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Small unilamellar vesicles are able to fuse with *Mycoplasma capricolum* cells

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We have investigated the fusion characteristics of intact *Mycoplasma capricolum* cells and small unilamellar vesicles (SUV). The rate and extent of fusion was monitored continuously by octadecylrhodamine B (R18) fluorescence dequenching assay, as well as by intracellular contents mixing, and by sucrose density gradient analysis. The fusion of SUV with *M. capricolum* cells was found to be dependent on poly(ethylene glycol) (PEG 8000), divalent cations in the medium, and on the cholesterol content of the lipid vesicles. Maximal levels of fusion were obtained with SUV containing 40 mol% cholesterol in the presence of 5% PEG. The rate and extent of fusion were affected by temperature, pH, osmotic pressure, and SUV/mycoplasma ratio. Under optimal fusion conditions, PEG did not increase the rate of exchange of either cholesterol or phospholipids between *M. capricolum* cells and SUV. Throughout the fusion process, *M. capricolum* cells remained intact as measured by the retention of [^{3}H]thymidine-labeled components, and viable. *M. capricolum* cells were rendered nonfusogenic by treatment with glutaraldehyde (> 0.01%) or chlorpromazine (> 10 μM). Fusion was partially inhibited by treating the cells with the uncoupler CCCP (5 μM) or proteolytic enzymes, suggesting that a proton gradient across the cell membrane is required for the fusion, and that the cells possess proteinase-sensitive receptors that are responsible for a tighter contact with the lipid vesicles.

Introduction

In recent years, there has been a great deal of interest in the use of liposomes as a model system for the study of the mechanism of membrane interactions, as well as in the use of liposomes for the delivery and introduction of macromolecules into cells [1–5]. Several methods have been used to monitor the extent and nature of liposome-cell fusion [2,6,7]. Among them are the fluorescence dequenching techniques [8,9]. Hoekstra and co-workers [10,11] introduced a sensitive assay to study membrane fusion by following the mixing of membrane lipids in both artificial and natural membranes. The method relies on the relief of fluorescence self-dequencing of the lipid probe octadecylrhodamine (R18) after the fusion of probe labeled membranes and non-labeled membranes.

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Mycoplasmas are simple prokaryotic cells that contain a single membrane and have no cell wall [12]. Although the lack of a cell wall would seem to favor genetic exchange by transformation, experiments performed so far to transform mycoplasmas gave inconsistant results, apparently due to the potent extracellular nuclease activity of these organisms [12]. There has been, therefore, much interest in a fusion-mediated delivery system of macromolecules into mycoplasmas [13–16]. Nevertheless, the low fusogenic activity of mycoplasmas hinders such an approach [17,18]. A breakthrough was achieved recently when we demonstrated that cholesterol-requiring mycoplasmas are able to fuse with intact Sendai virions or isolated Sendai envelopes [19].

In the present study, we show that *M. capricolum* cells are capable of fusing even with lipid vesicles, providing that the vesicles contain cholesterol and that divalent cations and low concentrations of poly(ethylene glycol) are present in the medium. This observation may open the way for the development of a fusion-mediated delivery system of genetic material into mycoplasma cells.

Materials and Methods

Organisms and growth conditions. Mycoplasma capricolum (California kid) was grown in a modified Edward medium supplemented with 4% horse serum [20]. To label membrane lipids, the cells were grown with either 0.02 μCi/ml of [9,10(n)-^{3}H]palmitic acid (40–60 Ci/mmol), 0.01 μCi/ml of [9,10(n)-^{3}H]oleic acid (2–10 Ci/mmmol), or 0.004 μCi of [4-^{14}C]cholesterol (60 mCi/mmol), all products of Amersham, U.K. To test for cell leakiness during the fusion process, 0.25 μCi/ml of [6-*methyl*-^{3}H]thymidine (35 Ci/mol), Nuclear Research Center, Negev, Israel) were added to the growth medium. The cultures were incubated at 37°C for 16–24 h and growth was followed by measuring the absorbance of the culture at 640 nm. Most experiments were performed with cells harvested at the late exponential phase of growth ($A_{640} = 0.25$). The organisms were collected by centrifugation at 12000 × *g* for 10 min, washed once, and resuspended in an isoosmotic solution of either NaCl (250 mM) or sucrose (500 mM) containing 10 mM $MgCl_2$, and 10 mM Tris-HCl (pH 7.4).

Preparation of lipid vesicles. Small unilamellar vesicles (SUV) were prepared from the total lipid fraction of *M. capricolum* in a solution containing 250 mM NaCl, 10 mM $MgCl_2$ and 10 mM Tris-HCl (pH 7.4) (to be referred to as A-buffer). Lipids were solubilized in diethyl ether and the solvent was then evaporated under N_2 to dryness, forming a thin film on the walls of a test tube. The dry lipids were rehydrated in A-buffer to a concentration of 2 mg/ml, vortexed for 2–3 min at room temperature and then sonicated for 10–15 min under nitrogen at 4°C in a W-350 Heat Systems Ultrasonic Disintegrator at 160 watts. The clear SUV dispersion thus obtained was stored at 4°C under nitrogen and was used within a week of its preparation.

Fusion measurements. Fusion of SUV and mycoplasma cells was monitored by the octadecylrhodamine B chloride (R18, Molecular Probes, Eugene, OR, U.S.A.) assay described before [19]. For the labeling of SUV with R18, 3–5 μl of an ethanolic solution of R18 (600 μg/ml) were rapidly injected into 200 μl of a SUV dispersion containing 0.4 mg/ml of lipids, incubated for 15 min at 37°C in the dark, and then rapidly cooled and stored at 4°C until used.

The R18 labeled SUV (about 20 μg) were mixed with 200 μl of intact mycoplasma cells (2 mg cell protein/ml) and 5% PEG 8000 (Merck, F.R.G.) 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 fluorescence dequenching was measured with excitation and emission wavelengths 560 nm and 590 nm, respectively [19], and with correction on light-scattering. The dequenching degree obtained in the presence of 0.1% Triton X-100 was taken to present 100% dequenching, i.e., infinite dilution of the probe. The fluorescence dequenching was calculated from the following equation:

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

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 and vesicles contents mixing measurements. For measuring the mixing of *M. capricolum* cell content and the content of SUV upon fusion, SUV were prepared in the presence of 10–40 mM of either 5,6-carboxyfluorescein (CF) or calcein. The SUV suspensions were passed through a Sephadex G-50 column to remove the fluorescent dyes outside the vesicles. Samples of the CF or calcein containing SUV were then incubated with *M. capricolum* cells at 37°C for 40 min, the suspension was diluted with 2.5 ml of cold A-buffer, and the fluorescence degree (excitation at 490 nm and emission at 520 nm) was measured before (experimental release) and after (total release) the addition of Triton X-100 (0.1% final concentration). The degree of fluorescence obtained in the presence of Triton X-100 was taken as 100% CF or calcein release.

Cholesterol and phospholipid exchange studies. M. capricolum cells (0.5–1 mg cell protein) containing [^{14}C]cholesterol and [^{3}H]phospholipids were incubated with lipid vesicles (90-fold excess with respect to lipid concentration) in 0.5 M sucrose containing 10 mM Tris-HCl (pH 7.4) and 10 mM $MgCl_2$ at 37°C, with gentle shaking for periods of up to 6 h. The lipid vesicles utilized were prepared from soybean L-α-phosphatidylcholine (3 mg/ml) and cholesterol (1.35 mg/ml). The cholesterol/phospholipid molar ratio of the vesicles was identical to that of the mycoplasma cell membrane. At defined time intervals, 0.5 ml aliquots for 3 min at room temperature in an Eppendorf centrifuge. The cell pellets were washed once, solubilized with 1% sodium dodecylsulfate, and radioactivity was determined.

Miscellaneous procedures. Labeling of *M. capricolum* cells with 12-(1-pyrene)dodecanoic fatty acid (P12, Molecular Probes, Eugene, OR, U.S.A.) was carried out by adding 5–10 μl of 2 mM P12 to 0.5 ml of a cell suspension in A-buffer (0.5 mg protein/ml) and incubating at 37°C for 20 min. The cells were then washed twice with A-buffer containing 0.4% bovine serum albumin (BSA), and resuspended in 1 ml volume of A-buffer. Fluorescence intensity of cells was measured in Perkin-Elmer LS-5B spectrofluorimeter with excitation and emission wavelengths 345 nm and 378 nm, respectively, with the correction on light-scattering.

Treatment of *M. capricolum* with proteolytic enzymes was performed by incubating 1 mg of *M. capricolum* cell protein with trypsin (30 μg) or pronase (25 μg) (both from Sigma) for 30 min at 37°C. Phospholipase A_2 treatment was performed by incubating 1 mg cell protein with 2–4 units of pancreatic phospholipase A_2 (Sigma) in A-buffer containing 1% BSA and 0.7 mM $CaCl_2$ for 1 h at 37°C.

For density gradient analysis, cells (0.5 mg cell protein) labeled with P12, intact SUV (40 μg) containing ^{3}H-labeled lipids, or SUV-cells fused material were layered over a 11 ml linear sucrose gradient (20–60%) in A-buffer and centrifuged for 2 h at 100000 × *g* in a Beckman SW41 rotor. Fractions (0.4–0.5 ml) were collected and assayed for radioactivity and fluorescence intensity. Intracellular water volume was determined in cell pellets, as previously described [21]. We used ^3H_2O to measure total pellet water and [^{14}C]poly(ethylene glycol) to measure the intercellular space in the cell pellet. The water space, minus the poly(ethylene glycol) space, was taken as the intracellular water volume.

Analytical procedures. Protein was determined by the method of Lowry et al. [22]. Lipids were extracted from membranes or cell preparations according to Bligh and Dyer [23]. The total lipid extract is composed mainly of: 42 mol% of cholesterol, 17 mol% phosphatidycholine, 14 mol% diphosphatidylglycerol, 12 mol% sphingomyelin acid, and 10 mol% phosphatidylglycerol [20,24,25]. To separate neutral from polar lipids, the lipid extracts were chromatographed on a silicic acid column [24]. Neutral lipids were eluted with 10 bed volumes of chloroform, and polar lipids were eluted with 10 bed volumes of chloroform/methanol (1 : 1, v/v).

Results

Fusogenic activity of PEG

In the present study, fusion between *M. capricolum* cells and small unilamellar lipid vesicles (SUV) was monitored by measuring the increase in fluorescence (fluorescence dequenching) of octadecylrhodamine B (R18) following incubation of fluorescently labeled SUV and *M. capricolum* intact cells. Fig. 1 shows that when *M. capricolum* cells were incubated with R18-labeled SUV in A-buffer for 40 min at 37°C, the degree of fluorescence dequenching was markedly affected by poly(ethylene glycol) (PEG 8000). The effect of PEG showed a concentration dependence with a dramatic enhancement of fluorescence dequenching obtained at relatively low PEG concentrations (4–6%). In the presence of 5% PEG, the extent of fusion was time-dependent (data not shown), and was virtually completed after 40 min of incubation. Other fusogenic substances, such as oleic acid (1–50 μM), spermidine (5 mM), or BSA (0.4–2.0%) did not enhance fluorescence dequenching.

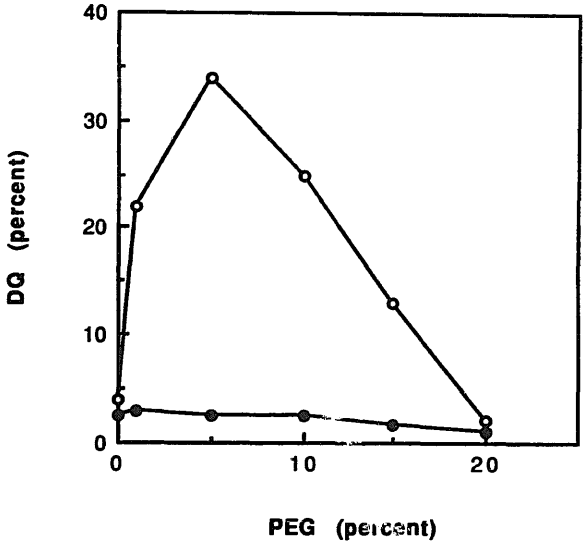

Fig. 1. The effect of PEG on the fusion of SUV with *M. capricolum* cells. R18-labeled SUV were incubated for 40 min at 37°C with *M. capricolum* cells in the presence of various concentrations of PEG. Intact cells (○); glutaraldehyde (0.1%) treated cells (●). The fluorescence dequenching measurements were performed as described in Materials and Methods.

The PEG-stimulated fluorescence dequenching was completely suppressed by treating the cell with low concentrations (0.01–0.1%) of glutaraldehyde or chlorpromazine (50 μM). Thus, glutaraldehyde (0.1%) or chlorpromazine (100 μM) treated cells wer included as negative controls in all subsequent experiments. With the increase of PEG concentration, the degree of fluorescence dequenching consistently decreased, reaching a low basic level at 20% PEG. Upon the completion of the SUV-*M. capricolum* cell fusion, the addition of Triton X-100 (0.1%) to the complete fusion mixture resulted in a 2–3-fold increase in the degree of fluorescence dequenching.

As was expected, the extent of R18 fluorescence dequenching directly correlated with the cell to SUV ratio in the incubation medium. Maximal fluorescence was obtained at a cell to SUV ratio of 20 (based on lipid content), implying that at such ratio an experimental infinitive dilution of the probe in *M. capricolum* membranes was achieved. Constant cell to SUV ratios were therefore used in the fusion assays throughout this study. During a 6-h incubation period of SUV and *M. capricolum* cells in the fusion medium, the cells remained intact, as was estimated by the retention of 92–97% of the [^{3}H]thymidine-labeled components within the cells [20] and showed only a small loss in viability (5%).

Divalent cations have been shown to be potent fusogenic agents, due to their ability to induce membrane aggregation and to dehydrate the intercellular space of adjacent membranes [2]. Fig. 2 shows that divalent cations are required for the PEG-stimulated SUV-

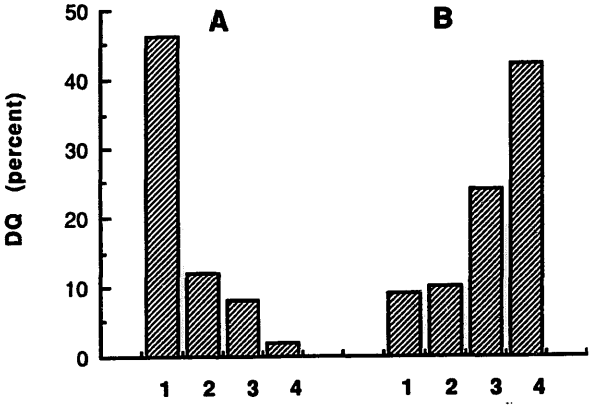

Fig. 2. The effect of EDTA and $MgCl_2$ on SUV-*M. capricolum* cell fusion. R18-labeled SUV were incubated with *M. capricolum* cells for 40 min at 37°C in a medium containing 5% PEG and various concentrations of EDTA, with or without $MgCl_2$. (A) Fusion in the presence of EDTA: (1), without EDTA; (2), 5 mM; (3), 10 mM; (4), 20 mM. (B) Fusion in the presence of EDTA (10 mM) and various concentrations of $MgCl_2$: (1), 5 mM; (2), 10 mM; (3), 20 mM; (4) 40 mM).

mycoplasma fusion. The addition of EDTA to the fusion medium (Fig. 2A) resulted in a marked inhibition of the fusion process, as revealed by the lower degree of fluorescence dequenching. The effect of EDTA was reversed by $MgCl_2$ (Fig. 2B) and, to a lesser extent, by $CaCl_2$ (data not shown).

To examine the possibility that PEG triggers not only the fusion process, but also lipid exchange between *M. capricolum* membranes and SUV, the exchange of cholesterol and phospholipids between *M. capricolum* cells and SUV was tested. In these experiments, we measured, under optimal fusion conditions (Fig. 3), the exchange of radioactive-labeled mycoplasma membrane cholesterol or phospholipids with a large excess (about

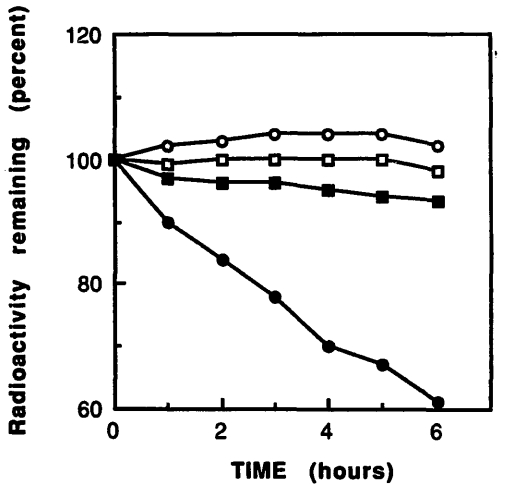

Fig. 3. Exchange of [^{14}C]cholesterol- and [^{3}H]palmitic acid-labeled lipids between *M. capricolum* cells and phosphatidylcholine-cholesterol liposomes. The exchange was performed at 37°C in sucrose-Tris-$MgCl_2$ buffer (pH 7.4) as described in Materials and Methods in the presence (open symbols) or absence (closed symbols) of 5% PEG. ○, ●, [^{14}C]cholesterol; □, ■, [^{3}H]palmitate-labeled lipids.

90 times) of nonradioactive cholesterol/egg phosphatidylcholine SUV. These vesicles contained approximately the same molar ratio of cholesterol to phospholipids as does the *M. capricolum* cells (0.9). This ratio was chosen to minimize the depletion of mycoplasma cholesterol during the incubation with SUV [25].

As seen in Fig. 3, in the absence of PEG, about 40% of *M. capricolum* [^{14}C]cholesterol underwent transfer to SUV, whereas the exchange of phospholipids was very low or nonexistent. In the presence of PEG (5%), the exchange of radioactive cholesterol was even lower. During the exchange period (6 h), cells remained intact, as was estimated by the retention of [^{3}H]thymidine-labeled components within the cells and the viability of the cells was fully preserved.

Further evidence of SUV-M. capricolum fusion

We have utilized the fluorescent probes 5(6)-carboxyfluorescein (CF) and calcein as internal aqueous markers. These probes were entrapped at self-quenching concentrations during SUV formation, with subsequent removal of their excess by gel filtration. Table I shows that when intact *M. capricolum* cells were incubated with SUV containing either carboxyfluorescein or calcein in the presence of 5% PEG, a pronounced increase in the fluorescence signal was obtained. Very little increase in fluorescence signal was observed in reaction mixtures without PEG, or in reaction mixtures containing either SUV alone or SUV plus glutaraldehyde-treated cells. These results indicate that a mixing of the SUV content with that of the mycoplasma cells occurred.

Almost the same increase in fluorescence signal was observed in experiments where cells, after the incubation with SUV and PEG, were separated from the reaction mixture by centrifugation, resuspended in fresh cold A-buffer and analyzed. These results exclude the possibility that the increase in the fluorescence signals

TABLE I

The release of carboxyfluorescein and calcein from SUV following the fusion with M. capricolum cells

SUV loaded with carboxyfluorescein or calcein were incubated with or without intact *M. capricolum* cells at 37°C for 40 min in the presence or absence of PEG. The extent of release was estimated as described in Materials and Methods.

Fusion system	% of total	
	carboxy-fluorescein	calcein
SUV with *M. capricolum* cells		
Without PEG	7.5	10.2
With 5% PEG	41.1	39.0
With 5% PEG and 0.1% glutaraldehyde	4.3	6.4
SUV without cells		
Without PEG	0.7	0.3
With 5% PEG	4.9	7.1

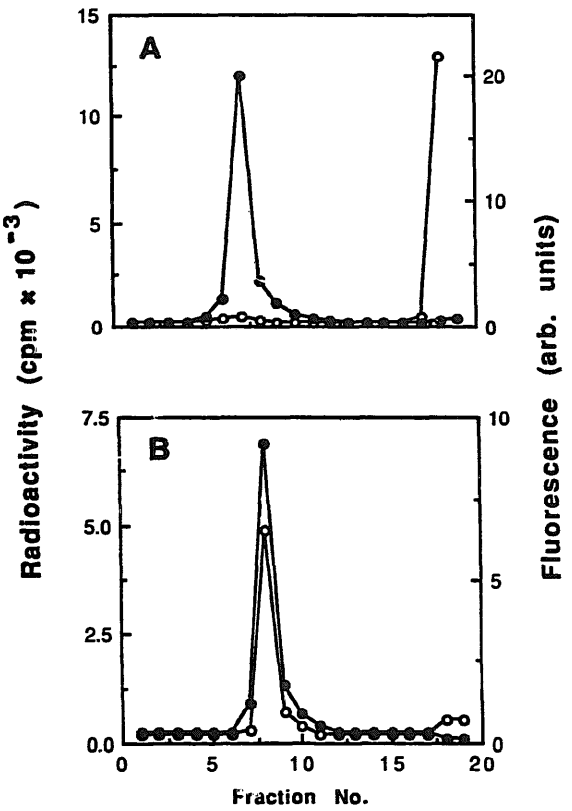

Fig. 4. Density gradient analysis of SUV-*M. capricolum* cell fusion. [^{3}H]Oleic acid labeled SUV (○) and P12-labeled cells (●) were incubated at 37°C for 40 min in the absence (A) or presence (B) of 5% PEG and then analyzed on 20–60% sucrose density gradients as described in Materials and Methods. The highest fraction number corresponds with the top of the gradient.

are due to the leakage of the SUV content to the reaction mixture upon SUV–cell interaction. Such leakage has been described in other fusion systems [2,6].

The results of a density gradient analysis of the fused SUV-mycoplasma material are shown in Fig. 4. The SUV utilized were composed of [^{3}H]palmitate-labeled *M. capricolum* lipids, whereas mycoplasma cells were fluorescently labeled with the fatty acid fluorescent analog P12, which was incorporated into the neutral lipid fraction (Tarshis, M., unpublished data). Thus, the position of the SUV band on the gradient was determined by measuring the distribution of radioactivity, and the position of the cells on gradient was determined by measuring the distribution of P12 derived fluorescence. Fig. 4a shows that when *M. capricolum* cells were incubated with SUV in the absence of PEG and analyzed on a 20–60% sucrose gradient, the cells sedimented at a density of 1.206 g/ml, whereas the SUV fraction formed a band at the top of the gradient. In the presence of PEG, cells and SUV preparation co-sedimented (Fig. 4B). Moreover, the cell-SUV band shifted to a new position corresponding to a low density (1.196 g/ml). These results suggest that the fusion results in bulk translocation of phospholipids from SUV into mycoplasma cell membranes.

Optimal fusion conditions. Fig. 5 shows that the fusion was a temperature dependent-process. In the absence of PEG, or when glutaraldehyde-treated cells were utilized in the complete fusion mixture, a low R18 fluorescence dequenching was observed. This fluorescence was much more pronounced above 37°C and may represent either a slow exchange of the label between membranes and SUV, leakage of the probe from SUV membranes, or spontaneous fusion. The effect of temperature on the net PEG-dependent process is shown in the insert to Fig. 5. The inset, obtained by subtracting the extent of fluorescence in the absence of PEG from that obtained in the presence of PEG shows the highest fluorescence level at 37°C. Maximal fusion activity was observed between pH 6.8 and 8.2. Above pH 8.2 and below pH 5.5, the fusion intensity was negligible.

Osmotic swelling of cells has been reported to affect lipid vesicle-cell fusion [27]. Fig. 6 shows that changes in R18 dequenching occurs when *M. capricolum* cells and SUV were incubated in fusion media of various osmolarities containing 5% PEG. Higher fluorescence dequenching levels were obtained in a medium of low osmolarity, whereas low levels were obtained in media of high osmolarity. Under these conditions, the wall-less mycoplasma cells swell or shrink, as monitored by measuring the intracellular cell water. The intracellular water volume of cells suspended in isoosmotic salt solutions (550 mosM) was about 1.6 μl/mg cell protein and was increased up to 2.9 μl/mg cell protein in a solution of 280 mosM.

Effect of inhibitors on mycoplasma-SUV fusion. The treatment of intact *M. capricolum* with either NH_4Cl

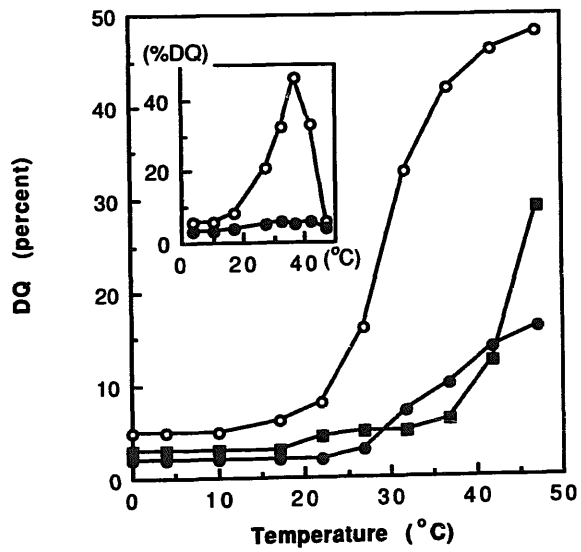

Fig. 5. Temperature dependence of SUV-*M. capricolum* cell fusion. SUV were incubated at various temperatures with native (open symbols), or glutaraldehyde treated (closed symbols) *M. capricolum* cells. ○, ●, with PEG; □, without PEG. Inset: net PEG-dependent fluorescent dequenching.

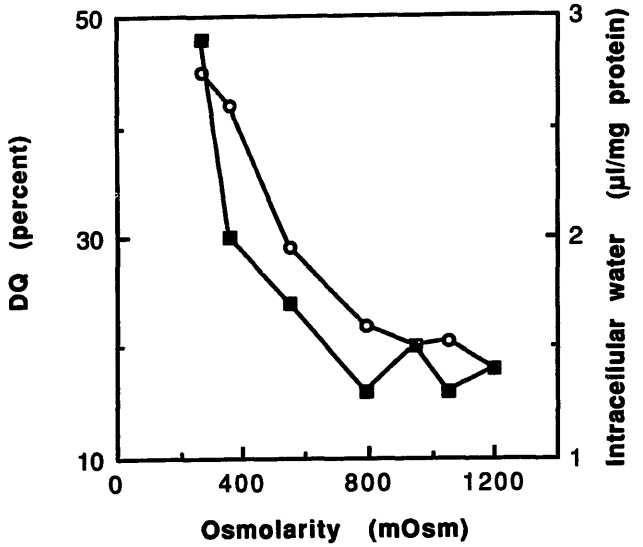

Fig. 6. The effect of the osmolarity of the incubation medium on the fusion of SUV with *M. capricolum* cells. R18-labeled SUV were incubated for 40 min at 37°C with cells in a medium of various osmolarities containing 5% PEG and then analyzed for fluorescence dequenching (○), and intracellular water content (■) as described in Materials and Methods.

(50 mM), methylamine (50 mM), or phenylmethylsulfonyl fluoride (PMSF, 7 mM), commonly used inhibitors of virus-cell fusion [19], has almost no effect on the R18 fluorescence dequenching. No effect on SUV-*M. capricolum* fusion was obtained with the reducing agent dithiothreitol (3 mM), known to perturb the fusogenic activity of Sendai virus. *M. capricolum* cells were rendered nonfusogenic by treatment with low concentrations of glutaraldehyde (0.05%) or chlorpromazin (100 µM). A partial decrease (45%) in the degree of fluorescence dequenching was obtained with the proton ionophore carbonyl cyanide-*m*-chlorophenylhydrazone (CCCP, 5 µM), whereas dicyclohexylcarbodiimide (DCCD, 100 µM), an inhibitor of the Mg^{2+}-dependent mycoplasmal ATPase, did not affect fusion. A marked decrease (44–53%) in the degree of fluorescence dequenching was obtained after *M. capricolum* cells were treated with trypsin (30 µg/ml 30 min at 37°C) or pronase (25 µg/ml, 30 min at 37°C). As after the proteolytic treatments, almost all of the [^{3}H]thymidine-labeled components were retained within the cells, it seems that such treatments did not affect cell intactness.

Effect of cholesterol on mycoplasma-SUV fusion. To test the effect of cholesterol on the PEG-induced mycoplasma-SUV fusion, intact *M. capricolum* cells were fused with SUV containing various amounts of cholesterol. Table II shows that a high degree of fluorescence dequenching was obtained with SUV made of total mycoplasal cell lipids, or with SUV containing the polar lipid fraction supplemented with 40 mol% of cholesterol, whereas a low extent of fluorescence dequenching was obtained with SUV containing the polar lipid fraction alone.

TABLE II

The effect of cholesterol on SUV-M. capricolum fusion

Total lipids were extracted from *M. capricolum* cells. The fractionation of lipids and preparations of SUV was conducted as described in Materials and Methods.

SUV made of	Dequenching (%)
Total lipid extract	54
Polar lipid fraction	12
Polar lipid fraction + 15 mol% cholesterol	23
Polar lipid fraction + 40 mol% cholesterol	47

Discussion

Our results show that when *M. capricolum* cells were incubated with small unilamellar vesicles in the presence of low concentrations of PEG and divalent cations, mixing of bilayer lipids can be demonstrated. We have used R18 dequenching assay to follow the fusion of *M. capricolum* cells and small unilamellar vesicles (SUV), as well as to explore the factors which influence it. This probe, once inserted into a membrane, will not dissociate from the membrane, either by spontaneous diffusion of the free monomers through the aqueous phase or by a collision-mediated transfer process [11]. Most important is that the fluorescence dequenching, representing the mixing of lipids, is not affected by the mere aggregation of membranes [28]. As the exchange of either cholesterol or membrane phospholipids was not enhanced under the optimal fusion conditions, and the incubation of SUV with glutaraldehyde treated cells resulted in a very low degree of fluorescence dequenching, it seems that the fluorescence dequenching observed is due to membrane fusion and not to lipid-lipid exchange processes.

Several proposals have been made to explain the ability of PEG to induce membrane fusion. Most proposals recognize the ability of high concentrations of PEG to alter the physical state of bulk water adjacent to the cell surface and for the water of hydration of phospholipid polar head groups in the cell membrane [1,27,28]. It has also been shown that PEG induces changes in the lipid phase transition [29], suggesting that PEG is capable of destabilizing the lipid backbone. In our study, enhanced fusion activity was obtained at rather low PEG concentrations (4–6%). At such concentrations, there are essentially no changes in medium polarity [29], or in the freedom of motion of the hydrocarbon chain of membrane phospholipids [1,29]. Hence, relative small or even undetectable perturbations in the surface water structure and/or the physical state of membrane lipids induced by low PEG concentrations

suffice to trigger the SUV-*M. capricolum* fusion process. The very low fusion activity observed at relatively high PEG concentrations may be due to a nonspecific aggregation of either *M. capricolum* or SUV that may prevent fusion [2].

As has been already mentioned, repulsive forces between negatively charged artificial or natural membranes have to be eliminated before fusion can occur [3,30]. The requirement for a divalent cation for the SUV-*M. capricolum* fusion was expected in view of the negatively charged cell surface of *M. capricolum* cells [25] and the SUV utilized. The SUV are made of the total lipid extract of *M. capricolum* containing negatively charged phospholipids, mainly phosphatidylglycerol and diphosphatidylglycerol [20,25]. It is well established that divalent cations or polycations are required for the fusion of negatively charged liposomes [31]. The divalent cations have to bring the bilayers into close proximity by overcoming the repulsive forces between the polar head groups of negatively charged phospholipids and to destabilize the attached bilayers [32]. Such destabilization may be due, in part, to the ability of certain phospholipids, e.g., diphosphatidylglycerol, to assume a non-bilayer configuration [8]. It has been proposed that the non-bilayer configuration is required to satisfy membrane-mediated processes such as fusion [2].

A casual relationship between cell swelling and fusion ability has been proposed previously [27]. Our data show that exposing *M. capricolum* cells to an hypo- or hyperosmotic environment in the presence of PEG resulted in changes in the extent of fluorescence dequenching. Thus, in hypoosmotic medium, fluorescent dequenching was increased, compared to cells exposed to isoosmotic conditions. This observation supports the suggestion that partial destabilization of the membrane bilayer is obtained by swelling cells in an hypoosmotic medium [32].

Liposome-cell fusion involves not only membrane bilayer intermixing, but also intermixing of internal aqueous contents to form a single compartment surrounded by one membrane. Düzgüneş et al. [31] pointed to the importance of utilizing this approach in conjunction with lipid mixing assay because probe mixing assay has to be interpreted cautiously, due to sensitivity to simple aggregation. Results obtained with SUV and mycoplasma cell mixing assay, which monitored dequenching of carboxyfluorescein and calcein incorporated into SUV internal space, were in good agreement with those obtained with R18 dequenching assay. These results were supported by density gradient analysis, indicating the fusion of the lipid vesicles with intact *M. capricolum* cells.

Our finding that proteolytic digestion of intact *M. capricolum* cells decreases the extent of SUV-*M. capricolum* fusion is of great interest. These findings support the notion that *M. capricolum* cells possess proteinase-sensitive receptors on its cell surface responsible for a tighter contact with the lipid vesicles. Proteinase-sensitive receptors have been detected on the surface of some prokaryotic and eukaryotic cells [9,33]. One cannot exclude the possibility that after the partial cleavage of surface proteins, membrane lipids tend to aggregate in protein-free membrane areas that may prevent them from fusion [34,35].

The presence of cholesterol in the mycoplasma cell membrane has been previously shown to be required for the fusion of mycoplasmas with Sendai or influenza viruses [19]. In the present study, we have shown, for the first time, that the presence of cholesterol in the SUV is obligatory to allow the PEG-stimulated SUV-mycoplasma fusion to occur. This conclusion is based on the following observations: (1) *M. capricolum* cells fused with SUV made of the total lipid fraction extracted from *M. capricolum* cells, but failed to fuse with SUV made of the isolated polar lipid fraction that contains no, or negligible amounts of, cholesterol, or with SUV made of azolectin (data not shown). (2) The fusogenic activity of SUV made of the polar lipid fraction was almost fully restored when supplemented with 40 mol% cholesterol. As traditional methods of introducing macromolecules into mycoplasma cells have so far failed, the fusion of mycoplasma with lipid vesicles may provide, for the first time, a precise approach for gene introduction, allowing study of gene expression and regulation in mycoplasmas.

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