside C, is shown in Figure 3. The effects of the four glycolipids are presented in Figure 4.

It appears that triglycosyl phenolphthiocerol and polyphthienoyltrehalose slightly inhibited dicarboxyfluorescein diffusion (Figure 4A). Dimycoloyltrehalose was without significant effect during the assay (up to 1 h) (Figure 4A); this could be due to slow diffusion kinetics of this glycolipid toward and inside liposomal membranes, since mycolic acids

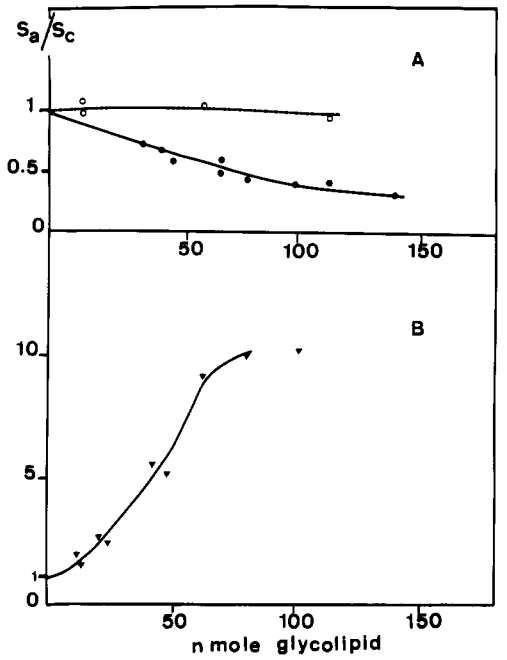

FIGURE 4: Dose dependence of leakage induced by glycolipids. Leaks of liposome-entrapped carboxyfluorescein were followed by measuring the slopes of recorded fluorescence intensity. This figure presents the slope assay/slope control ratios (S_a/S_c) calculated from the type of assay presented in Figure 3; curves shown were drawn from results of two independent experiments. (A) (Empty circles) Dimycoloyltrehalose (Cord factor); (full circles) polyphthienoyltrehalose or triglycosyl phenolphthiocerol (identical curves). (B) Glycopeptidolipid (mycoside C).

have very long chains. In contrast, mycoside C had a rather dramatic effect (Figure 4B), seen directly on the recorder trace (Figure 3); it immediately induced a dose-dependent increase of the passive permeability of liposomes.

Effects on liposome permeability have been reported (Stewart-Tull et al., 1978) in experiments performed with crude extracts either from the growth medium or from mycobacteria cells, but to our knowledge, defined cell wall compounds have not been tested previously on membrane passive permeability.

Effects on Mitochondrial Oxidative Phosphorylation. Dimycoloyltrehalose (Cord factor) has long been known to inhibit active respiration in the presence of ADP, and to uncouple it from phosphorylation, particularly at coupling site II (Kato, 1970). It was active both in vitro and in vivo (Kato, 1970; Kato & Fukushi, 1969).

The activity of polyphthienoyltrehalose was tested in our laboratory in the course of the structural study of this compound (Daffé et al., 1988): it had an activity on isolated rat liver mitochondria like that of dimycoloyltrehalose, but it was less active. The two other lipids have not been tested previously.

Triglycosyl phenolphthiocerol was apparently completely inactive, up to the highest tested dose (133 nmol/mg of protein).

Mycoside C was active immediately after addition to a mitochondrial suspension in the assay cuvette, while for the two trehalose derivatives a preincubation with mitochondria in a small volume (usually 200 μL) was needed to observe an effect. Mycoside C had effects resembling that of an uncoupler (Figure 5), since it did not change active respiration significantly (except at high doses), but it strongly increased the controlled respiration rate. A large dose-dependent effect was observed on controlled respiration measured just after addition of the glycolipid suspension (Figure 5B). Moreover, doses

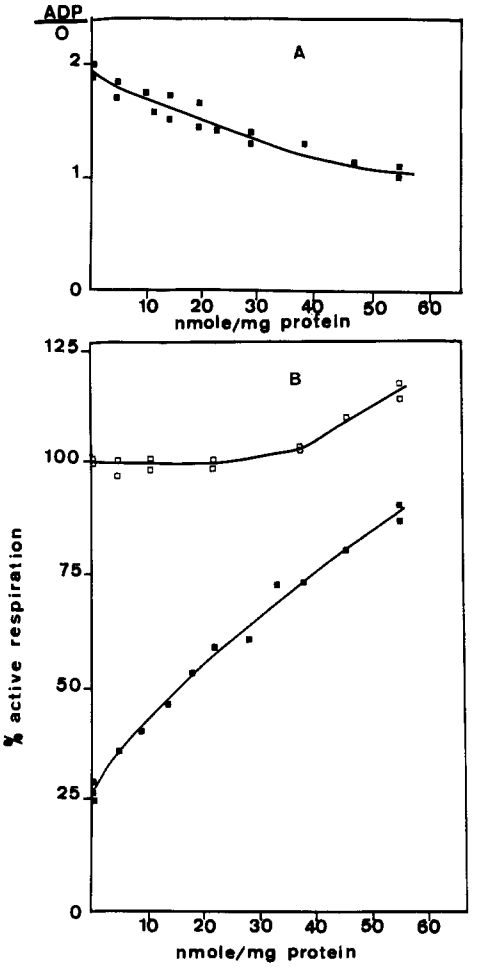

FIGURE 5: Dose-dependent effects of glycopeptidolipid (mycoside C) on oxidative phosphorylation in isolated rat liver mitochondria (succinate as substrate). Curves are the results of two independent experiments. (A) ADP/O. (B) Respiration. The reference value (100%) was the active respiration (in the presence of ADP) of non-inhibited mitochondria. (Empty squares) Active respiration; (full squares) controlled respiration (in the absence of ADP) measured 3 min after mycoside C addition.

around 50 nmol of glycolipid/mg of protein (i.e., 5×10^{-5} M mycoside C) induced the loss of 1 ADP/O unit (Figure 5A).

The two very active molecules tested, namely, dimycoloyltrehalose and mycoside C, acted on mitochondrial oxidative phosphorylation in vitro for similar doses, but they had distinct conditions of action and clearly different effects:

First, mycoside C was active without preincubation, while dimycoloyltrehalose was active after preincubation with mitochondria. This could be due to differences in the diffusion kinetics of these lipids toward mitochondrial membrane, since it was shown with model molecules that glycolipids were able to diffuse from their suspension and to penetrate within the mitochondrial membranes (Lanéelle & Tocanne, 1980). Pathogenic mycobacteria provoke long-lasting diseases, and it is likely that diffusion kinetics of glycolipids is not an important parameter in vivo.

Second, dimycoloyltrehalose had a rather specific activity, since studies with model molecules have shown that the nature of the sugar greatly influenced the activity on mitochondria (Kato & Asselineau, 1971; Asselineau & Kato, 1973; Gillois et al., 1984). This indicated that a specific interaction could participate in the inhibitory mechanism, suggesting an effect of Cord factor on proteins but not an effect on membrane passive permeability, in agreement with the results of the above assays on liposome permeability. Mycoside C inhibited oxi-

dative phosphorylation in a manner resembling that of classical uncouplers; therefore, the observed large increase of liposome permeability strongly suggested that mycoside C could act on mitochondria by increasing the proton passive permeability.

Conclusion

Glycolipids discovered in pathogenic mycobacteria during the last few years, i.e., polar mycosides C from *M. avium* and the triglycosyl phenolphthiocerol from *M. leprae*, were first studied for their antigenic properties, and then they were tested on cells of the immune system. Both glycolipids were found to modify the mitogenic response of lymphocytes (Brownback & Barrow, 1988). *M. leprae* phenolic glycolipid was first found to cause nonspecific inflammation (Brett et al., 1984) and to specifically suppress the oxidative response of monocytes (Vachula et al., 1989) and lymphocyte mitogenesis (Mehra et al., 1984).

It was proposed that at least some of these effects were specific to the tested glycolipid. However, it was recently shown (Fournié et al., 1989) that phenolic glycolipids from *M. leprae*, *Mycobacterium bovis*, and *Mycobacterium kansasii* were nearly equally effective in suppressing the lymphoproliferation in vitro, in spite of their different sugar epitopes. Thus it had to be considered that some of the observed effects could be due to nonspecific glycolipid–membrane interactions, as was found for those described here. For instance, one can postulate that membrane rigidifcation induced by trehalose derivatives might disturb the complex information exchanges occurring between cells of the immune system since it has been shown that membrane rigidification could inhibit leukocyte functions (Roozemond & Bonavida, 1985; Roozemond et al., 1985), and it is likely that the drastic effects observed with mycoside C cannot be without deleterious consequences on macrophages that have phagocyted *M. avium* cells.

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