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Freeze-etch electron microscopy of erythrocytes, *Acholeplasma laidlawii* cells and liposomal membranes after the action of filipin and amphotericin B*

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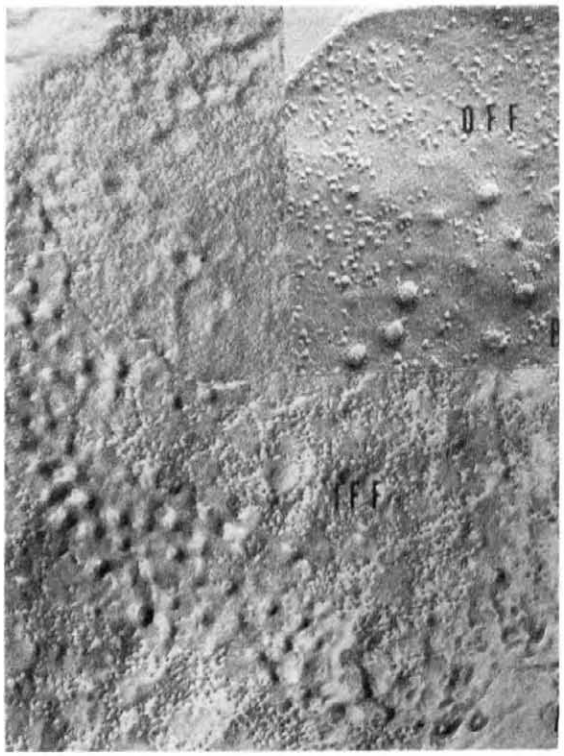
SUMMARY

Freeze-etch electron microscopy demonstrated that filipin induces the formation of aggregates 150–250 Å in diameter, in the membranes of rat erythrocytes, in cholesterol-containing membranes of *Acholeplasma laidlawii* cells and in egg lecithin–cholesterol liposomes. No change in fracture faces was observed when cholesterol was absent in the membranes of *A. laidlawii*, and lecithin liposomes.

Amphotericin B does not visibly affect the freeze-etch morphology of erythrocytes, cholesterol-containing *A. laidlawii* cells and lecithin–cholesterol liposomes.

The polyene antibiotics filipin and amphotericin B can interact with a variety of biological and artificial membranes. It was established by studies on *Acholeplasma laidlawii* membranes[1,2], liposomes[3], monomolecular layers[4], bimolecular films[5–7] and by ultraviolet spectroscopy[8,9] that the presence of sterol is a prerequisite for the interaction. It was also shown that a 3β-OH group, a planar sterol nucleus and the sterol side-chain are involved and that the interaction is primarily hydrophobic in nature[8]. Differential scanning calorimetric studies demonstrated that polyene antibiotics can withdraw sterol from its association with phospholipids[8]. Negative staining of erythrocytes and cholesterol-containing liposomal membranes revealed that the interaction of filipin and cholesterol in the membrane results in the formation of "pits"[10]. Limitations of this method did not warrant the conclusion that these pits are through and through holes in the membrane, although this possibility had to be seriously considered[10]. In this paper we report that

*This report is dedicated to Miss Marijke Sanderse, cooperator in this work, who died tragically in an air crash.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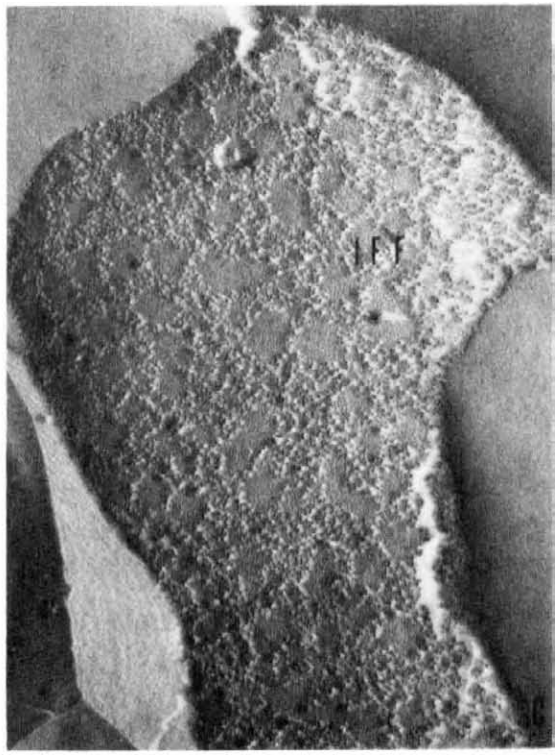
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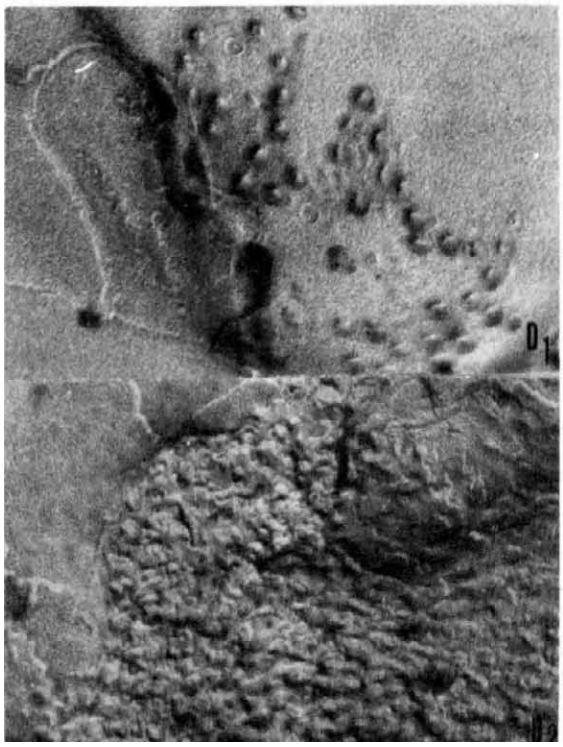

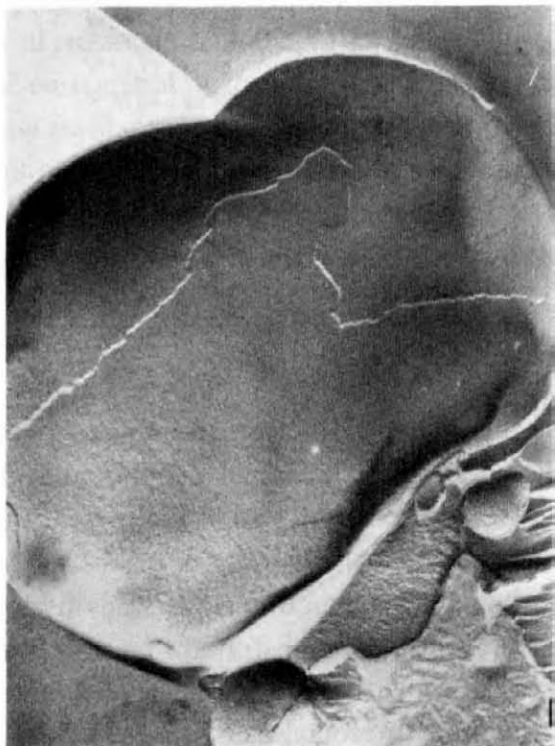

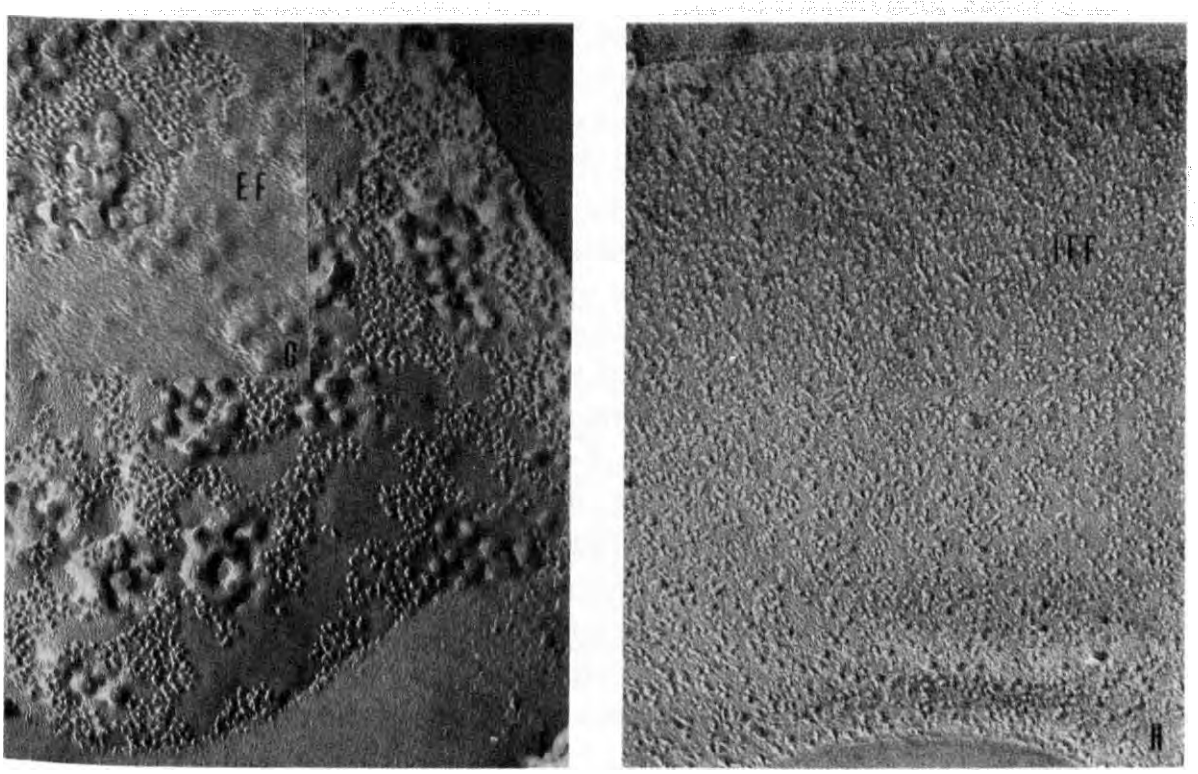

Fig. 1. Freeze-etch micrographs of *A. laidlawii* cells, liposomes and rat erythrocytes incubated with filipin or amphotericin B. A and B: *A. laidlawii* cells, grown in the presence of cholesterol, treated with filipin. C: *A. laidlawii* cells grown in the absence of cholesterol, treated with filipin. D: Cholesterol–egg lecithin liposomes incubated with filipin; 1, after 1 min; 2, after 10 min. E: Egg lecithin liposomes incubated with filipin. F and G: Rat erythrocytes, treated with filipin. H: Rat erythrocytes, control. IFF, inner fracture face; OFF, outer fracture face; EF, etch face. Magnification, approx. 60 000 ×

freeze-etching shows that filipin forms aggregates in the cholesterol-containing membranes of *A. laidlawii* cells, erythrocytes and lecithin–cholesterol liposomes; no pores could be detected after etching the membrane. *A. laidlawii* strain B cells were grown in the lipid-poor medium supplemented with 0.05 mM oleic acid with or without 25 mg cholesterol per l of culture medium, as described before[8,11]. Cells in the logarithmic phase were washed and suspended in 100 mM $CaCl_2$ – 10 mM Tris–HCl, pH 7.5, giving a final concentration of 60 μg cell protein per ml. Freshly collected rat erythrocytes (from acid–citrate–dextrose-treated blood: cells packed for 10 min at 3000 × *g*) were washed once in 150 mM NaCl–10 mM Tris–HCl, pH 7.5, and twice in 100 mM $CaCl_2$ – 10 mM Tris–HCl, pH 7.5, and finally 250 μl packed cells were suspended in 150 ml of 100 mM $CaCl_2$ – 10 mM Tris–HCl, pH 7.5. Liposomes were formed by dispersing 40 μM egg lecithin and 1.6 μM phosphatidic acid (derived from egg lecithin) with or without 7.75 μM cholesterol (15.7 mole %) in 1.0 ml 150 mM KCl at room temperature. The lipid dispersion was sonicated for 2 min under N_2 at maximum power with a Branson sonifier. The liposomes were dialysed 3 times against 200 ml $CaCl_2$ –Tris–HCl, pH 7.5, buffer at 0 °C. Before use, the liposomes were diluted 100 times in the same buffer. Filipin (obtained from the Upjohn Company, Kalamazoo, Michigan) and amphotericin B (obtained from the Squibb Institute for Medical Research, New Brunswick, N.Y.) were initially dissolved in dimethylformamide and

dimethylsulfoxide, respectively. The final concentration of the polyene antibiotics was 20 μg/ml in the case of *A. laidlawii* cells and liposomes; in the experiments on erythrocytes the concentration of the polyene antibiotics was 10 times less. After incubation of the cells with the polyenes for 10 min at 37 °C, the *A. laidlawii* cells and liposomes were centrifuged at 15 000 x *g* and the erythrocytes at 3000 x *g*. Liposomes and cells were quenched from 37 °C in a mixture of liquid and solid nitrogen and prepared further in a Denton freeze-etch apparatus as described before[12]. Micrographs were made with a Siemens Elmiskop IA.

The phenomena observed after treatment of *A. laidlawii* cells, grown in the presence of cholesterol, with filipin are shown in Fig. 1A and B. Besides the normal fracture pattern with small particles in a lace-like distribution on a smooth background, large aggregates and excavations of 150–250 Å in diameter are visible on the fracture faces of the membrane. The aggregates are predominantly at the outer (Fig. 1B) and the excavations (also without etching) on the inner fracture face (Fig. 1A). Etching reveals that these aggregates are lying in the membranes and cannot represent pores (etched face Fig. 1A)*. No particular structures were seen on the freeze-fracturing faces of *A. laidlawii* cells, grown in the presence of cholesterol, when treated with amphotericin B (Fig. 1C). Cells grown in the absence of cholesterol showed no aggregate formation after incubation with either filipin or amphotericin B.

Because of the small distances between the lipid layers in the liposomes, the fracture face of each bilayer is more complicated than in *A. laidlawii* cells. Otherwise the observations on liposomes are comparable with those on *A. laidlawii* cells. Treatment of lecithin–cholesterol liposomes with filipin showed aggregates on the fracture faces of the outermost layers of the liposomes of the same dimensions as found for *A. laidlawii* cells (Fig. 1D). Amphotericin B causes no change of the fracture faces of lecithin–cholesterol liposomes. No effects are found for lecithin liposomes without cholesterol, after addition of filipin or amphotericin B. Controls were taken of cells and liposomes in the absence of polyene antibiotics. No particular structures were observed. Erythrocytes (Fig. 1F) exhibit the same aggregates and excavations on the fracture faces after action with filipin, as found with cholesterol-containing *A. laidlawii* membranes. Also after etching no holes are visible on the etch face (Fig. 1G). The control erythrocytes and those treated with amphotericin show the normal fracture faces (Fig. 1H).

It can be concluded from the freeze-etch pictures that filipin forms large aggregates in the hydrophobic core of cholesterol-containing membranes of *A. laidlawii* cells, lecithin–cholesterol liposomes and erythrocytes. As mentioned above, negative staining showed that filipin forms "pits" in membranes of cholesterol-containing liposomes and erythrocytes[10]. The freeze-etch studies described here demonstrate that aggregates formed in cholesterol-containing membranes of *A. laidlawii*, lecithin–cholesterol liposomes and erythrocytes cannot be considered as pores because no through and through holes are seen at the etch face. Recent differential scanning calometry measurements showed that all the cholesterol could be withdrawn by filipin from its association with lecithins in

*Negative staining of cholesterol-containing *A. laidlawii* cells treated with filipin demonstrated the same pattern as reported already for erythrocytes and liposomes.

liposomes consisting of 1-oleoyl-2-stearoylphosphatidylcholine and 20 mole % of cholesterol[8]. Therefore we suggest that aggregates in the hydrophobic core, visualized by freeze-etch electron microscopy, must be interpreted as cholesterol–filipin complexes. It is unlikely that solutes will permeate through these aggregates formed, but that membrane disorganisation takes place because of a rearrangement of the membrane lipids[13], followed by lysis.

With amphotericin B no aggregates or clusters could be observed in erythrocytes and cholesterol-containing *A. laidlawii* and liposomes. The induction of pores by amphotericin B (5–10 Å in diameter) has been suggested by several authors[6,7] but pores of such a small diameter cannot be detected by freeze-etch electron microscopy.

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