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ELECTRON MICROSCOPIC LOCALIZATION OF CHOLESTEROL IN BOVINE MILK FAT GLOBULES

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Abstract

An electron microscopic method designed for the detection of cholesterol in milk fat was evaluated for reliability. This method is based on the incubation of cream from raw milk with filipin (a polyene antibiotic) which has a specific affinity for cholesterol followed by freeze fracturing and electron microscopic examination of fat globules. Cholesterol was localized within the membrane and the triglyceride core of milk fat globules. Cholesterol was highly organized within membrane portions and less organized within triglyceride portions of milk fat globules. The configuration of localized cholesterol was similar to configurations reported for plasma membranes.

Introduction

The origin of cholesterol in milk products is partially from milk serum and to a greater extent from the milk fat globule (MFG). Bovine MFGs are about 1% protein and 99% lipid of which 96-99% is triacylglycerol (Timmen and Patton, 1988). MFGs possess a central core of lipid surrounded by a thin protein inner coat and enclosed by the outer milk fat globule membrane bilayer (Mather, et al., 1977; Freudenstein, et al., 1979; Franke, et al., 1981; Keenan, et al. 1982; Buchheim, 1982, 1986). There currently exist no reports of cholesterol localization in milk fat globules using electron microscopic techniques.

Cholesterol is a major constituent of animal plasma membranes, plasma lipoproteins, and animal fat. Several recent studies have utilized electron microscopic techniques to localize cholesterol in animal cells and tissues (Elias, et al., 1979; Andrews and Cohen, 1979; Friend and Bearer, 1981; Behnke, et al., 1984; Miller, 1984; Harris, 1988). A technique used in cellular localization of cholesterol using electron microscopy has been freeze fracture following incubation with filipin. Filipin is a polyene antibiotic that has a specific affinity to the β -hydroxysterol molecule. The specific binding of cholesterol with filipin creates a complex that is observable by freeze fracture electron microscopy and enables localization information.

There exist several hypotheses for the appearance of filipin/cholesterol complexes within biological membranes. DeKruyff and Demel (1974) suggested that a polymer with equal molar fractions of filipin and cholesterol was formed in the hydrophobic core of the membrane bilayer. Kitajima, et al. (1976) proposed that the location of the filipin/cholesterol complexes may not specify the exact location of cholesterol. Filipin was assumed to reorient cholesterol from a vertical orientation in the bilayer to a horizontal position at the interfaces of the hydrophobic core with inter- and extracellular environments. Elias, et al. (1979) theorized that filipin induced membrane buckling could be caused by the formation of two groups of four filipin/cholesterol complexes that would account for hemispherical as well as circular complexes.

Miller (1984) used the published diffusion coefficients of cholesterol and calculated that the binding of cholesterol and filipin occurred in

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less than 10 sec. He suggested a resolution of localization ranging from 200 nm to 4 μ m. Miller (1984) further suggested that filipin may serve as a nucleation site for non-membrane cholesterol thus yielding polymorphic images when viewed with freeze fracture.

Behnke, et al. (1984) stated that reports of membrane perturbation or fragmentation referring to the physico-chemical effects of filipin may mislead researchers to believe that filipin destroys plasma membranes. Their studies of red blood cells showed that approximately 10% of the cholesterol of the total cell mass was removed during filipin incubation. They further showed that cholesterol-rich microdomains are dependent on filipin concentrations and the number of cells present in the system as well as on the extent of membrane shedding.

The purpose of the present investigation is to evaluate the feasibility of freeze fracture techniques to localize cholesterol in bovine milk fat globules and compare the results with existing studies regarding biological membranes. The data will be used to theorize possible configurations of cholesterol within MFGs.

Materials and Methods

Raw milk, from Holstein cattle, was received within 24 h after expression and cooled to 4°C in a bulk holding tank. Raw milk samples (100 g) were placed in centrifuge tubes and centrifuged at 1900 rpm for 5 min. on a Clay Adams #0011 centrifuge. Experimental cream samples (35 - 0.25 ml replicates) were removed and placed in separate depressions of ceramic spot plates with 0.25 ml filipin solution (10 mg. filipin-Sigma/1.0 ml. dimethylformamide) and incubated at 40°C for 1.0 h. Control samples (0.25 ml cream) were incubated at 40°C in 1.0 ml dimethylformamide. Samples were placed on Balzers specimen supports, cryofixed in Freon 22 cooled in liquid nitrogen, placed in a Balzers 400 K freeze fracture apparatus, fractured at -110°C, etched for 1 min at 1×10^{-7} mbars, and coated with 20 nm platinum at a 45° angle and stabilized with 200 nm of carbon at a 90° angle. Replicas were rinsed in distilled water, 10% sodium hypochlorite, methanol/acetone (1:1), placed on gold 300 mesh grids and viewed in a Zeiss EM 10 transmission electron microscope operating at 60 kV.

Results

Freeze fracture replicas of control cream samples were characterized by MFGs with varying amounts of liquid and solid triglyceride and smooth membranes surfaces (Figures 1 and 2). Figure 3 represents a replica from an experimental cream sample following filipin incubation. Casein submicelles (m) were found to adhere to the outer milk fat globule membrane (MFGM), the surface of which was pitted with depressions interpreted as filipin/cholesterol complexes within the membrane. The pattern of filipin/cholesterol complexes was a consistent feature within MFGMs.

Figure 4 shows a MFG that was fractured resulting in a removal of the triglyceride and portions of the inner bilayer of the MFGM. The partial membrane fracture accounted for the raised effect of the 25 nm bumps (fc in micrographs) and

the presence of casein submicelles in the same region. Figure 5 represents a replica with a fracture plane just below the protein inner coat of an experimental MFG. The spatial arrangement of filipin/cholesterol complexes is similar to the arrangement observed in the membrane region of MFGs. The radiating lines visible in the triglyceride are interpreted as arrays of crystalline lipid, a consistent feature in all treatments.

Figure 6 shows an experimental cream replica with patches of MFGM over the triglyceride core of a MFG. The spatial arrangement of filipin/cholesterol complexes is similar to those presented earlier. Figure 7 represents a fracture through a similarly treated MFG seen in Figure 6. The fracture plane was just below the protein inner coat and clearly within the triglyceride core. The spatial arrangement was identical to the arrangement observed in the MFGMs. Figure 8 shows filipin/cholesterol complexes localized within the triglyceride core of experimental MFGs. Complexes within these vicinities were arranged in a less organized manner.

Discussion

Although there are pros and cons to the use of filipin as a cholesterol probe many researchers from different laboratories have obtained very similar results with variations of this technique. Miller (1984) points out that the consistency between individual researchers utilizing this technique cannot be ignored and may signify properties of plasma membranes not yet understood. The following discussion should be weighed carefully due to the controversy that exists using filipin methods.

It is difficult to compare existing accounts of cholesterol localization in cells and tissues to the present investigation concerning bovine MFGs. This is due to the origin of the MFGM. It is generally accepted that milk fat is expressed via the Golgi apparatus of secretory cells in mammary epithelia (Mather and Keenan, 1983; Wooding, 1971). The complex secretion mechanism results in the milk fat being surrounded by protein components (inner protein coat) as well as the outer MFGM (Keenan and Dylevski, 1985; Keenan, et al. 1982;). This is in contrast with most plasma membranes that can be replenished by organelles found in living cells.

The highly organized pattern of filipin/cholesterol complexes within the MFGM are strikingly similar to reports for plasma membranes of animal cells and tissues (Elias, et al., 1979; Friend and Bearer, 1981; Behnke, et al., 1984; Andrews and Cohen, 1979). The main morphological difference between the filipin/cholesterol pattern in MFGMs and animal cells is the higher density of filipin/cholesterol complexes within plasma membranes of living cells. This is not unexpected due to the origin of MFGMs. The cholesterol configuration observed within MFGMs is possibly similar to the configuration within the apical plasma membrane of the mammary secretory cell or the secretory vesicles.

The difference in cholesterol localization from the triglyceride core to the outer membrane suggests an organization of cholesterol in MFGMs.

Cholesterol localization

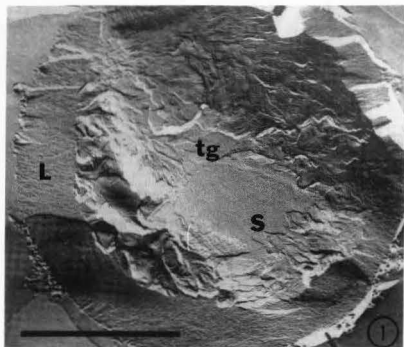


Figure 1. Freeze fracture replica of control milk fat globule incubated at 40°C. The triglyceride (tg) exhibits solid (S) and liquid (L) regions within the milk fat. Scale bar equals 0.5 μ m.

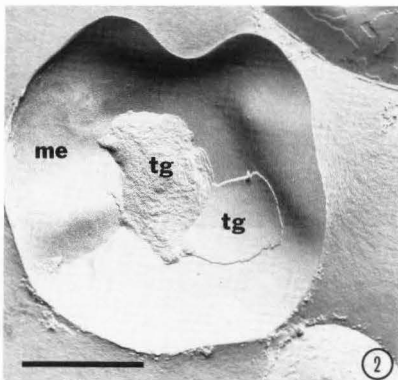


Figure 2. Freeze fracture replica of control milk fat globule with fracture plane through outer membrane (me). Note smooth appearance of membrane. Triglyceride (tg). Scale bar equals 0.5 μ m.

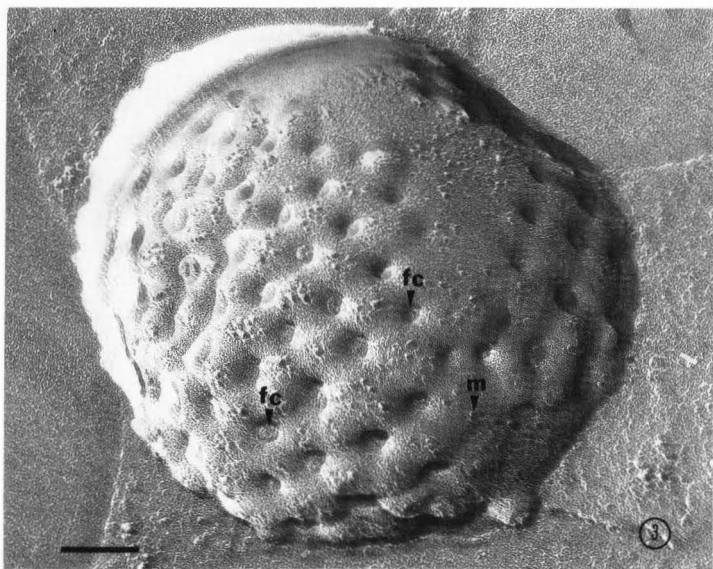


Figure 3. Freeze fracture replica of milk fat globule incubated with filipin. The depressions within the outer membrane represent filipin/cholesterol complexes (fc). Casein micelles (m) are apparent on the outer membrane. Scale bar equals 0.2 μ m.

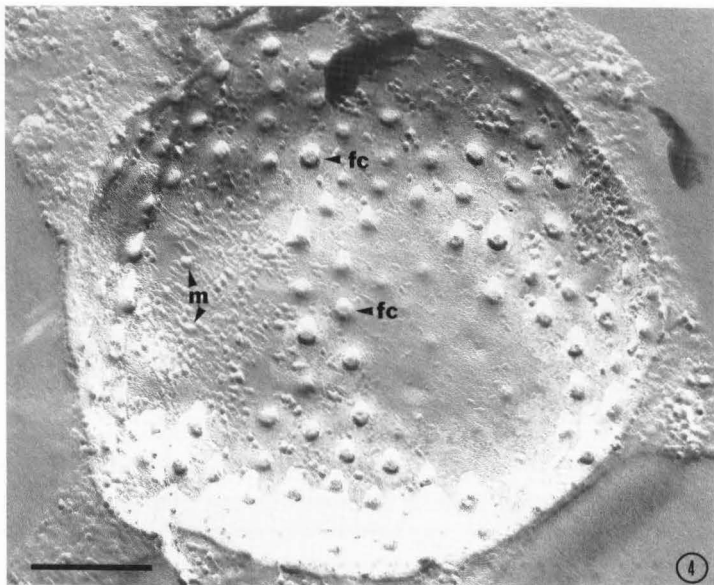


Figure 4. Freeze fracture replica of milk fat globule incubated with filipin. The fracture plane is between the outer membrane bilayers and displays filipin/cholesterol complexes (fc). Partial fracture through the outer membrane displays casein micelles (m). Scale bar equals 0.2 μ m.

It is inviting to propose that cholesterol has a random arrangement within the lipid core and becomes highly organized in the surrounding membrane. However, the random nature of fracture planes through triglyceride regions makes it difficult if not impossible to visualize the arrangement of filipin/cholesterol complexes. The distance between complexes within the core is such that a judgment concerning the looseness of the complexes can be made. Timmen and Patton (1988) state that bovine triglyceride is primarily liquid at bovine body temperature. Since milk samples were incubated at 40° C a majority of the milk fat was considered liquid. This, in turn, increases molecular motion and may increase the probability of a random arrangement of cholesterol within the core. Hence, filipin/cholesterol complexes of the triglyceride core appear to be less organized than those observed in the surrounding membrane. The closer the complexes to the inner coat and outer membrane the more organized they become. This is supported by the data shown in Figures 6 and 7 when compared to Figure 8.

In conclusion, it is apparent that cholesterol within MFCMs has a very different arrangement from cholesterol within the triglyceride core. Immunocytochemical locali-

zation with TEM could improve localization resolution and may contribute a quantitative microscopic method for measuring the amount of cholesterol in milk fat.

Acknowledgments

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Cholesterol localization

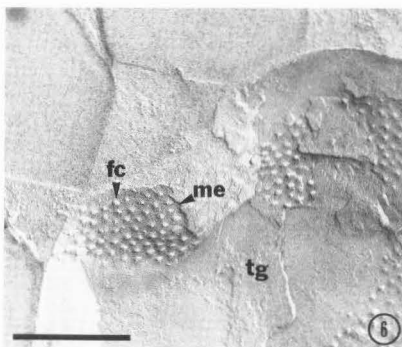
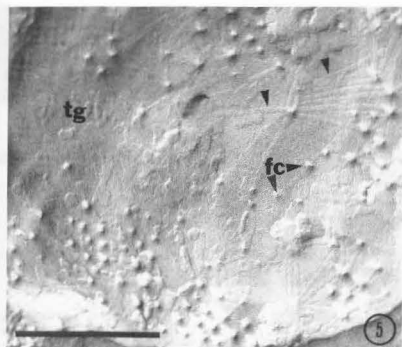


Figure 5. Freeze fracture replica of filipin incubated milk fat with fracture plane within triglyceride (tg). Filipin/cholesterol complexes (fc). Note arrays of crystalline lipid at small arrowheads. Scale bar equals 0.5µm.

Figure 6. Freeze fracture replica of filipin incubated milk fat with fracture plane revealing membrane patches (me) adhering to triglyceride (tg). Note pattern of filipin/cholesterol complexes (fc). Scale bar equals 0.5µm.

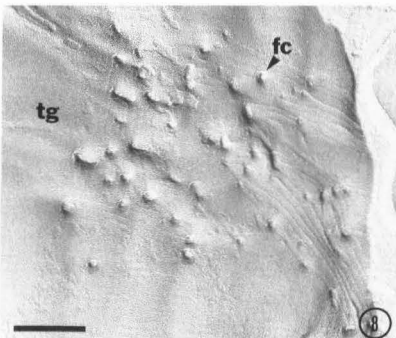
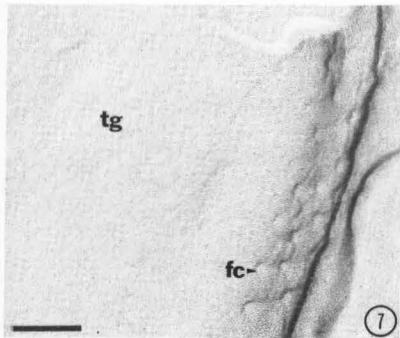


Figure 7. Freeze fracture replica near outer membrane region of filipin incubated milk fat globule. Note pattern of filipin/cholesterol complexes (fc) embedded within the triglyceride (tg). Scale bar equals 0.1µm.

Figure 8. Freeze fracture replica of filipin incubated milk fat globule showing arrangement of filipin/cholesterol complexes (fc) within triglyceride (tg). Scale bar equals 0.2µm.

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Discussion with Reviewers

B E Brooker: In several figures of both control and experimental samples, casein micelles are present at the surface of globules. Is this an effect of the DMF? If so, is it not possible that there are changes induced in the MFGM by this treatment that affect the nature and/or distribution of filipin/cholesterol complexes?

R.G. Miller: As I understand it, experimental samples (i.e. filipin-treated) contained 50% DMF as a cryoprotectant, whereas control samples contained 80% DMF. Is there a reason for this difference in DMF treatment? Since a portion of the MFG sample is non-aqueous, the final concentration of DMF within the aqueous phase might be substantially higher than these values. Do you feel that incubation in this high concentration of an organic solvent have an effect upon the morphology of the MFG? Might this have an effect on filipin cholesterol binding?

Author: It is certainly possible DMF may cause a biochemical perturbation not detectable by freeze fracture methodology. DMF was used primarily to solubilize filipin not as a cryoprotectant for

freeze fracture methodology. The difference in DMF concentration between control and experimental treatments was used to determine possible artefacts caused by the DMF treatments. It is very important to confirm the data regarding the behavior of cholesterol with filipin using other methods such as computer imaging or immunocytochemistry. Work in progress shows the presence of milk protein components on the surface of MFGs a common feature of raw milk from bulk storage. Of course a quantification of MFG surface protein from bulk storage versus DMF treatments may indicate characteristics of solvent treatments pertinent to the adherence of milk proteins and other possible artefacts.

B E Brooker: Filipin/cholesterol complexes appear at the end of the focus of several 'triglyceride arrays'. What is the significance of this clear spatial relationship?

Author: There are several possible interpretations for the location of filipin/cholesterol complexes at the focus of crystalline lipid arrays. The configuration may be indicative of cholesterol associated lipids that are highly organized with milk fat. Another possibility is that these are fixation artefacts introduced by too low freezing velocities. These possibilities are purely speculative and should be studied by controlled experimentation.

M Kalab: In this paper, the localization of cholesterol in the fat globule membrane is based on the assumption that cholesterol is present in the membrane and reacts with filipin. Evidently, it would be useful to confirm the findings using an experimental system under controlled conditions. Such a system would consist of an oil/water emulsion which would contain protein and in which the concentration of cholesterol would be controlled. Was an attempt made to study such a system?

I Heertje: Have controlled experiments with filipin ever been performed on membrane systems containing no cholesterol?

Author: The idea of using model emulsion systems to confirm the binding of filipin and cholesterol is a good one. Preliminary results from freeze fracture studies of synthetic liposomes exhibit similar results as presented in the current paper.

I Heertje: May the filipin technique be considered as a localization technique in the strict sense, considering the mobility of cholesterol and the aggregation of filipin?

D Holcomb: It is thought that filipin causes reorientation of the cholesterol. Could the author give more explanation of why this is not a serious problem in this work?

Author: Due to the apparent mobility of cholesterol, filipin localization with freeze fracture methodology should be used only as a qualitative measure in conjunction with biochemical analyses. Hence, the presence of cholesterol would first be determined by freeze fracture and then confirmed by further analyses.

I Heertje: Are proper antibodies available for localization of cholesterol by immuno EM? If so, are such studies envisaged?

Cholesterol localization

R.G. Miller: Indeed, there are many problems with localization of a mobile substance such as cholesterol. The solution which you propose in the last paragraph is raising antibodies which are specific to cholesterol. First, I don't see that the use of antibodies is going to help in localization of highly mobile species such as cholesterol. Do you feel that an antibody sort of probe would fare any better than filipin? Secondly, in order to make such an antibody, an antibody-producing animal must be found which does not have a substantial amount of endogenous cholesterol. Have you found such a beast?

Author: Currently there are no commercial antibodies available for cholesterol localization using immuno EM. Immuno fracture labeling using such an antibody would be very important to our overall understanding of cholesterol localization in membranes. The advantage to antibody labelling is that cholesterol may not be reoriented by the antibody during binding as has been proposed for filipin/cholesterol binding. A number of laboratories are presently attempting to produce polyclonal and monoclonal antibodies to cholesterol.

I Heertje: Do other sterols present in milk give rise to the same reaction?

Author: To my knowledge the filipin molecule reacts only with β -hydroxysterol (cholesterol) which composes more than 99% of the sterols in milk fat.

D Holcomb: How do you know where the fracture plane is? Maybe the author could explain how those locations were determined?

Author: The interpretation of fracture planes through membranes and triglyceride portions of MFGs are based upon repeatable morphological characteristics found during the course of this study, relevant published micrographs, and relevant theories of freeze fracture methodology.

R.S. Miller: Although many have used filipin in order to provide some information concerning the localization of cholesterol in membranes, to my knowledge, this is the first report which strives to localize cholesterol in the bulk phase of a liquid. A priori, there's no reason to expect that filipin cholesterol complexes in bulk phase should have any morphological resemblance to a filipin cholesterol complex in a bilayer membrane. How sure are you that you are indeed picking up filipin cholesterol complexes within the tri-acyl glyceride core of the milk fat globule? What is the measured concentration of cholesterol in the milk fat globule membrane compared to the concentration within the tri-acyl glyceride core; does this correlate in any way with the number of filipin cholesterol complexes that are in the two regions?

Author: The morphological characteristics of filipin cholesterol complexes in membranes versus triglyceride regions are strikingly different. There really is no reason to believe they would be the same based upon the structural differences of the two regions. Work in progress on aqueous systems shows the filipin cholesterol complexes may take on a variety of morphological

characteristics dependant upon the nature of the system. Hence, I am relatively confident that the structures isolated within triglyceride regions of MFGs are indeed filipin cholesterol complexes. The concentration of cholesterol in MFGs is approximately 2.5 mg/g fat. The total cholesterol within the core ranges between 97-83% and 1.5-7.8% in the membrane portion dependant upon the season the milk was collected. It is difficult to correlate the number of filipin cholesterol complexes detected by freeze fracture to these concentration values as the current method is strictly a qualitative measure.