Two cholesterol pools in *Acholeplasma laidlawii* membranes

P.J. Davis, H. Efrati, S. Raxin* and S. Rottem

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

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Cholestrol exchange kinetics between [^{14}C]cholesterol-labeled *Acholeplasma laidlawii* and *Mycoplasma gallisepticum* cells and phosphatidylcholine-cholesterol vesicles followed a biphasic curve, with faster exchange rates for *A. laidlawii*. The same biphasic curve was obtained with isolated membranes. Cholesterol exchange between lipid vesicles and *A. laidlawii* cells depleted of phospholipids by phospholipase A_2, fitted a monophasic linear curve. The data support the hypothesis that the biphasic cholesterol exchange kinetics do not result from the transbilayer distribution of cholesterol, but reflect the presence in the membrane of two cholesterol pools associated with lipids of high and low affinity for cholesterol.

Cholesterol exchange *Mycoplasma membrane* Acholeplasma laidlawii Mycoplasma gallisepticum *Phospholipase A_2* *Lipid vesicle*

1. INTRODUCTION

The kinetics of [^{14}C]cholesterol transfer between *Mycoplasma gallisepticum* cells and lipid vesicles exhibited a biphasic curve [1]. This was interpreted to represent two cholesterol pools, a rapidly exchangeable pool representing cholesterol located in the outer leaflet of the bilayer, and a slower exchangeable pool that represents cholesterol in the inner leaflet of the bilayer [1]. Doubts about the validity of this interpretation were raised in light of recent data indicating the rapid transbilayer mobility of cholesterol in biological membranes [2,3] and the finding of a similar biphasic cholesterol exchange curve with isolated *M. gallisepticum* membranes, in which both membrane leaflets are accessible to the lipid vesicles [4]. The recent hypothesis put forward [4] to interpret the two cholesterol pools in the membrane is based on the notion that cholesterol shows different affinities to various membrane lipids. Thus, the faster exchangeable pool in *M. gallisepticum* represents cholesterol molecules in weak association with certain membrane lipids.

* To whom correspondence should be addressed

To obtain further support for the above hypothesis *A. laidlawii* was chosen as a test organism. This mycoplasma differs radically in lipid composition from *M. gallisepticum* in having a high content (about 60% of total membrane lipids) of glycolipids and phosphoglycolipids [5,6]. *A. laidlawii* is also distinguished by its low cholesterol binding capacity, as compared to *Mycoplasma* species [7]. A possible explanation for the low cholesterol content can be based on the assumption that membrane glycolipids have a low affinity for cholesterol, as compared to phospholipids. Support for this explanation was recently obtained [5] by analysis of cholesterol binding ability of various *A. laidlawii* strains differing in glycolipid content. It seemed worthwhile to determine cholesterol exchange between *A. laidlawii* and lipid vesicles, and test how depletion of the cell membranes of phospholipids will affect cholesterol exchange. The data presented in this paper show that as in *M. gallisepticum* [^{14}C]cholesterol exchange between *A. laidlawii* cells and lipid vesicles follows a biphasic curve but the exchange rates of both the fast and slow exchangeable pools are much faster in *A. laidlawii*. Cholesterol exchange between *A. laidlawii* cells

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depleted of phospholipids and lipid vesicles was, however, fitted into a monophasic linear curve, supporting the hypothesis that the biphasic cholesterol exchange kinetics result from membrane lipid pools differing in their affinity for cholesterol.

2. MATERIALS AND METHODS

2.1. *Organisms and growth conditions*

Acholeplasma laidlawii (strain 1012, obtained from Dr M.F. Barile, FDA, Bethesda, MD) and *M. gallisepticum* (strain A5969) were grown in a modified Edward medium [8] containing 0.5% (w/v) delipidated bovine serum albumin. For growth of *A. laidlawii* the medium was supplemented with elaidic acid (20 μg/ml) and cholesterol (0.2 μg/ml), while for growth of *M. gallisepticum*, a mixture of cholesterol (20 μg/ml), oleic and palmitic acids (10 μg/ml each) and egg phosphatidylcholine (egg PC; 25 μg/ml) in methanol was added to the medium. In early experiments *A. laidlawii* was also grown in Edward medium containing 4% (v/v) of horse serum replacing all the above supplements. Membrane cholesterol was labeled by adding 0.002 μCi/ml of [^{14}C]cholesterol to the medium. The organisms were harvested at the mid-exponential phase of growth (A_{640} = 0.17–0.22) by centrifugation at 12000 × *g* for 15 min, washed once in 0.25 M NaCl and resuspended in a buffer solution containing 0.4 M sucrose, 50 mM Tris–HCl (pH 7.5), and 25 mM $MgCl_2$ (to be referred to as sucrose–Tris–$MgCl_2$ buffer). Cell membranes of *A. laidlawii* were isolated by osmotic lysis of the organisms, as described in detail in [8].

2.2. *Phospholipase A_2 treatment*

A. laidlawii cells (2 mg cell protein/ml) were suspended in a buffer solution containing 0.25 M NaCl, 25 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$ and 5 mM $CaCl_2$. After 30 min of incubation at 37°C phospholipase A_2 from porcine pancreas (Sigma) was added to a final concentration of 100 μg/ml, and incubation was continued for 2 h. Cells were collected at 12000 × *g* for 15 min, washed with 0.25 M NaCl and resuspended in the sucrose–Tris–$MgCl_2$ buffer.

2.3. *Preparation of lipid vesicles*

Egg PC–cholesterol vesicles (molar ratio 1:1) were prepared by mixing cholesterol (13.5 mg) and egg PC (30 mg) in chloroform solutions. The solvent was evaporated under nitrogen and the dried lipid dispersed in 10 ml of the sucrose–Tris–$MgCl_2$ buffer by ultrasonic irradiation for 60 min at 4°C, using a W350 Heat Systems sonicator operated at 50% duty cycle with a large probe at 160 W. Large vesicles and debris were eliminated by centrifugation at 34000 × *g* for 30 min at 4°C.

2.4. *Cholesterol exchange*

[^{14}C]Cholesterol-labeled *A. laidlawii* and *M. gallisepticum* cells, or isolated *A. laidlawii* membranes were incubated at 37°C in the sucrose–Tris–$MgCl_2$ buffer supplemented with 2% bovine serum albumin and egg PC–cholesterol vesicles. The vesicles contained approximately 100-fold more unesterified cholesterol than that found in the cells or membranes. To prevent cell aggregation due to liberated DNA, 20 μg/ml of deoxyribonuclease (Sigma) was added to the cell suspensions. At various time intervals, duplicate 0.5-ml samples (each containing 0.2–0.4 mg cell or membrane protein) were transferred into plastic microfuge tubes. The cells or membranes were collected by centrifugation at 12000 × *g* for 4 min in the microfuge. To facilitate sedimentation of the membranes an antiserum to *A. laidlawii* was added at a final 10^{-3} dilution. The pellets were washed once with 0.25 M NaCl at 4°C. The tips of the microfuge tubes containing the cell or membrane pellet were cut, placed in 5 ml of scintillation liquid (40% Lumax, Lumac, Schaesberg, The Netherlands) and counted in a Packard Tri-Carb liquid scintillation spectrometer.

2.5. *Analytical methods*

Cell protein was determined by the method of [9]. Lipids were extracted by the procedure of [10] and the extracts applied to silica gel HR (Merck) thin-layer plates. The plates were developed with chloroform–methanol–water (65:25:4, v/v) at 4°C, and the lipid spots detected by iodine vapor. Phospholipid spots were scraped off the thin-layer plates and total phosphorus in the material was determined by the method of [11] after digestion with magnesium nitrate. Colorimetric measurement of cholesterol was done according to [12].

3. RESULTS AND DISCUSSION

Our exchange system comprised *A. laidlawii* and *M. gallisepticum* cells containing labeled cholesterol incorporated during growth, and a large excess of phosphatidylcholine vesicles containing unlabeled cholesterol at a 1 : 1 molar ratio. The excess of vesicle cholesterol prevented a decrease in the cholesterol content of the cell membranes during the incubation period and ensured that when the system reaches an equilibrium, the maximal amount of labeled cholesterol will be exchanged from the cells. Determination of cholesterol content of cells at the end of the incubation period showed, in fact, no significant change from zero time, indicating the lack of net cholesterol transfer. Bovine serum albumin was added to the exchange mixture, as this compound was shown to enhance considerably cholesterol exchange, possibly by increasing cholesterol solubility in the aqueous medium [1]. Although the exchange experiments lasted for about 12 h cell integrity was retained, as indicated by minimal changes in absorbance of the cell suspension at 500 nm, and by viability tests based on colony counts [13].

Fig.1 compares cholesterol exchange from *A. laidlawii* and *M. gallisepticum* cells. While the two exchange curves resemble each other in being biphasic, suggesting the existence of two cholesterol pools, the exchange rates of both pools from *A. laidlawii* cells were much faster. As cholesterol exchange rates were found to increase with a decrease in the cholesterol to phospholipid molar ratio [14] it is feasible to assume that the fast cholesterol exchange rates in *A. laidlawii* are due to the low cholesterol content of *A. laidlawii* membranes.

The cholesterol exchange curve of intact *A. laidlawii* cells was essentially identical in shape and in rate to that obtained with isolated membranes of this organism (not shown). The isolated membrane preparations used in the exchange experiments failed to show any swelling in glycerol or erythritol solutions by the procedure of [15] indicating that the membrane ghosts were open and not sealed.

The identity of the cholesterol exchange curves of intact *A. laidlawii* cells and isolated membranes is in accord with recent data obtained with *M. gallisepticum* [4].

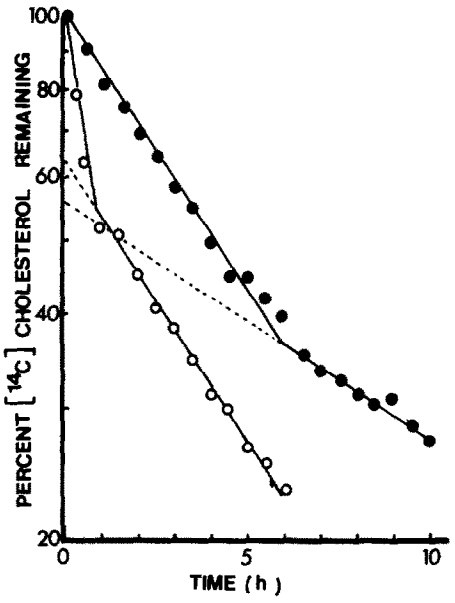

Fig.1. Exchange of [^{14}C]cholesterol between (○) *A. laidlawii* and (●) *M. gallisepticum* cells and phosphatidylcholine-cholesterol vesicles. The organisms were grown in the presence of [^{14}C]cholesterol, while the vesicles contained unlabeled cholesterol. For experimental details see section 2.

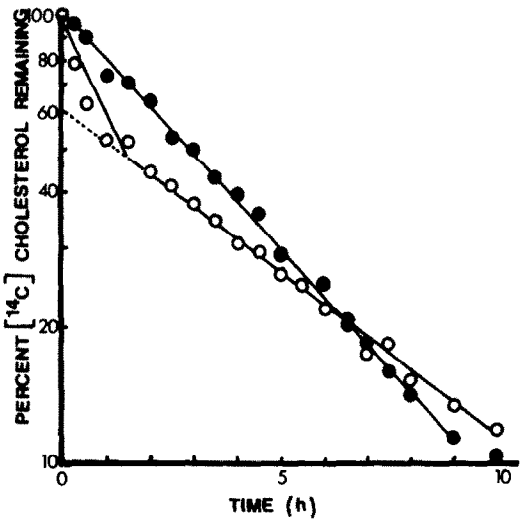

Fig.2. Exchange of [^{14}C]cholesterol between phosphatidylcholine-cholesterol vesicles and (●) *A. laidlawii* cells treated with phospholipase A_2 and with (○) untreated control cells.

To test the hypothesis that the biphasic exchange kinetics results from the presence in the membrane of lipid classes differing in their affinity for cholesterol, the *A. laidlawii* membrane phospholipids, phosphatidylglycerol and diphosphatidylglycerol were hydrolyzed by phospholipase A_2. These phospholipids comprise ~30–35% of the total polar lipids in this organism, the rest being glycolipids and phosphoglycolipids [5,6]. Although phospholipids of *A. laidlawii* cells grown with 4% horse serum were resistant to phospholipase A_2 treatment, apparently due to the relatively high cholesterol content of the membranes [16], the phospholipids of cells grown in the medium in which the serum was replaced by albumin, elaidic acid and a low concentration of cholesterol were highly susceptible to phospholipase A_2. This treatment resulted in a decrease of 86% in the phosphatidylglycerol content of the cell membrane. As can be seen in fig.2 the cholesterol exchange kinetics of the phospholipase A_2-treated cells could be fitted into a linear exchange curve. However, the rate of cholesterol exchange from the treated cells was intermediate between that of the fast and slow rates shown with untreated cells. One cannot rule out the possibility that the change observed in cholesterol exchange rates results from changes in the transbilayer distribution of cholesterol in the phospholipase-treated cells. Yet, our finding that isolated unsealed *A. laidlawii* membranes show a cholesterol exchange pattern identical with that of intact cells contradicts this interpretation. The intermediate exchange rate may be explained, according to our view, by changes in the affinity for cholesterol of the lipids remaining in the membrane after removal of the glycerophospholipids by the phospholipase.

In conclusion, the data can be taken to support the hypothesis that the biphasic cholesterol exchange curve reflects two cholesterol pools associated with lipids of low and high affinity for cholesterol rather than a result of transbilayer distribution of cholesterol in the membranes. In the case of *A. laidlawii* it appears that the lipids of low affinity for cholesterol are glycolipids, while phospholipids constitute lipids of high affinity for cholesterol.

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