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Fusion of Phospholipid Vesicles with Viable *Acholeplasma laidlawii*

(membrane fusion/spin labels/mycoplasma)

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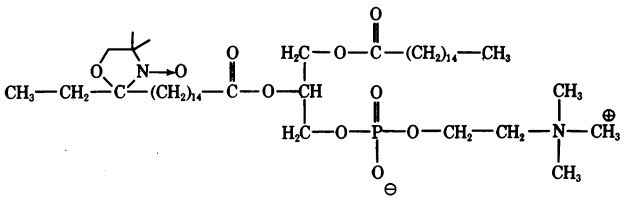
ABSTRACT When vesicles of dipalmitoyl phosphatidylcholine produced by sonication are mixed with *Acholeplasma laidlawii* in neutral buffer, a phenomenon occurs that is most readily explained in terms of fusion of lipid vesicle membrane with the mycoplasma cytoplasmic membrane. The mycoplasma can readily accumulate large quantities of the foreign lipid without loss of viability. The added lipids do not remain in patches but diffuse laterally throughout the mycoplasma membrane.

Previous workers in this laboratory have shown that phospholipid vesicles can fuse with various biological membranes (1, 2) and that after fusion the introduced lipids diffuse rapidly throughout the biological membrane. An obvious extension of this work is to viable cells. In particular, we report here the fusion of vesicles of dipalmitoyl phosphatidylcholine with the pleuropneumonia-like organism, *Acholeplasma laidlawii*, which lacks a cell wall and is bounded by a plasma membrane.

MATERIALS AND METHODS

L-α-Dipalmitoyl phosphatidylcholine (DPPC) and L-α-dimyristoyl phosphatidylcholine (DMPC) used for fusion experiments were obtained from Calbiochem and were used without further purification. They contained traces of impurities (less than 1%).

Tempophosphate was prepared by the method of Weiner (3) and purified as described by Kornberg *et al.* (4). It was used as the disodium salt. DPPC spin-labeled in the choline head group (5) was a gift of M. G. McNamee, ^{14}C-labeled egg phosphatidylcholine of Dr. C. J. Scandella, and the spin label, tempo, of K. L. Wright, all of this laboratory. ^{14}C-labeled sucrose and 2-deoxy-D-glucose were obtained from New England Nuclear Corp. The spin label, **I**, was prepared by



I

acylation of pure DPPC (Schwarz/Mann) lysolecithin with the spin-label fatty acid anhydride, as described by Hubbell and McConnell (6).

A. laidlawii, strain B, was initially obtained from Dr. E. Stanbridge and Dr. L. Hayflick of the Stanford Department of Medical Microbiology and was subsequently grown on a modified Edward medium (7). Cultures were harvested after 17–22 hr at 34.5° by centrifugation at 9000 rpm (9750 × *g*) for 30 min. Such mycoplasma were fully viable and actively reduced spin labels at 36°. Furthermore, they possessed a phospholipase B activity (8). These properties were inconvenient for certain experiments. Treatment with 30% sucrose at 0° for several hours greatly retarded their ability to reduce water-soluble spin labels (i.e., collection on a 30/70% sucrose step gradient and subsequent standing at 0° before being washed with neutral buffer). Nevertheless, such treatment left the mycoplasma intact; they retained their phospholipase B activity and the ability to reduce lipid-soluble spin labels. Subsequent treatment of the mycoplasma with 4 mM $HgCl_2$ effectively inhibited both their remaining reducing capability and also their lipase, but at the same time made them very leaky.

Leakiness of the treated *A. laidlawii* could be gauged by incubation in neutral buffer containing 1 mM tempophosphate at 36° for up to 1 hr or more. This treatment permitted the water-soluble spin label to permeate the membrane. If the suspension was then cooled to 0° and a 10-fold excess of ascorbate was added (5), any spin label that was not protected within the mycoplasma was reduced within 1 min.

In general, vesicles of the various phospholipids used were prepared by sonication under argon for up to 40 min in a Branson Sonicator (model W185D) at power setting 3.5. This treatment produces single-compartment, bilayer vesicles (9). DPPC was sonicated at 50°; spin label **I** was sonicated at 40°.

DPPC vesicles were loaded with the water-soluble, lipid-insoluble substances, tempophosphate, [^{14}C]sucrose, or [^{14}C] 2-deoxy-D-glucose (which are all relatively slow at penetrating lipid membranes at 0°) by the following procedure: DPPC was sonicated in 2.0 ml of neutral buffer containing the desired label. After centrifugation to remove titanium powder (produced during sonication), the vesicles were passed through a column of fine Sephadex G-25 (Pharmacia) to remove any label not contained in the vesicles.

Experiments were performed in 0.15 M NaCl buffered with 0.05 M phosphate at pH 7.0.

After a fusion experiment, vesicle and mycoplasma fractions were readily separated by centrifugation at 36° on a 0–30% sucrose step gradient, followed by washing the mycoplasma with 36° buffer. Thin-layer chromatography of the extracted *A. laidlawii* lipids and the remaining vesicle lipids

Abbreviations: DPPC, dipalmitoyl phosphatidylcholine; DMPC, dimyristoyl phosphatidylcholine; tempo, 2,2,6,6-tetramethylpiperidine-1-oxyl; tempophosphate, 2,2,6,6-tetramethylpiperidine-1-oxyl-4-phosphate.

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indicated that relatively little, if any, *A. laidlawii* lipid became associated with the vesicles of DPPC.

RESULTS

A. laidlawii readily take up DPPC (to a concentration of up to 50% of their total lipid content)

Normal *A. laidlawii* band sharply at a position corresponding to a density of 1.21–1.22 g/cm³ on a 30–70% sucrose density gradient. After treatment with vesicles of DPPC in neutral buffer, the *entire population* bands almost equally sharply at a new position corresponding to a lower density. Depending on the ratio of vesicle lipid to mycoplasma lipid and the incubation time, the density of the mycoplasma can be decreased dramatically (the *fraction* of added DPPC that is incorporated does, however, decrease with increasing vesicle concentration). Thus, when *A. laidlawii* representing about 0.6 mg of membrane lipid in 0.40 ml of buffer were incubated with 0.40 ml of a DPPC vesicle suspension containing 9.25 mg of DPPC per ml for 30 min at 36° followed by separation on a 36° sucrose gradient and subsequent washing, the density of the mycoplasma had decreased to 1.17 g/cm³.

Normal *A. laidlawii* contain no phosphatidylcholines (8, 10). Thus, when their lipids are extracted with 2:1 chloroform–methanol and chromatographed on silica gel (65:25:4 chloroform–methanol–water eluent), no major lipids appear at the R_F value characteristic of phosphatidylcholines. However, after fusion with vesicles of DPPC, separation of the extraneous DPPC, and subsequent lipid extraction, thin-layer chromatography yields a spot corresponding to DPPC.

When DPPC vesicles are prepared by sonication in the presence of a small amount of ^{14}C-labeled egg phosphatidylcholine, the latter is incorporated in the lipid bilayers. Fusion of such vesicles with *A. laidlawii* and subsequent separation of extraneous lipid from the mycoplasma yields cells containing radioactive lipid. Thus, when 0.20 ml of a suspension of DPPC vesicles containing 5% radioactive egg phosphatidylcholine (total DPPC, 0.60 mg) was incubated with *A. laidlawii* equivalent to about 1.1 mg of mycoplasma lipid in 0.76 ml of buffer for 30 min at 36°, 45% of the radioactivity was found associated with the mycoplasma and 55% remained associated with the vesicles. Thus, the mycoplasma now contained some 25% exogenous lipid. In this particular experiment the mycoplasma had been previously treated with 30% sucrose and 4 mM $HgCl_2$, as described in *Methods*. Similar experiments have been performed with viable *A. laidlawii* that yielded analogous results. Thorough washing of the mycoplasma before a fusion experiment had little effect on the extent of fusion.

Uptake of large quantities of DPPC has no detectable effect on *A. laidlawii* viability

Freshly grown *A. laidlawii* were collected, rinsed, and resuspended in neutral phosphate buffer at 0°. Equal portions (0.30 ml) corresponding to about 0.4 mg of mycoplasma lipid were placed in each of two small tubes. The tubes were warmed to 36° and to one was added 0.30 ml of buffer and to the other 0.30 ml of a sonicated dispersion of DPPC containing 37 mg of DPPC per ml. Both tubes were incubated at 36° for 60 min before being plated out on mycoplasma agar. The viability of the mycoplasma seemed unaffected by the vesicle addition. Medium titer was 1.2×10^8 viable cells per ml, as established by this experiment.

The added phospholipid diffuses laterally throughout the mycoplasma membranes

At first sight it would seem possible that the phosphatidylcholine found associated with *A. laidlawii* after a fusion experiment might be present in patches; however, we have concluded from two types of experiment that this is not so, but rather that the new lipid diffuses throughout the mycoplasma lipids to become part of the cell membranes.

When vesicles are prepared from the spin-labeled phospholipid, **I**, the paramagnetic resonance signal is a single broad line due to the proximity of the unpaired electrons to one another. Diffusion of these spin-labeled phospholipids into unlabeled lipid leads to the appearance of the three-line spectrum characteristic of nitroxide radicals in dilute solution. This phenomenon has been fully discussed by Scandella *et al.* (1) and Devaux *et al.* (11).

When vesicles of **I** produced by ultrasonic irradiation are mixed with viable *A. laidlawii*, the fusion process is reflected in the paramagnetic resonance spectrum by: (*a*) gradual appearance of a sharper, 3-line component; (*b*) gradual spin-label reduction by the mycoplasma; and (*c*) formation of some free fatty acid 1,14 spin label, presumably through the action of a phospholipase (8). Prior treatment of the mycoplasma with 30% sucrose and 4 mM mercuric chloride virtually eliminates the processes (*b*) and (*c*) but does not affect (*a*).

Furthermore, pure DPPC exhibits a very sharp phase transition at about 41° (12, 13), which can be readily measured by monitoring the distribution of a spin label, tempo, between aqueous and lipid regions (6, 13). This distribution has been measured for *A. laidlawii* that have been fused with DPPC vesicles and is plotted as the "tempo spectral parameter" (13) in Fig. 1 along with data for ordinary *A. laidlawii* and DPPC liposomes. Pure DPPC displays a sharp transition at 41.2°, and the ordinary *A. laidlawii* show no transition at all. The *A. laidlawii* that have been fused with DPPC show a rather broad transition with an inflection point several degrees lower than that of pure DPPC (about 38° in this case). This behavior is characteristic (13) of the lateral phase separation of a region relatively rich in DPPC, or of a noncooperative phase transition of pure DPPC (9, 12). Our experiments with the spin-labeled phospholipid, **I**, preclude the latter possibility.

The nature of the fusion process

Other members of this laboratory (9) have studied a process whereby vesicles of pure DPPC fuse with one another in such a way that there is mixing of internal contents and a new, larger, composite vesicle is formed. We have attempted to discover whether or not the aqueous contents of vesicles become associated with those of the mycoplasma upon fusion.

When DPPC vesicles are prepared containing a high concentration of tempophosphate (0.1–0.2 M) the EPR signal is greatly broadened due to spin exchange interactions between unpaired electrons (i.e., the tempophosphate is localized in pockets of high concentration). Addition of such loaded vesicles to viable *A. laidlawii* in neutral buffer leads to a rapid decrease in the paramagnetic resonance signal linewidth with a concomitant rise in amplitude. The decrease in linewidth is due to dilution of the spin label upon release from the vesicles. This released spin label is not associated with the mycoplasma but is free in solution. The same observation was made with mycoplasma pretreated with sucrose, the only difference being

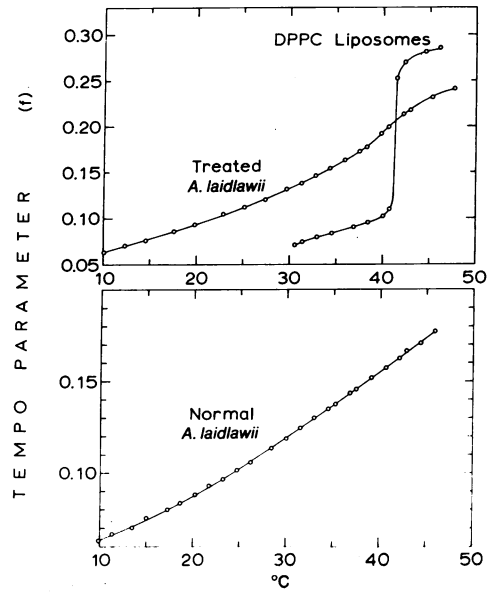

FIG. 1. Plots of the tempo spectral parameter (f) as a function of temperature for: DPPC liposomes; *A. laidlawii* containing DPPC introduced by fusion with vesicles as about 25% of their total lipid (treated *A. laidlawii*); normal *A. laidlawii*. The tempo spectral parameter is approximately equal to the fraction of the spin label, tempo, dissolved in the lipid phase.

A. laidlawii were pretreated with 30% sucrose and 4 mM $HgCl_2$ to minimize their lipase action on DPPC and their reduction of the spin label. All samples were slurries in 0.15 M NaCl buffered with 0.05 M phosphate at pH 7.0 and contained about 2.5% lipid by weight.

that the untreated mycoplasma were eventually able to reduce the released label.

Analogous experiments have been performed with [^{14}C] sucrose or 2-deoxy-D-glucose in place of tempophosphate. In a typical experiment 1.5 ml of a DPPC vesicle preparation (1.6 mg of DPPC per ml) with a total of 8473 cpm of contained 2-deoxy-D-glucose was incubated with 1.8 ml of *A. laidlawii* equivalent to a total of about 2.2 mg of mycoplasma lipid. After 10 min at 36° the mycoplasma were centrifuged through 30% sucrose at 36° in order to separate them from the mixture, and the resulting pellet was analyzed for radioactivity. Only 1.7% of the total counts added were found associated with the mycoplasma. Complete transfer of vesicle contents upon fusion in this experiment would be expected to leave 50% or more of the radioactive sugar inside the mycoplasma. Hence, all but at most a few percent of the vesicle aqueous contents are lost upon fusion with the mycoplasma.

None of the experiments so far described tells how the added DPPC partitions between the inside and the outside of the mycoplasma membrane. DPPC spin-labeled in the choline head group does not form vesicles upon sonication. But a 50/50 mixture with DMPC does. Each spin label is then exposed to one of two aqueous environments, the inside or the outside of a vesicle. When ascorbate is added at 0°, only the outside labels are reduced (5). Preliminary experiments performed by fusing vesicles containing head-group-labeled DPPC with *A. laidlawii* pretreated with sucrose indicate that less than 10% of the label associated with the mycoplasma is protected from ascorbate after 1 min at 0°. This result may reflect an asymmetric distribution of the head-group-labeled phospholipid. However, the same observation may be accounted for by rapid flip-flop (half time about 15 sec or less) of the labeled phospholipid.

DISCUSSION

The technique of mixing phospholipid vesicles with membranes in order to introduce exogenous lipid or lipid-associated material seems to be applicable to viable organisms, at least in the case of *A. laidlawii*. Presumably, the same approach will work with other mycoplasmas and bacterial L-forms where no cell wall exists.

It is interesting that the viability of an organism that ordinarily contains no DPPC is unaffected by fusion with relatively large amounts of this exogenous foreign lipid. However, it is already known (8) that this organism can tolerate a variety of fatty acid compositions.

The fact that exogenous DPPC readily diffuses laterally throughout the mycoplasma membrane does not necessarily imply complete lipid homogeneity. In fact, the data shown in Fig. 1 suggest that at the growth temperature of *A. laidlawii*, at least part of the added DPPC tends to be involved in a lateral phase separation of regions relatively rich in DPPC.

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