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CHOLESTEROL-PHOSPHATIDYLCHOLINE DISPERSIONS AS DONORS OF CHOLESTEROL TO *MYCOPLASMA* MEMBRANES

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Summary

Growing cells of sterol-requiring *Mycoplasma hominis* and sterol non-requiring *Acholeplasma laidlawii* were used to test the ability of cholesterol-dipalmitoyl phosphatidylcholine dispersions to serve as cholesterol donors to these organisms. Dispersions with high cholesterol to phosphatidylcholine ratios were more effective than dispersions with low cholesterol to phosphatidylcholine ratios in donating cholesterol to the membranes of both mycoplasmas and in promoting growth of the sterol-requiring species. *M. hominis* took up almost three times as much cholesterol as did *A. laidlawii*. In addition, significant quantities of the phosphatidylcholine component of the dispersions were found to be associated with *M. hominis* membranes as against none in the *A. laidlawii* membrane preparations. In all cases, the percentage of cholesterol taken up by *M. hominis* from the dispersions exceeded that of phosphatidylcholine by a factor of 3—5. These results were interpreted to suggest that all the cholesterol taken up by *A. laidlawii* is transferred from the dispersion to the membranes by a process which involves only a transient contact between the organisms and the lipid dispersions, whereas a certain amount of the cholesterol taken up by *M. hominis* may also be derived from lipid dispersions adhering to or fusing with the cell membranes.

Introduction

Mycoplasmas offer several unique advantages for studying the incorporation and role of cholesterol in biological membranes. The majority of mycoplasmas require exogenous cholesterol for growth and incorporate large quantities of it into their plasma membrane. The inability of mycoplasmas to synthesize or esterify cholesterol, to hydrolyze cholesteryl esters, and to pinocytose lipoprotein particles, facilitates their use in investigating the factors controlling the transfer of cholesterol from an exogenous cholesterol source to the plasma

membrane [1,2]. The purpose of the present investigation was to test the efficiency of cholesterol-phospholipid dispersions as donors of cholesterol to mycoplasmas. The major effort was directed at answering two questions: (a) does the molar ratio of cholesterol to phospholipid in the lipid dispersions determine its effectiveness as a cholesterol donor? and (b), is the phospholipid component of the lipid dispersions incorporated into the membrane together with cholesterol?

Materials and Methods

Organisms and membranes. Mycoplasma hominis (ATCC 15056) and *Acholeplasma laidlawii* (oral strain) were grown in a modified Edward medium [3] in which the serum supplement was replaced by 0.5% bovine serum albumin, 20 μg/ml elaidic acid, and various cholesterol-phosphatidylcholine dispersions prepared as described below. For growth of *M. hominis* the medium was also supplemented with 20 mM L-arginine and its pH was adjusted to 6.5. The organisms were grown for 17—24 h at 37°C. Growth was estimated by measuring the absorbancy of the cultures at 640 nm. The organisms were harvested by centrifugation at 10000 × *g* for 15 min, washed in 0.25 M NaCl and osmotically lysed; the cell membranes were isolated and washed as described in detail by Razin and Rottem [3].

Lipid dispersions. The dispersions were prepared by a modification of the technique described by Cooper et al. [4]. Various quantities of cholesterol (8—60 mg) in chloroform were added to 40 mg of L-α-dipalmitoyl phosphatidylcholine (Sigma, St. Louis, Mo., U.S.A.) in a glass scintillation vial. The solvent was evaporated under nitrogen, leaving a thin lipid film on the bottom of the vial. 10 ml of 0.155 M NaCl were added and the suspension was sonicated in ice at maximum output of an M.S.E. ultrasonic disintegrator (60 W, 20 kHz) for four 15 min periods with 5 min intervals for cooling. The resulting dispersions were centrifuged at 22000 × *g* for 30 min to sediment undispersed lipid. The supernatant fluid was separated and kept at 4°C. Before being added to the growth medium, the lipid dispersion was centrifuged again as above, and in order to determine the exact molar ratio of cholesterol to phosphatidylcholine, the supernatant fluid was analysed for its cholesterol and phospholipid content as described below. For some experiments labeled cholesterol-phosphatidylcholine dispersions were prepared by the addition of small amounts of either [4-^{14}C]cholesterol (50 Ci/mol) or phosphatidyl[*Me*-^{14}C]choline (50 Ci/mol, New England Nuclear, Boston, Mass.) to the unlabeled cholesterol or phosphatidylcholine solutions used for preparing the lipid dispersions.

Density-gradient centrifugation. Cell membranes of organisms grown with [4-^{14}C]cholesterol-phosphatidylcholine dispersions were isolated, washed and subjected to centrifugation on freshly prepared discontinuous sucrose gradients consisting of 4-ml layers made of 20%, 30% and 60% (w/v) sucrose. After 2 h of centrifugation at 40 000 rev./min in the SW41 rotor of a Spinco preparative ultracentrifuge, the membranes which sedimented on top of the 60% sucrose layer were collected, washed twice in dilute β-buffer [3] and their protein content and radioactivity were determined. Radioactivity was also determined in samples of the two upper sucrose layers.

Analytical procedures. Protein was determined according to Lowry et al. [5]. Lipids were extracted from membranes with chloroform/methanol (2 : 1, v/v; ref. 6). The membrane lipid extracts or the lipid dispersions were analysed for cholesterol by the colorimetric technique of Rudel and Morris [7], and for lipid phosphorus by the method of Ames [8]. Thin-layer chromatography of membrane lipids was carried out as described before [6] using chloroform/methanol/acetic acid/water (100 : 60 : 16 : 8, by vol.) as the developing system. Phosphatidylcholine spots were detected on the plates by the molybdate spray reagent [9]. Radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer using a Triton X-100/toluene scintillation mixture composed of two parts of toluene scintillation liquor (4 g 2,5-diphenyloxazole and 100 mg 1,4-bis (5-phenyloxazolyl-2) benzene in 1 l of toluene) and one part of Triton X-100 (scintillation grade).

Results

The cholesterol requirement of *M. hominis* was met by the addition of cholesterol-phosphatidylcholine dispersions to the serum-free medium. The extent of growth depended on both the cholesterol to phospholipid molar ratio of the dispersions and on the concentration of cholesterol in the growth medium (Fig. 1). The higher the molar ratio of cholesterol to phospholipid, the better was the growth response, but for best growth the cholesterol concentration in the medium had to be greater than 5 μg/ml. As expected, the sterol non-requiring *A. laidlawii* grew very well in the serum-free medium without the

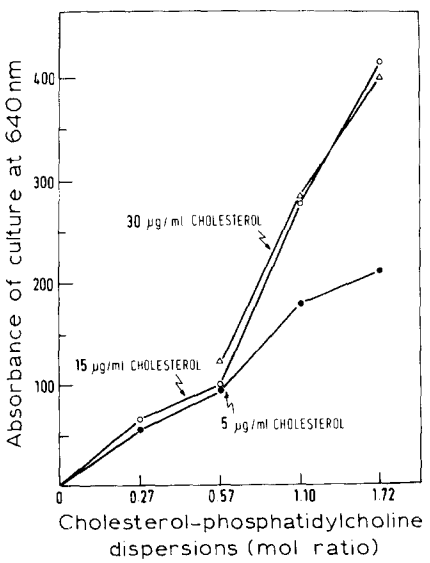

Fig. 1. Growth response of *M. hominis* to various concentrations of cholesterol added to the growth medium as cholesterol-phosphatidylcholine dispersions of different molar ratios. Absorbancy of cultures was determined after 21 h of incubation at 37°C. Data for 30 μg/ml cholesterol are not available for the 0.27 molar ratio dispersions as it was impractical to reach such a high concentration of cholesterol with this dispersion.

TABLE I

CHOLESTEROL AND PHOSPHOLIPID CONTENT IN MEMBRANES OF *M. HOMINIS* AND *A. LAIDLAWII* GROWN WITH CHOLESTEROL-PHOSPHATIDYLCHOLINE DISPERSIONS OF DIFFERENT MOLAR RATIOS

The data represent the mean of the results obtained in five experiments with different batches of cholesterol-phosphatidylcholine dispersions which explains the range in the cholesterol/phospholipid molar ratio in the 4 classes of dispersions used.

Cholesterol in medium		Organism					
μg/ml	Cholesterol/ phospholipid in dispersion (molar ratio)	*M. hominis*			*A. laidlawii*		
		Cholesterol		Phospholipid	Cholesterol		Phospholipid
		μg/mg protein	μmol/μmol lipid Pi	μg lipid Pi/ mg protein	μg/mg protein	μmol/μmol lipid Pi	μg lipid Pi/ mg protein
5	0.20—0.35	53 ± 9	0.42 ± 0.10	13 ± 3	13 ± 6	0.12 ± 0.04	11 ± 3
	0.52—0.62	68 ± 15	0.61 ± 0.14	10 ± 3	23 ± 4	0.18 ± 0.06	11 ± 2
	0.87—1.30	71 ± 10	0.89 ± 0.11	7 ± 2	23 ± 1	0.18 ± 0.06	11 ± 3
	1.50—1.94	76 ± 13	1.12 ± 0.10	6 ± 0.5	27 ± 7	0.23 ± 0.05	11 ± 2
15	0.20—0.35	72 ± 15	0.53 ± 0.10	13 ± 1	14 ± 6	0.10 ± 0.04	12 ± 2
	0.52—0.62	74 ± 3	0.62 ± 0.11	11 ± 1	21 ± 7	0.12 ± 0.02	11 ± 1
	0.87—1.30	75 ± 13	1.00 ± 0.11	6 ± 1	31 ± 6	0.23 ± 0.03	10 ± 2
	1.50—1.94	71 ± 10	1.09 ± 0.12	3 ± 0.5	40 ± 3	0.30 ± 0.03	11 ± 2
30	0.52—0.62	82 ± 12	0.61 ± 0.10	11 ± 1	27 ± 5	0.20 ± 0.03	13 ± 4
	0.87—1.30	72 ± 12	1.10 ± 0.03	6 ± 0.6	31 ± 2	0.27 ± 0.04	12 ± 3
	1.50—1.94	85 ± 8	1.76 ± 0.09	3 ± 0.4	37 ± 5	0.28 ± 0.03	10 ± 1
90	1.50—1.94	112 ± 3	1.67 ± 0.18	6 ± 0.9	45 ± 5	0.33 ± 0.06	10 ± 1

cholesterol-phosphatidylcholine dispersions. Moreover, when lipid dispersions were added to give cholesterol concentrations of 30 μg/ml and higher, growth was partially inhibited, with the cultures reaching absorbancy values at 640 nm of only 0.4 as against values of about 0.6 with little or no cholesterol.

Table I shows that the cholesterol to membrane protein values in *M. hominis* membranes showed some increase on increasing the cholesterol to phospholipid molar ratio of the lipid dispersions and on increasing the cholesterol concentration in the growth medium from 5 μg/ml to 30 μg/ml. A more significant increase in membrane cholesterol content was obtained by adding the dispersion with the highest cholesterol to phospholipid molar ratio to give a final concentration of 90 μg/ml in the medium. The ratio of cholesterol to membrane phospholipids, however, increased with the increase in the cholesterol to phospholipid molar ratio of the lipid dispersions while the ratio of membrane phospholipid to protein decreased. When related to membrane protein, the amounts of cholesterol taken up by *M. hominis* were almost three times as high as those taken up by *A. laidlawii* grown with the same lipid dispersions. Unlike the findings with *M. hominis*, the cholesterol content of *A. laidlawii* membranes increased on raising the cholesterol to phospholipid molar ratio of the lipid dispersion, this increase being accompanied by an almost parallel increase in the molar ratio of cholesterol to membrane phospholipids as the ratio of membrane phospholipids to protein showed little change. This trend was particularly pro-

TABLE II

BINDING OF PHOSPHATIDYLCHOLINE TO MEMBRANES OF *M. HOMINIS* GROWN WITH CHOLESTEROL-[^{14}C]PHOSPHATIDYLCHOLINE DISPERSIONS

Experiment	Cholesterol in medium		Membrane lipids			Dispersed phosphatidylcholine taken up (% total in medium)	Dispersed cholesterol taken up (% total in medium)
	µg/ml	Cholesterol/phosphatidylcholine in dispersion (molar ratio)	Cholesterol (µg/mg protein)	Total lipid Pi (µg/mg protein)	Phosphatidylcholine/total phospholipid * (mol %)		
1	10.9	0.76	67	12.20	11.4	1.6	4.9
	14.4	1.96	83	4.70	13.8	6.0	27.5
2	12.4	0.32	44	11.20	15.1	1.4	8.2
	17.4	1.94	65	8.70	6.4	2.4	10.2

* Calculation of the phosphatidylcholine content was based on the radioactivity values found in the membrane lipid extract compared to the specific radioactivity value of phosphatidylcholine added to the medium.

nounced with 15 µg cholesterol/ml (Table I).

To examine the possibility that the phosphatidylcholine component of the lipid dispersions is also incorporated by the cells, the organisms were grown with dispersions containing cholesterol and labeled phosphatidylcholine. Table II shows that significant quantities of labeled phosphatidylcholine were found in *M. hominis* membranes. Thin-layer chromatography of the extracted membrne lipids demonstrated the presence of phosphatidylcholine, and essentially all the radioactivity detected in the membranes could be recovered in the phosphatidylcholine spot scraped off the plate. More phosphaitdylcholine was detected in membranes of *M. hominis* cells grown with lipid dispersions having lower cholesterol to phospholipid molar ratios, probably due to the much higher phosphatidylcholine concentration in the medium in this case. Nevertheless, as Table II shows, the percentage of the dispersed cholesterol taken up by the organisms during growth exceeded that of the dispersed phosphatidylcholine by a factor of 3—5. In sharp contrast to the findings with *M. hominis*, no phosphatidylcholine was taken up by *A. laidlawii* grown with the same labeled phosphatidylcholine dispersions.

The possibility that the phosphatidylcholine detected in *M. hominis* membranes originated from dispersed lipids co-sedimenting with the cells and membranes during cell harvest and membrane isolation was tested by growing *M. hominis* with labeled cholesterol-phosphatidylcholine dispersions having cholesterol-phospholipid molar ratios of 0.55 and 2.0. The washed membranes isolated from the above cultures were subjected to centrifugation on a discontinuous sucrose gradient. All radioactivity of the membrane preparation was associated with the membrane band sedimenting on top of the 60% sucrose layer, with no radioactivity detected in the 20% or 30% sucrose layers. The labeled lipid dispersions by themselves were shown to concentrate in and on top of the 20% sucrose layer.

Discussion

Our results show that the higher the cholesterol to phospholipid ratio of a lipid dispersion, the more effective it is in serving as a cholesterol donor to mycoplasma membranes, corroborating the findings of Cooper et al. [4,10] with eukaryotic cell membranes. Furthermore, with the sterol-requiring mycoplasmas the better performance as cholesterol donors of lipid dispersions having a high cholesterol to phospholipid molar ratio was also expressed by improved growth. Our results may explain the recent observations of Slutzky et al. [2], that the low-density lipoproteins from human serum are more effective cholesterol donors to mycoplasmas than the high-density lipoproteins. The much higher cholesterol to phospholipid molar ratio of low-density lipoproteins (0.70—1.11) as compared with high-density lipoproteins (0.11—0.30) has to be considered as a major factor responsible for the higher efficiency of low-density lipoproteins as cholesterol donors to mycoplasmas.

The finding of significant quantitites of phosphatidylcholine in *M. hominis* membranes deserves some consideration. The amounts of phosphatidylcholine associated with the membranes increased as the concentration of phosphatidylcholine in the growth medium increased (i.e., with the lipid dispersions having low cholesterol to phospholipid molar ratios, Table II). The binding of exogenous phosphatidylcholine, therefore, may be one of the factors responsible for the lower cholesterol to phospholipid molar ratio in membranes of cells grown with the phosphatidylcholine-rich dispersion and for the higher phospholipid to protein ratio in the membranes (Table I). The possibility that the phosphatidylcholine detected in the membrane preparation represents co-sedimentation of cholesterol-phosphatidylcholine dispersions with the membranes during centrifugation, was excluded by the sucrose-density gradient centrifugation analysis, and by the absence of phosphatidylcholine from membranes of *A. laidlawii* grown with the same lipid dispersions. Nevertheless, the results of the sucrose-density gradient centrifugation do not rule out the possibility that in *M. hominis* preparations, phosphatidylcholine constituted part of lipid dispersions which either fused with the membrane, or adhered to its surface. Phosphatidylcholine and sphingomyelin have been detected in lipid extracts of the sterol-requiring *Mycoplasma pneumoniae* grown with 20% horse serum [11,12]. As these lipids cannot be synthesized by the mycoplasmas, they must necessarily originate from the serum lipoproteins. Since lipoprotein particles appear to bind very poorly if at all to mycoplasma membranes (ref. 2 and unpublished data of G.M. Slutzky), the exogenous phospholipids could have been transferred to the membrane by a process which involves only transient contact between the organisms and the lipid dispersions. More data on this subject are needed before definite conclusions can be drawn.

Our results show that membranes of growing *A. laidlawii* cells do not bind phosphatidylcholine from lipid dispersions, thus excluding the possibility that the dispersions fuse with the cell membrane. These results are in disagreement with those reported by Grant and McConnell [13] claiming the incorporation of large quantities of dipalmitoyl phosphatidylcholine into *A. laidlawii* membranes by fusion with lipid vesicles. The experimental conditions of Grant and McConnell were, however, totally different from ours. They incubated heavy

suspensions of non-growing cells with very large quantities of dispersed dipalmitoyl phosphatidylcholine, whereas we used growing cells with much lower concentrations of dipalmitoyl phosphatidylcholine.

The inability of the *A. laidlawii* membrane to bind phosphatidylcholine from lipid dispersions during growth is another characteristic distinguishing it from the *M. hominis* membrane. The capacity of the *A. laidlawii* membrane to incorporate exogenous cholesterol is much lower than that of *M. hominis* as shown with different cholesterol donors in this and in previous studies [2,6, 14]. In addition, exogenous cholesteryl esters and triglycerides are not incorporated into *A. laidlawii* membranes, as against significant incorporation of these lipids into *M. hominis* membranes [2,6,14]. It appears, therefore, that the *M. hominis* membrane is much less restricted than that of *A. laidlawii* with the respect to the capacity to incorporate exogenous lipids. The reasons for this difference are presently under investigation.

In conclusion, our results suggest that all the cholesterol taken up by *A. laidlawii* is transferred from the dispersions to the membranes by a process which involves only transient contact between the organisms and the lipid dispersions, whereas a certain amount of cholesterol taken up by *M. hominis* may constitute part of the lipid dispersions adhering to or fusing with the cell membrane.

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