Cholesterol is required for the fusion of single unilamellar vesicles with *Mycoplasma capricolum*

Mark Tarshis, Michael Salman and Shlomo Rottem

Department of Membrane and Ultrastructure Research, The Hebrew University—Hadassah Medical School, POB 1172, Jerusalem 91010, Israel

ABSTRACT Small unilamellar vesicles (SUV) were prepared from the total lipid extract of *Mycoplasma capricolum*. The SUV were labeled with the fluorescent probe octadecylrhodamine B chloride (R18) to a level at which the R18 fluorescence was self-quenched. At pH 7.4 and 37°C, and in the presence of 5% polyethylene glycol, an increase in the R18 fluorescence with time was observed when the R18-labeled SUV were introduced to a native *M. capricolum* cell suspension. The fluorescence dequenching resulting from dilution of the R18 into the unlabeled membranes of *M. capricolum*, was interpreted as a result of lipid mixing during fusion between the SUV and the mycoplasma cells. The presence of cholesterol in the SUV was found to be obligatory to allow SUV-mycoplasma fusion to occur. Adaptation of *M. capricolum* cells to grow in a medium containing low cholesterol concentration provided cells in which the unesterified cholesterol content was as low as 17 μg/mg cell protein. The fusion activity of the adapted cells was very low or nonexistent. Nonetheless, when an early exponential phase culture of the adapted cells was transferred to a cholesterol-rich medium, the cells accumulated cholesterol and regained their fusogenic activity. The cholesterol requirement for fusion in the target mycoplasma membrane was met by a variety of planar sterols having a free β-hydroxyl group, but differing in the aliphatic side chain, e.g., β-sitosterol or ergosterol, even though these sterols, having a bulky side chain, are preferentially localized in the outer leaflet of the lipid bilayer. It is suggested that the role of cholesterol in mycoplasma-SUV fusion is not at the level of bulk bilayer viscosity but rather, affecting local lipid-lipid or lipid-protein interactions that are relevant to the fusion event.

INTRODUCTION

An unresolved problem in mycoplasma research is the nature of mycoplasma-eukaryotic cell interaction. In spite of extensive past studies showing that mycoplasmas are ectoparasites interacting with the cell surface of eukaryotic cells, there are a growing number of recent studies showing that mycoplasmas can penetrate into host cells (1) or fuse with them (2). Fusion of mycoplasmas with eukaryotic cells may result in delivering of mycoplasma components into the host cell and thus affecting their normal functions (3). Therefore, characterizing the factors controlling the fusogenic activity of mycoplasmas is of great interest. In previous studies, it was suggested that cholesterol in target mycoplasma cells is crucial for the fusion of intact Sendai virus or Sendai virus envelopes with mycoplasma (4) and for the formation of intergeneric hybrids of various *Mycoplasma* and *Spiroplasma* species (5).

Mycoplasmas are unique among prokaryotes in requiring cholesterol for growth (6). These cells lack the ability to synthesize cholesterol and therefore require an exogenous source of cholesterol supplied in the growth medium (7). Mycoplasmas incorporate cholesterol from the growth medium into their cell membranes without any modification to a level of ~30–40 mol% of the total membrane lipids (8). The ability to alter the cholesterol content of several *Mycoplasma* species by adapting the cells to grow with very low cholesterol (7) provided a most useful way of exploring the relationship between cholesterol and the physical state of membrane lipids and numerous cell functions (9). In this study, we investigated the role of cholesterol in an *in vitro* fusion system of *Mycoplasma capricolum* and small unilamellar vesicles (SUV). We have used sterol depleted, adapted *M. capricolum* cells to examine the role of cholesterol in the recipient target cell and reconstitution studies to determine the role of cholesterol in the donor SUV. We have shown that planar sterols having a 3 β-hydroxyl group are required both at the level of the recipient mycoplasma membrane and at the level of SUV membranes to allow fusion. The possible role(s) of cholesterol in the fusion event are discussed.

Address correspondence to Professor S. Rottem.

MATERIALS AND METHODS

Organisms and growth conditions

Mycoplasma capricolum (California kid) cells were grown in a modified Edward medium (10) in which the horse serum had been replaced with 1.0% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO), 20 μg/ml of cholesterol, and 10 μg/ml each of oleic and palmitic acids (11). *M. capricolum* cells adapted to grow in a low cholesterol-containing medium were grown in the medium described above which contained a lower amount of cholesterol (1 μg/ml). The cultures were incubated at 37°C and growth was followed by measuring the absorbance of the culture at 640 nm and pH shifts. The cells were harvested at the exponential phase of growth by centrifugation at 12,000 *g* for 10 min, washed twice and resuspended in A-buffer (250 mM NaCl, 10 mM $MgCl_2$, and 10 mM Tris-HCl, pH 7.4). Viability of the cells was determined by the colony counting technique.

Lipid analysis

Lipids were extracted from intact cells by the method of Bligh and Dyer (12). The solvents were evaporated under a stream of nitrogen and the lipids were redissolved in 0.5–1 ml of chloroform. Cholesterol and cholesterol esters were separated on Supelco Ready Coat G silica gel plates

and developed at room temperature with benzene-ethyl acetate (5:1 vol/vol). The free and esterified cholesterol spots were scraped off the plates, extracted with chloroform at 45°C for 30 min and sterol content was determined by the phthaldialdehyde method (13). Thin layer chromatography of total membrane lipids was performed using silica gel HR plates. The plates were first developed at room temperature with petroleum ether (bp 40–60°C)-acetone (3:1 vol/vol) and then at 4°C with chloroform-methanol-water (65:25:4 vol/vol). Lipid spots were detected by iodine vapor. For determination of radioactivity in the lipid spots, the components were scraped from the plates into scintillation vials containing 3 ml of scintillation liquor and radioactivity was measured in a Beckman scintillation spectrometer. To determine phospholipid content in the phospholipid spots, the components were scraped from the plates into test tubes and organic phosphorus was determined by the method of Ames (14) after digestion of the samples with an ethanolic solution of $Mg(NO_3)_2$.

Methyl esters of fatty acids were prepared by heating lipid samples in 14% boron trifluoride in methanol (Sigma Chemical Co.) at 72°C for 15 min. The resulting methyl esters were extracted with n-hexane and subjected to gas-liquid chromatography in a Tractor 540 gas chromatograph equipped with a polar column (100 × 0.2 cm, packed with 10% SP 2330 on 100/120 mesh chromosorb W from Supelco, Belfonte, PA). Fatty acids were identified by their retention time relative to those obtained with standard mixtures of fatty acid methyl esters (Supelco).

Lipid vesicle preparation and fusion measurements

SUV were prepared from the total lipid fraction of *M. capricolum* in A-buffer by sonication under nitrogen at 4°C in a W-350 Heat Systems ultrasonic disintegrator at 160 W, as previously described (15). For reconstitution experiments, 30 mol% of the tested sterol dissolved in chloroform was added to the total lipid fraction extracted from adapted *M. capricolum* cells and then SUV were prepared. Fusion of SUV and *M. capricolum* cells was monitored by the octadecylrhodamine B chloride (R18; Molecular Probes) dequenching assay previously described (15). For the labeling of SUV, 3–5 μl of an ethanolic solution of R18 (600 μg/ml) were rapidly injected into 200 μl of a SUV dispersion containing 0.5 mg/ml of lipids and incubated for 15 min at 37°C in the dark. The labeled SUV were freed of unincorporated label by passing 100 μl samples through Sephadex G-50 (Pharmacia) in 1 ml tuberculin syringes. For fusion measurements, the R18 labeled SUV (~20 μg) were mixed with 200 μl of intact *M. capricolum* cells (2 mg cell protein/ml) and 5% PEG 8000 (Merck, Germany) and incubated at 37°C for various periods of time. 2 ml of cold A-buffer were then added to the reaction mixture and the intensity of fluorescent dequenching was measured in a Perkin-Elmer LS-5B fluorimeter with excitation and emission wave lengths of 560 and 590 nm, respectively, and with correction for light scattering. The dequenching degree obtained in the presence of 0.1% Triton X-100 was taken to represent 100% dequenching, i.e., infinite dilution of the probe. The fluorescent dequenching was calculated from the following equation:

$$DQ(\%) = [F - (F_1 \times I/I_{tr})]/[F_1 - (F_1 \times I/I_{tr})],$$

where F is the fluorescence obtained from the reaction mixture at the end of the incubation period; F_1 is the same, but after solubilization with Triton X-100; I and I_{tr} are the fluorescence of the reaction mixture before incubation (zero time) and after solubilization with Triton X-100, correspondingly. Cells were rendered nonfusogenic by treatment with either 10 μM chlorpromazine or 0.1% glutaraldehyde (15) and these treatments were used as a negative control in all subsequent experiments.

Analytical procedures

Protein was determined by the method of Bradford (16). Merocyanin 540 binding was essentially performed according to Del Buono et al. (17). In brief, *M. capricolum* cells (0.4–0.5 mg protein/ml) were incubated for 15 min at 37°C in the dark with 5 μl/ml of merocyanin (stock solution 1 mg/ml in 50% ethanol; Sigma Chemical Co.). The cells were pelleted, washed twice with A-buffer containing 0.4% BSA, resuspended in 2 ml of A-buffer and examined within 10 min in Perkin-Elmer LS-5B spectrofluorimeter with excitation and emission wave lengths 540 and 585 nm, respectively, and with the correction for light scattering. Organic peroxides were measured according to Silverman and Santucci (18) with slight modifications. 300–400 μg of *M. capricolum* cells in 1.0 ml of A-buffer were incubated in the dark at 37°C for 10 min with 4 μl of a stock ethanol solution (0.4 μM) of the fluorescence probe 5-(and 6-)carboxy-2',7'-dichlorofluoresceine diacetate (CDFD; Molecular Probes). After incubation, cells were washed twice with A-buffer and 2 ml of 0.1% of Nonidet P-40 (Sigma Chemical Co.) were added to the pellet to lyse the cells. The fluorescence of the samples was measured with excitation and emission at 505 and 535 nm respectively, and with the correction on background fluorescence. Since the conversion of CDFD to the fluorescent compound dichlorofluoresceine requires the presence of both peroxides and peroxidase, in control experiments horseradish peroxidase (4 U/ml, Sigma Chemical Co.) was added to the cell extracts prior to the measurements of fluorescence. This addition resulted in a four-to-fivefold increase of fluorescence, thus indicating the specificity of the reaction.

RESULTS

Adaptation of *M. capricolum* to a low cholesterol medium

Serial passages of *M. capricolum* cells in a modified serum-free Edward medium containing 1% bovine serum albumin, palmitic and oleic acids (10 μg/ml of each) and a decreasing concentration of cholesterol (from 20 μg to 1.0 μg/ml) were required in order to adapt the cells to grow in a low cholesterol medium. The cells adapted to 1.0 μg/ml cholesterol (adapted cells) grew slower than the cells grown with 20 μg/ml (native strain) and reached the stationary phase of growth at a much lower absorbance (640 nm) than the native strain. Table 1 shows that the native cells contain three to four times more cholesterol than the adapted strain and had a cholesterol to phospholipid molar ratio threefold higher than the adapted cells. The adaptation to a low cholesterol containing medium was associated with a pronounced decrease in the microviscosity of the lipid backbone as indicated by the increase in the binding of merocyanin 540, a lipid soluble fluorescent probe that is partitioning preferentially into fluid-state lipid bilayers (19). These results are in agreement with previous results utilizing diphenylhexatriene fluorescence polarization (20, 8). The adaptation was also accompanied by an elevated generation of free radicals and as a result the accumulation of higher levels of organic peroxides (Table 1). The phospholipids phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG) accounted for almost 80% of the total polar lipids of *M. capricolum* (21). The relative amounts of PG and DPG measured in exponential phase cells were affected by adapting the cells to a low cholesterol containing medium. The DPG to PG molar ratio was decreased from 1.00 in the native cells to 0.52 in the adapted cells (data not shown).

TABLE 1 **Lipid composition and characteristics of native and adapted *M. capricolum* cells**

Cells	Phospholipids	Cholesterol	FC/PL	Merocyanin binding	Organic peroxides
	μg/mg protein		*molar ratio*	*a.u./mg protein*	
Native	130 ± 15	56 ± 5	0.79	192 ± 27	121 ± 10
Adapted	101 ± 10	17 ± 2	0.29	330 ± 23	193 ± 17

Native and adapted strains were grown on a medium containing 20 and 1 μg/ml cholesterol, respectively. The cells were grown to the mid-exponential phase of growth (native cells, A_{640} = 0.32, pH = 6.4; adapted cells, A_{640} = 0.13, pH = 6.0) harvested and analyzed as described in Materials and Methods.

Fusogenic activity of native and adapted cells

The fusogenic activity was determined by fusing small unilamellar vesicles (SUV) prepared from the total lipid fraction of native *M. capricolum* cells, with intact *M. capricolum* cells. The SUV were labeled with the fluorescent probe octadecylrhodamine B chloride (R18) to a level at which the R18 fluorescence was self-quenched. When the labeled SUV were mixed with *M. capricolum* cells at 37°C, an increase in the fluorescent intensity of the R18 was observed. This increase was interpreted as the dilution of the R18 in the membrane of *M. capricolum* upon fusion (15). Table 2 shows that the adaptation to a low cholesterol containing medium was associated with a marked decrease in the fusogenic activity of the cells. The fusogenic activity of the native cells was dependent on the growth phase of the culture, being high with exponential phase cells and substantially lower with stationary phase cells.

Adaptation of *M. capricolum* cells to a low cholesterol-containing medium also affected the homologous *M. capricolum* cell-cell fusion. The extent of fusion was quantitatively evaluated by following dequenching of R18 incorporated into the cell membrane of donor *M. capricolum* cells after their incubation with an excess of nonlabeled recipient *M. capricolum* cells in the presence of PEG 8000 (5%) and Mg^{2+} ions. Fig. 1 shows that high fluorescent dequenching was observed upon interaction of native donor cells with native recipient cells. Interaction of adapted donor cells with native recipient cells resulted in a much lower fluorescent dequenching, whereas nonsignificant fluorescence values were observed upon interaction of adapted donor cells with adapted recipient cells or native donor cells with adapted recipient cells.

Table 3 shows that the addition of 5 mM α-tocopherol (Sigma Chemical Co.) to the low cholesterol containing growth medium reduced the accumulation of organic peroxides by the adapted cells by ~60% without affecting the growth characteristics and cell viability. Nonetheless, the fusion of α-tocopherol-treated cells with SUV was almost the same as the fusion of control cells grown without the antioxidant. Likewise, the treatment of native cells with $FeCl_3$ (0.5 mM) resulted in a more than twofold increase in the organic peroxides accumulated

TABLE 2 **Fusion of SUV with native and adapted *M. capricolum* cells**

Cells	Additives to medium	Fusion activity (%) at various phases of growth: Early exponential	Stationary
Native	4% HS	26.6 ± 4.0	10.9 ± 2.1
	1% BSA plus 20 μg/ml cholesterol	22.1 ± 1.7	18.2 ± 2.0
Adapted	1% BSA plus 1 μg/ml cholesterol	3.3 ± 0.1	3.0 ± 0.1

Cells were grown as described in the legend to Table 1. Fusion analyses were performed as described in Materials and Methods.

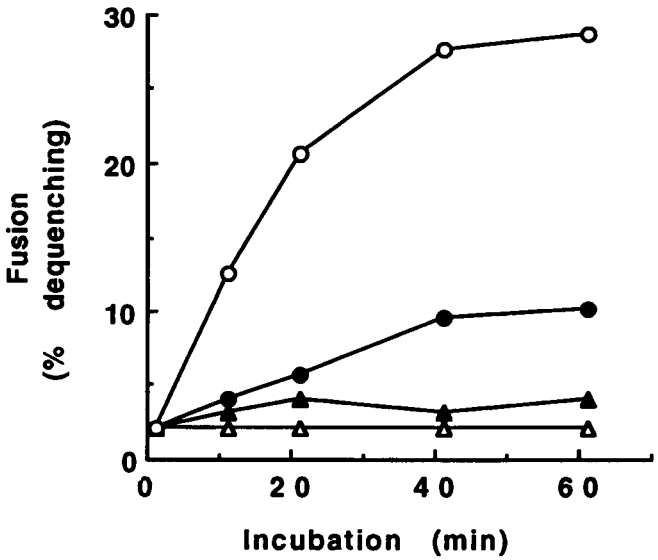

FIGURE 1 The kinetics of *M. capricolum* cell-cell fusion. Native and adapted cells were grown in Edward medium containing 20 and 1 μg/ml cholesterol, respectively. For measuring fusion activity, R18-labeled donor cells were incubated at 37° in the presence of 5% PEG with recipient cells (1:10 ratio) and fusion was followed as described in Materials and Methods: (○) fusion of native donor cells with native recipient cells; (▲) fusion of native donor cells with adapted recipient cells; (●) fusion of adapted donor cells with native recipient cells; (△) fusion of adapted donor cells with adapted recipient cells.

TABLE 3 **The effect of α-tocopherol on the fusion activity and organic peroxide level of native and adapted *M. capricolum* cells**

Cells	Organic peroxides	Fusion activity
	a.u./mg protein	%
Native	242	24.0
Native with α-tocopherol	184	22.7
Adapted	376	3.7
Adapted with α-tocopherol	256	5.8

Cells were grown as described in the legend to Table 1. α-tocopherol (Sigma Chemical Co.) dissolved in ethanol was added to the growth medium to the final concentration of 5 mM. The addition of α-tocopherol did not affect the viability of the cells. Fusion and organic peroxide analyses were performed as described in Materials and Methods.

in the cell membrane but had only a small effect on the fusogenic activity of the cells (data not shown).

Since *M. capricolum* cells can neither synthesize nor modify long chain fatty acids, these cells depend on an exogenous supply of fatty acids in the growth medium. This requirement is usually met by a mixture of palmitic and oleic acids. All the results so far described were obtained in a medium containing a palmitic to oleic acid ratio of 1.0 (10 μg of each/ml of medium). Increasing the palmitic to oleic acid ratio in the low cholesterol containing medium containing 1 μg cholesterol per ml without changing the total fatty acid concentration (20 μg/ml) resulted in an increase in the microviscosity of the membranes as indicated by the merocyanin binding capacity (Table 4). As expected, the fusion activity of cells grown with the high palmitic to oleic acid ratio was lower than that of cells grown with the low palmitic to oleic ratio.

Transfer of the adapted cells to a cholesterol rich medium

Fig. 2 shows that transferring an early exponential culture of adapted *M. capricolum* cells to a rich cholesterol

TABLE 4 **Fusion of SUV with adapted *M. capricolum* cells grown with various concentrations of fatty acids**

Fatty acids added	Fusion	Merocyanin binding	DPG/PG ratio
μg/ml	%	*a.u./mg protein*	
Oleic (5) + palmitic (15)	0	253	0.52
Oleic (10) + palmitic (10)	6.1	305	0.54
Oleic (15) + palmitic (5)	15.9	318	0.41

Adapted *M. capricolum* strain was grown in a medium containing various concentrations of oleic and palmitic acids. Fusion acitivity and merocyanin binding were determined as described in Materials and Methods. The DPG to PG molar ratio was calculated from the phosphorus content of the DPG and PG spots resolved by thin layer chromatography.

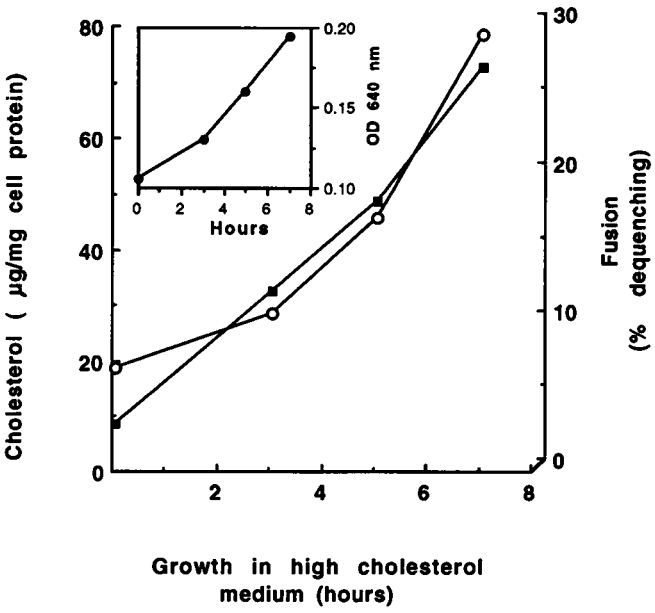

FIGURE 2 Fusion activity of adapted *M. capricolum* cells transferred to a cholesterol-rich medium. Adapted cells were grown for 24–26 h in a low cholesterol medium (1 μg/ml cholesterol) and then transferred to a cholesterol-rich medium (20 μg/ml cholesterol) and incubated for 7 h at 37°C. Cholesterol and fusion analyses were performed as described in Materials and Methods. (○) cholesterol content; (■) fusion activity of cells. The inset shows the change in the absorbance of the culture (●) after the transfer to the cholesterol-rich medium.

medium and incubating the culture at 37°C for 7 h was accompanied by a marked increase in the unesterified cholesterol content from 20 μg/mg cell protein to 80 μg/mg cell protein. At various time intervals after the transfer of the cells to a cholesterol-rich medium, the fusion activity of the cells with R18-labeled SUV was determined. The figure shows that the increase in the cholesterol content was accompanied by a concomitant increase in the ability of the cells to fuse. When the cells were transferred to the high cholesterol medium and were incubated at 4°C, neither an increase in the cholesterol content nor an increase in the fluorescent dequenching occurred.

We have previously shown that inhibitors affecting a membrane potential gradient have a pronounced effect on the bilayer leaflet distribution of cholesterol in *Mycoplasma capricolum* cells indicating that cholesterol translocation from the outer to the inner leaflet is facilitated in actively growing cells where a membrane potential takes place (22). It was, therefore, of interest to study the effect of such inhibitors on the fusogenic activity of the cells. When the adapted cells were transferred to a cholesterol-rich medium containing carbonyl cyanide m-chlorophenyl-hydrazone (CCCP, 5 or 10 μM), dicyclohexylcarbodiimide (DCCD, 10 μM) or gramicidin (3 μM) and incubated at 37°C for 4 h, cholesterol uptake was partially inhibited (by ~30%), but the fusogenic activity of the inhibitor-treated cells was almost identical

TABLE 5 **Fusion activity of adapted *M. capricolum* cells transferred to a growth media with different sterols**

Sterol added to medium	A_{640}	Sterol incorporated	Fusion with SUV
		μg/mg protein	%
None	0.11	17	3.8 ± 0.7
Cholesterol	0.19	72	25.8 ± 2.9
β-sitosterol	0.14	55	10.6 ± 1.6
Dihydrocholesterol	0.17	65	21.4 ± 2.3
Ergosterol	0.17	43	20.6 ± 2.0

Adapted cells grown for 24-26 h with 1 μg/ml of cholesterol were transferred to the growth media containing different sterols (20 μg/ml). After 7 h of growth at 37°C, cells were harvested and sterol content and fusion activity were analyzed as described in Materials and Methods.

TABLE 6 **Fusion of donor SUV, derived from native or adapted cells with recipient *M. capricolum* cells**

	Fusion activity (% dequenching) with:	
SUV made of:	Native cells	Adapted cells
Lipids from native cells	22.0 ± 1.9	5.7 ± 0.4
Lipids from adapted cells	6.1 ± 0.9	2.7 ± 0.6
Lipids from cells grown with 4% HS	26.9 ± 3.0	5.5 ± 0.5

SUV were fused with native (grown with 20 μg/ml of cholesterol) or adapted (grown with 1 μg/ml of cholesterol) cells as described in Materials and Methods.

to the fusogenic activity of control untreated cells that contain an equivalent amount of cholesterol (data not shown).

The sterol requirement of mycoplasmas could be met by a planar 3β-hydroxy sterol that contains an aliphatic side chain. *M. capricolum* cells grew well with a sterol having a more saturated steroid nucleus (dihydrocholesterol), an identical steroid nucleus but a bulky aliphatic side chain, such as β-sitosterol, or a more unsaturated steroid nucleus and a bulky side chain, such as ergosterol (8, 23). Table 5 shows that transferring the adapted *M. capricolum* cells to a medium containing the various sterols and incubating the cultures at 37°C resulted in an almost identical growth rates with a concomitant increase in the content of the various sterols from 17 μg/mg cell protein at zero time to 34–56 μg/mg cell protein after 7 h of incubation. The table also shows that at the end of the incubation period the ability of the cells, incubated with β-sitosterol, to fuse with R18-labeled SUV was only partially restored whereas cells incubated with ergosterol or dihydrocholesterol showed a fusogenic activity almost identical to that of cells grown with cholesterol.

Cholesterol is required in the SUV membrane

Table 6 shows that the effect of cholesterol is not only at the level of the mycoplasma membrane itself, but is also required in the SUV membranes. Thus, native *M. capricolum* cells grown with 20 μg/ml of cholesterol fused efficiently with SUV made from lipids of the native cells or lipids of cells grown with 4% horse serum, but not with SUV made of lipids of the adapted cells. As expected, adapted *M. capricolum* cells fused neither with SUV made of lipids of the adapted cell nor with SUV made of lipids of the native cells. To test the effect of the sterols incorporated in the SUV membrane on the fusion, SUV were prepared from the lipid fraction of the adapted cells supplemented with 30 mol% of various sterols. The extent of the fluorescence dequenching obtained by fusing the various SUV preparations with native *M. capricolum* cells is shown in Fig. 3. The figure shows that highest fluorescent dequenching was observed with SUV containing cholesterol, dihydrocholesterol and ergosterol. Lower fluorescent dequenching was observed with β-sitosterol containing SUV, whereas SUV containing epicoprostanol (5β-cholestan-3α-ol) or cholesteryl laurate showed practically no fluorescent dequenching.

DISCUSSION

We have shown in our study that cholesterol is required both in the mycoplasma cell membrane and in the membrane of the small unilamellar vesicle (SUV) to permit the fusion of *M. capricolum* cells with the SUV. It has been suggested previously that cholesterol in the target mycoplasma cell is required for the fusion of mycoplasmas with Sendai or influenza viruses (4) since choles-

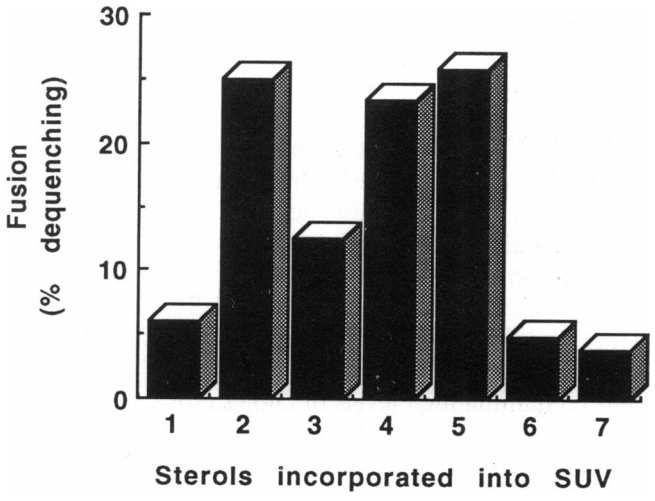

FIGURE 3 The effects of sterols incorporated into SUV on the fusion of native *M. capricolum* cells. Sterols were added to lipid extracts of adapted *M. capricolum* cells to a final concentration of 30 mol%. SUV-mycoplasma fusion was performed as described in Materials and Methods: (1) no sterol; (2) cholesterol; (3) β-sitosterol; (4) dihydrocholesterol; (5) ergosterol; (6) epicoprostanol; (7) cholesteryl laurate.

terol containing Mycoplasma species were capable of fusing with the viruses, whereas the cholesterol-free Acholeplasma species did not fuse. In this study, the requirement for cholesterol in the target mycoplasma cell membrane was directly demonstrated by showing that adapting *M. capricolum* to grow with low levels of cholesterol resulted in a marked decrease in the fusion activity of the cells.

The adaptation to a low cholesterol medium was associated with the accumulation of high amounts of organic peroxides (see Table 1). This may be due to the role cholesterol plays as an intramembraneous lipophilic antioxidant, preventing the formation of lipid peroxides as the degradation products of free radicals attack of polyenoic fatty acids (24). Peroxides formation depends also on the microviscosity of membrane lipid bilayer and on the unsaturated/saturated fatty acid ratio in the membrane (25, 26). Radicals may also affect oxidizable amino acids of transmembrane proteins, thus increasing the membrane permeability for ions and nonelectrolytes (27), or oxidize protein sulfhydryl groups, leading to protein dysfunctions (28).

When the adapted *M. capricolum* cells were transferred to a cholesterol rich medium, cholesterol content was increased by more than threefold during 7 h of incubation. The increase in the cholesterol level was associated with a drastic increase in the fusogenic activity of the cells from a very low activity of the adapted cells at zero time (3.5% dequenching) to a high fusogenic activity at the end of the incubation period (25% dequenching), comparable to the fusion activity of native cells. The increase in the fusogenic activity was not affected by inhibitors such as gramicidin, CCCP or DCCD. These inhibitors had only a small effect on the cholesterol uptake from the growth medium, but profoundly inhibited the translocation of the sterol from the outer leaflet to the inner leaflet of the lipid bilayer, e.g., in the presence of gramicidin, almost 90% of the cholesterol incorporated into *M. capricolum* cells was located in the outer leaflet (22). Therefore, it seems that a symmetrical distribution of cholesterol between the outer and inner leaflets is of no importance for the fusion process.

The requirement for cholesterol for *M. capricolum*-SUV fusion is not only in the target mycoplasma cell membrane, but also at the level of the SUV membrane. Thus, fusion of SUV made of the total lipid fraction of adapted *M. capricolum* cells with native *M. capricolum* cells containing high levels of cholesterol was very low. The fusogenic activity of the SUV was restored when supplemented with 30 mol% of a planar sterol having a free 3β-hydroxyl group.

What is the role of cholesterol in mycoplasma-SUV fusion? The effect of cholesterol on the packing of the bulk membrane lipids is well established (29, 30). Nonetheless, it is unlikely that cholesterol exerts its action by increasing the microviscosity of bulk membrane lipids since increasing the microviscosity of the adapted *M. capricolum* cell membranes by increasing the saturated to unsaturated fatty acid ratio decreased rather than increased the fusion activity of the cells. Furthermore, upon ageing of *M. capricolum* cells, the microviscosity of the cell membrane was dramatically increased, accompanied by a marked decrease in the fusogenic activity of the cells. Besides an effect on the packing of the bulk lipids several other factors may be responsible for the requirement for cholesterol for the fusion process. Among them is the ability of cholesterol to act as a spacer or filler molecule separating phospholipid head groups (31). Since acidic phospholipids, mainly PG and DPG, are the major phospholipids synthesized by *M. capricolum* (8), cholesterol will induce a decrease in the density of surface charges. Such decrease would be expected to modify intermolecular interactions between polar head groups directly affecting fusion (32).

As nonbilayer forming lipids have been proposed to be involved in a fusion process (33, 34), it could be expected that destabilization of the membrane lipid bilayer by nonbilayer-forming lipids causes the formation of fusogenic intermediates with subsequent membrane fusion (35). It has been previously shown that *M. capricolum* membrane undergoes a Ca^{2+}-dependent bilayer to hexagonal II transition which depends on the DPG level in the membrane (36). Our observation that in native *M. capricolum* cells grown with cholesterol the DPG to PG molar ratio was higher than in adapted cells may suggest that localized regions of bilayer destabilization are formed in the cell membrane of the native strain. In this view these regions, which are crucial for the fusion process, are not formed in the adapted cells.

One cannot exclude the possibility that cholesterol may lead to conformational changes in membrane protein that participate in the fusion event. It was suggested that proteins are involved in the initiation of membrane fusion by locally producing or activating a fusogen, or by acting as a fusogen (37). Indeed, we have previously shown that partial proteolysis of surface proteins decreased the ability of *M. capricolum* cells to fuse with derived SUV, suggesting the involvement of a membrane protein in mycoplasma-SUV fusion (15). Cholesterol was also shown to interact with hydrophobic regions of the fusogenic protein which participates in the fusion of Sendai virus with host cells (38).

This work was supported by the Israel-United States Binational Agriculture Research and Development Fund (grant No. 1902-90R) and by grant No. 3221 (to M. Tarshis) from the Israeli Ministry of Science and Technology.

Received for publication 31 August and in final form 9 November 1992.

REFERENCES

1. O'Toole, C., and M. Lowdell. 1990. Infection of human T-cells with mycoplasma, inhibition of CD4 expression and HIV-1 gp 120 glycoprotein binding and infectivity. *Lancet.* 336:1067.

2. Franzoso, G., D. S. Dimitrov, R. Blumenthal, M. F. Barile, and S. Rottem. 1992. Fusion of *Mycoplasma fermentans* strain incognitus with T-lymphocytes. *FEBS Lett.* 303:251–254.
3. McGarrity, G. J., and H. Kotani. 1985. Cell culture mycoplasmas. *In* Mycoplasmas, Vol. IV. S. Razin and M. F. Barile, editors. Academic Press, Inc., Orlando, FL. 353–391.
4. Citovsky, V., S. Rottem, D. Nussbaum, Y. Laster, R. Rott, and A. Loyter. 1988. Animal viruses are able to fuse with prokaryotic cells. *J. Biol. Chem.* 263:461–467.
5. Tarshis, M., M. Salman, and S. Rottem. 1991. Fusion of mycoplasmas: the formation of cell hybrids. *FEMS Lett.* 82:67–72.
6. Razin, S. 1982. Sterols in mycoplasma membranes. *In* Current Topics in Membranes and Transport, Vol. 17. F. Bronner and A. Kleinzeller, editors. Academic Press, New York, London. 187–205.
7. Dahl, J. S., C. E. Dahl, and K. Bloch. 1980. Sterols in membranes: growth characteristics and membrane properties of *Mycoplasma capricolum* on cholesterol and lanosterol. *Biochemistry.* 19:1467–1472.
8. Rottem, S. 1980. Membrane lipids of mycoplasmas. *Biochim. Biophys. Acta.* 604:65–90.
9. Yeagle, P. L. 1985. Cholesterol and the cell membrane. *Biochim. Biophys. Acta.* 822:267–287.
10. Razin, S., and S. Rottem. 1976. Techniques for manipulation of mycoplasma membranes. *In* Biochemical Analysis of Membranes. A. Maddy, editor. Chapman and Hall, London. 3–26.
11. Gross, Z., and S. Rottem. 1984. The preservation of *Mycoplasma capricolum* cell intactness after phospholipase A_2 treatment. *Biochim. Biophys. Acta.* 778:372–378.
12. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37:911–917.
13. Rudel, L. L., and M. D. Morris. 1973. Determination of cholesterol using o-phthaldialdehyde. *J. Lipid Res.* 14:364–366.
14. Ames, B. N. 1966. Assay of inorganic phosphate, total phosphate, and phosphatases. *Meth. Enzymol.* 8:115–116.
15. Salman, M., M. Tarshis, and S. Rottem. 1991. Small unilamellar vesicles are able to fuse with *Mycoplasma capricolum* cells. *Biochim. Biophys. Acta.* 1063:209–216.
16. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.
17. Del Buono, B. J., S. M. White, P. L. Williamson, and R. A. Schlegel. 1989. Plasma membrane lipid organization and the adherence of differentiating lymphocytes to macrophages. *J. Cell. Physiol.* 138:61–69.
18. Silverman, D. J., and L. A. Santucci. 1988. Potential for free radical-induced lipid peroxidation as a cause of endothelial cell injury in Rocky Mountain spotted fever. *Infect. Immun.* 56:3110–3115.
19. Schlegel, R. A., J. A. Reed, L. McEvoy, L. Algarin, and P. W. Williamson. 1987. Phospholipid assymetry of loaded red cells. *Meth. Enzymol.* 149:281–243.
20. Rottem, S., V. P. Cirillo, B. De Kruyff, M. Shinitzky, and S. Razin. 1973. Cholesterol in mycoplasma membranes. Correlation of enzymic and transport activities with physical state of lipids in *Mycoplasma mycoides* var. *capri* adapted to grow with low cholesterol concentrations. *Biochim. Biophis. Acta.* 323:509–519.
21. Gross, Z., and S. Rottem. 1986. Lipid interconversions in aging *Mycoplasma capricolum* cultures. *J. Bacteriol.* 167:986–991.
22. Clejan, S., R. Bittman, and S. Rottem. 1978. Uptake, transbilayer distribution, and movement of cholesterol in growing *Mycoplasma capricolum* cells. *Biochemistry.* 17:4579–4583.
23. Clejan, S., R. Bittman, and S. Rottem. 1981. Effect of sterol structure and exogenous lipids on the transbilayer distribution of sterols into membrane of *Mycoplasma capricolum. Biochemistry.* 20:2200–2204.
24. Hegner, D. 1980. Age-dependence of molecular and functional changes in biological membrane properties. *Mechanisms Ageing Develop.* 14:101–118.
25. Sies, H. 1986. Biochemistry of oxidative stress. *Angew. Chem. Int. Ed. Engl.* 15:1058–1071.
26. Watanabe, H., A. Kobayashi, T. Yamamoto, S. Suzuki, H. Hayashi, and N. Yamazaki. 1990. Alterations of human erythrocyte membrane fluidity by oxygen-derived free radicals and calcium. *Free Radical Biol. Med.* 9:507–514.
27. Halliwell, B., and J. M. C. Gutteridge. 1984. Lipid peroxidation, oxygen radicals, cell damage, and antioxidant therapy. *Lancet.* 1:1396–1398.
28. Davies, K. J. A. 1987. Protein damage and degradation by oxygen radicals. *J. Biol. Chem.* 262:9895–9901.
29. Melchior, D. L. 1982. Lipid phase transition and regulation of membrane fluidity in prokaryotes. *Curr. Top. Membr. Transp.* 17:263–316.
30. Huang, T.-H., A. J. DeSiervo, and Q.-X. Yang. 1991. Effect of cholesterol and lanosterol on the structure and dynamics of the cell membrane of *Mycoplasma capricolum. Biophys. J.* 59:691–702.
31. Wieslander, A., A., Christiansson, L. Rilfors, and G. Lindblom. 1980. Lipid bilayer stability in membranes. Regulation of lipid composition in *Acholeplasma laidlawii* as governed by molecular shape. *Biochemistry.* 19:3650–3655.
32. Duzgunes, N. 1988. Cholesterol and membrane fusion. *In* Biology of Cholesterol. P. L. Yeagle, editor. CRC Press, Boca Raton, FL. 197–212.
33. Blumenthal, R. Membrane fusion. *Curr. Topics Membr. Transp.* 29:203–254.
34. Bentz, J., and H. Ellens. 1988. Membrane fusion: kinetics and mechanisms. *Coll. Surf.* 30:65–112.
35. Burger, K. N. J., and A. J. Verkleij. 1990. Membrane fusion. *Experientia.* 46:631–644.
36. Bittman, R., S. Clejan, and S. W. Hui. 1990. Increased rates of lipid exchange between *Mycoplasma capricolum* membranes and vesicles in relation to the propensity of forming nonbilayer lipid structures. *J. Biol. Chem.* 265:15110–15117.
37. Stegman, T., R. W. Doms, and A. Helenius. 1989. Protein-mediated membrane fusion. *Annu. Rev. Biophys. Chem.* 18:187–211.
38. Asano, K., and A. Asano. 1988. Binding of cholesterol and inhibitory peptide derivatives with the fusogenic hydrophobic sequence of F-glycoprotein of HVJ (Sendai virus): possible implication in the fusion reaction. *Biochemistry.* 27:1321–1326.