Electron Spin Resonance Studies on Intact Cells and Isolated Lipid Droplets from Fatty Acid-modified L1210 Murine Leukemia

Ido Simon, C. Patrick Burns, and Arthur A. Spector

ABSTRACT

It has been suggested that the formation of cytoplasmic lipid droplets may produce an artifact and be responsible for the differences in membrane physical properties detected in lipid-modified cells using fluorescence polarization or spin label probes. To investigate this, the electron spin resonance spectra of lipid droplets isolated from the cytoplasm of L1210 leukemia cells were compared with spectra obtained from the intact cell. Mice bearing the L1210 leukemia were fed diets containing either 16% sunflower oil or 16% coconut oil in order to modify the fatty acid composition of the tumor. A microscope-rich fraction prepared from L1210 cells grown in animals fed the sunflower oil-rich diet contained more polyenoic fatty acids (52 versus 29%), while microsomes from L1210 cells grown in animals fed the coconut oil-rich diet contained more monoenic fatty acids (37 versus 12%). The order parameter calculated for lipid droplets labeled with the 5-nitroxyxystearic acid spin probe was only about one-half that of intact cells, whereas it was similar to that obtained for pure triolein droplets suspended in buffer. Order parameters of the inner hyperfine splittings calculated from the spectra of cells grown in the sunflower oil-fed animals [0.543 ± 0.001 (S.E.)] were lower than those from the cells grown in animals fed the coconut oil diet (0.555 ± 0.002) (p < 0.005). In contrast, the order parameters of the lipid droplets isolated from the cells grown in animals fed sunflower oil (0.303 ± 0.029) or coconut oil (0.295 ± 0.021) were not significantly different, indicating that motion of a spin label probe in the highly fluid cytoplasmic lipid droplets is not affected by these types of modifications in membrane fluidity. The fatty acid modification produces a probe-specific behavior that can be modified by feeding the tumor-bearing mice diets enriched with different fats (5, 14). Plasma membrane fractions isolated from homogenates of these lipid-modified tumor cells have considerably different fatty acid compositions (1, 4). Since membrane fluidity can be affected by changes in fatty acid composition, the question arose as to whether the fatty acid modifications that were produced vivo were sufficient to alter this physical property. This was investigated by the technique of ESR. Spin label probes provide information regarding the physical state of the membrane (8, 16, 22), and the relative values of the order parameters calculated from the ESR spectra indicate the comparative molecular ordering within the membrane lipid bilayer (8, 16, 21). When used in low concentrations, the spin label does not affect the viability of cells (11), and it is a reproducible and valid method for studying the physical properties of the outer surface of intact cells or model membranes (22). Based upon data obtained when nitroxyxystearic acid spin probes were inserted into plasma membranes isolated from the tumor cells, it was concluded that membrane fluidity was appreciably altered by the types of fatty acid modification produced vivo (4, 13).

A recent study in which the fluorescent probe diphenylhexatriene was incorporated into lymphocyte membranes indicated very little effect on the rotational relaxation time, even though the membrane fatty acid composition was modified considerably (27). Another study with normal lymphocytes and malignant lymphoblasts which also used the diphenylhexatriene probe showed appreciable fluorescence changes in the malignant cells (25). When purified plasma membranes were isolated, however, no differences in fluorescence polarization were observed relative to the membrane of normal lymphocytes. It was concluded that the polarization differences in the intact cells are due to the presence of cytoplasmic lipid droplets, the fluorescent probe accumulating in these droplets and reporting differences in the physical properties of this structure, not the membrane. Furthermore, it was suggested that previous positive effects obtained with isolated plasma membrane preparations were due to contamination of these preparations with the lipid droplets that were present in the cell cytoplasm (25, 27). This conclusion has been emphasized by letters published recently in Cancer Research regarding the lipid fluidity of malignant lymphoid cell membranes (26, 30).

In the present ESR study, we have included a direct assessment of lipid droplets isolated from fatty acid-modified L1210 cells. In addition, we have examined intact cells in which the spin probe was allowed to decay and was subsequently reactivated with ferricyanide. Our results indicate that the previously observed ESR changes cannot be due to the presence of cytoplasmic lipid droplets in the fatty acid-modified cells. Based upon these data, we conclude that membrane fluidity, as indicated by ESR measurements using nitroxyxystearic acid spin probes, is indeed altered by the fatty acid compositional changes that can be produced in ascites tumor cells.

INTRODUCTION

The fatty acid composition of ascites tumors, such as the Ehrlich ascites carcinoma and L1210 lymphocytic leukemia, can be modified by feeding the tumor-bearing mice diets enriched with different fats (5, 14). Plasma membrane fractions isolated from homogenates of these lipid-modified tumor cells have considerably different fatty acid compositions (1, 4). Since membrane fluidity can be affected by changes in fatty acid composition, the question arose as to whether the fatty acid modifications that were produced in vivo were sufficient to alter this physical property. This was investigated by the technique of ESR. Spin label probes provide information regarding the physical state of the membrane (8, 16, 22), and the relative values of the order parameters calculated from the ESR spectra indicate the comparative molecular ordering within the membrane lipid bilayer (8, 16, 21). When used in low concentrations, the spin label does not affect the viability of cells (11), and it is a reproducible and valid method for studying the physical properties of the outer surface of intact cells or model membranes (22). Based upon data obtained when nitroxyxystearic acid spin probes were inserted into plasma membranes isolated from the tumor cells, it was concluded that membrane fluidity was appreciably altered by the types of fatty acid modification produced in vivo (4, 13).

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In the present ESR study, we have included a direct assessment of lipid droplets isolated from fatty acid-modified L1210 cells. In addition, we have examined intact cells in which the spin probe was allowed to decay and was subsequently reactivated with ferricyanide. Our results indicate that the previously observed ESR changes cannot be due to the presence of cytoplasmic lipid droplets in the fatty acid-modified cells. Based upon these data, we conclude that membrane fluidity, as indicated by ESR measurements using nitroxyxystearic acid spin probes, is indeed altered by the fatty acid compositional changes that can be produced in ascites tumor cells.

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The abbreviations used are: ESR, electron spin resonance; PBS, phosphate-buffered saline (in g/liter: NaCl, 8.0; Na2HPO4·7H2O, 2.16; KCl, 0.2; KH2PO4, 0.2; CaCl2, 0.1; MgCl2·6H2O, 0.1).
MATERIALS AND METHODS

Male DBA/2J mice (14 to 16 g) (Harlan-Sprague-Dawley, Madison, Wis.) were fed a predominantly polyunsaturated fat diet [basal fat-deficient mixture (Teklad, Inc., Madison, Wis.) containing 16% sunflower oil (Arrowhead Mills, Inc., Hereford, Texas)] or a predominantly saturated fat diet containing 16% coconut oil (Ruger Chemical Co., Hillside, N. J.). The exact fatty acid composition of the diets has been reported (3); briefly, the sunflower oil-rich diet contained 88% unsaturated, mostly linoleate, and the coconut oil-rich diet contained 90% saturated fats. L1210 cells (1 × 10⁵) were injected i.p. into the mice after they were fed the diets for 4 weeks, and the cells were allowed to grow for an additional week during which the diets were continued. The cells were harvested and the erythrocytes were lysed with 0.83% NH₄Cl for 10 min at 4°C. After washing the cells twice with PBS at 4°C, the cells were used immediately.

The cells were disrupted by nitrogen cavitation (29) at 4°C using 900 psi. Phase microscopy was used to monitor cell breakage. The homogenates were then layered onto 20% sucrose and centrifuged for 60 min at 100,000 × g to obtain lipid droplets, which floated to the top. The pellets were resuspended and centrifuged at 10,000 × g for 15 min to remove nuclei and mitochondria, and then the supernatant was centrifuged at 100,000 × g for 60 min to obtain a microsomal fraction. Triolein (Nu Chek Prep, Elysian, Minn.) lipid droplets were formed by vigorous shaking in PBS.

The microsomal fraction was extracted with CHCl₃:CH₃OH (2:1, v/v) (7). Phospholipids and neutral lipids in the Folch-washed CHCl₃ extract were separated by thin-layer chromatography on Silica Gel G using a solvent system of hexane:diethyl ether:acetic acid:methanol (85:20:2:2). The phospholipid fraction was saponified with 0.5 N KOH in 80% methanol for 20 min at 80°C. Fatty acids in the saponifiable fraction were methylated (18), and the methyl esters were separated using a Hewlett-Packard 5710A gas chromatograph equipped with a 1.8-m column packed with 10% SP-2330 on Chromosorb WAW. The individual fatty acid peaks were identified by comparison of their retention times to those of standard fatty acid methyl esters (Supelco, Inc., Bellafonte, Pa.). Cholesterol and triglycerides were determined chemically (19).

For electron microscopy, washed fresh cells were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 2 hr and postfixed for 1 hr in 2% osmium tetroxide. The pellets were stained for lipid distribution (31) and embedded in Epon 812 and Araldite. Ultrathin sections were cut, placed in 300 to 400 mesh copper grids, stained with 1% uranyl acetate and 1% lead acetate, and examined using a Hitachi H-600 electron microscope at 50 kV.

The ESR measurements were carried out in a Varian E-104 spectrometer, equipped with a variable temperature-controlled unit. The temperature was monitored throughout the ESR recording with a Hewlett-Packard 3466B-Digital Multimeter equipped with a teflon copper-constantin thermocouple that was placed above the sample cavity. The spectra were recorded as the first derivative of the absorption curve, with the use of a Nicolet Model 535-4 signal averager. The field modulation was 100 kHz, microwave power was 10 milliwatts, scanning time was 4 min, constant was 0.0 to 0.032 sec, and modulation amplitude was 1 G. The ESR spectrometer was operating in the x-band that was linear and accurate to ±0.1 G.

The fatty acid spin label probe, 5-nitrooxystearate [2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinylxoyl], was purchased from Syva Associates (Palo Alto, Calif.). A stock solution containing 0.1 μM probe in 100% ethanol was prepared and kept in the dark at −20°C. L1210 cells or lipid droplets were incubated in PBS with 3 μl of the spin label solution for 15 min at 37°C with gentle shaking. The L1210 cells were washed twice with PBS to remove the free spin label and were studied immediately.

The observed values of the outer (2T₀) and inner (2T₁) hyperfine splitting were used to calculate the order parameters. (a) Order parameter (S) (8) was calculated as follows:

\[ S = \frac{T₂ - T₁ - C}{T₁ + 2T₂ + 2C} \times 1.723 \]

where C = 1.4 G − 0.053 (T₁ − T₂). (b) The order parameter of the outer hyperfine splittings S(T₀) (21) was calculated by the following equation:

\[ S(T₀) = \frac{3(T₁ - T₀)}{(T₂ - T₁)} \]

RESULTS

Cell Morphology and Lipid Composition. Fig. 1 is an electron micrograph of an L1210 cell grown in a mouse fed the diet containing 16% coconut oil. A number of large lipid droplets are present in the cytoplasm. Similar droplets were noted with cells grown in the mice fed the sunflower oil diet, and there were no obvious morphological differences in cells from the 2 diet groups.

Droplets isolated by flotation from homogenates of both types of cells contained a similar lipid composition: 93% triacylglyceride; 7% cholesterol; and no measurable phospholipid. When the lipids contained in the droplets were studied by thin-layer chromatography, there was a large band corresponding to standard triolein and a small band corresponding to cholesterol.

Microsomes also were isolated from the homogenates of both types of cells, and their phospholipid fraction was analyzed. As shown in Table 1, differences in the microsomal fatty acid compositions were observed. The polyenoic fatty acid content of the phospholipids was 75% larger in microsomes isolated from the cells grown in the mice fed the sunflower oil diet. In these microsomes, the polyene:monoene ratio was 4.4, as compared with 0.77 in the microsomes isolated from the cells grown in mice fed the coconut oil diet. Most of the increase in polyunsaturated fatty acid in the microsomes from...
Lipid Modification and ESR of Tumor Cells

the sunflower oil-grown cells was accounted for by linoleic and arachidonic acids.

ESR Analysis. The ESR spectra of the spin label embedded in the membrane bilayer can be used to detect changes of the freedom of motion in lipid membranes (8, 11, 16). The order parameter of the spin label depends on the temperature, position of the oxazolidine ring in the hydrocarbon chain, degree of unsaturation, length of the phospholipid acyl chains, cholesterol:phospholipid ratio, lecithin:sphingomyelin ratio, and lipid:protein ratio (6, 10, 22). Chart 1 shows typical ESR spectra obtained at 37° with the 5-nitroxystearate spin probe. Spectra are shown for intact L1210 cells and for lipid droplets isolated from these cells. The spectra for the cells (c and d) exhibit major qualitative differences as compared with the spectra for the lipid droplets (a and b).

In the cells, the spin label signal decayed after 30 min of incubation at 37°. After complete decay, the signal could be restored to 98% of the original intensity by exposure to 1 mM potassium ferricyanide. Since mammalian cells, including cultured mouse L-cells, are relatively impermeable to ferricyanide (11, 17, 21), the rapid restoration of the ESR signal following exposure to 1 mM ferricyanide suggests that the spin probe is located primarily near the outer surface of the cell and, therefore, that the ESR spectra are derived for the most part from the plasma membrane.

The order parameter S is the most common one reported for ESR studies, but the order parameter of the outer hyperfine splitting $S(T_i)$ was found to be more sensitive to changes in lipid flexibility, and its values are more stable with time (21). Greater degrees of freedom of the spin label in the bilayer are associated with the smaller values of the order parameter. Table 2 contains the order parameter S and the order parameter of the outer hyperfine splittings $S(T_i)$, calculated from the ESR spectra of cells grown in the mice fed the coconut and sunflower oil diets. Both order parameters were significantly higher at all temperatures tested, except $S(T_i)$ at 27°, for the cells grown in the animals fed coconut oil. These results are in complete agreement with the previous findings of Burns et al. (4) in which plasma membrane fractions isolated from homogenates of these 2 types of lipid modified L1210 cells were compared. The absolute values of the order parameters for the intact cells were similar to those for the corresponding plasma membrane preparations (4).

Table 1

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<thead>
<tr>
<th>Fatty acid composition of phospholipids from L1210 microsomes</th>
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<tr>
<td>Microsomes prepared from L1210 cells grown in mice fed the experimental diets were extracted with CHCl₃:CH₃OH, the phospholipids were separated with the use of silicic acid chromatography, and the saponifiable lipids were obtained by alkaline hydrolysis. Fatty acids in the saponifiable lipids were methylated and separated by gas chromatography.</td>
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<td><strong>Composition (%)</strong></td>
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<td>Polynenic:monoenic ratio</td>
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<sup>a</sup> Fatty acids are abbreviated as number of carbon atoms:number of double bonds. 6 Mean of 2 experiments.

Table 2

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<th>Order parameters of 5-nitroxystearate-labeled L1210 cells</th>
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<td>L1210 cells grown in animals fed the experimental diets were harvested and spin labeled with 5-nitroxystearic acid. The ESR spectra at 4 temperatures were recorded, and the order parameters S and order parameters of outer hyperfine splittings $S(T_i)$ were calculated.</td>
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<tr>
<td><strong>Temperature</strong></td>
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<td>37°</td>
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<sup>a</sup> Mean ± S.E. of 4 separate preparations of cells. 6 NS, not significant.
In order to show the isotropic motion, ESR spectra of the lipid droplets also were obtained at 4°. These are shown in Chart 2, which also contains spectra of the 5-nitroxystearate probe in triolein droplets suspended in phosphate buffer. The spectra obtained for the lipid droplet preparations (a and b) are almost identical to that for the triolein (c). By contrast, the spectra of the spin probe in phosphate buffer contained 3 sharp peaks, indicated by arrows, as is typical for a rapidly tumbling probe. Because it was difficult to determine the outer hyperfine splittings in these spectra, they were compared by calculating the order parameter of the inner hyperfine splittings \( S(T,J) \). The values are given in Table 3. No appreciable differences were observed between the lipid droplets isolated from the cells grown in mice fed sunflower oil as compared with coconut oil. At both 4° and 37°, the values for the lipid droplets isolated from the cells were in the same range as those obtained for the triolein preparation. For comparison, \( S(T,J) \) at 37° for cells grown in mice fed the 2 diets also are included. These values are roughly 80% higher than those for the lipid droplets. In agreement with the \( S \) and \( S(T,J) \) parameters, the \( S(T,J) \) value for the cells grown in the mice fed coconut oil is slightly larger than that for the cells grown in the mice fed sunflower oil.

Through the use of a signal-averaging computer program, the ESR spectra of the lipid droplets obtained from the cells grown in the mice fed coconut oil was subtracted from the ESR spectra of the droplets from the cells grown in the mice fed sunflower oil. The result is shown in Chart 3. Only the 3 peaks associated with free spin probe motion remain, indicating that the ESR spectra of both types of droplets are identical. Taken together, these findings suggest that the differences in ESR spectrum of the cells grown in mice fed coconut oil as opposed to sunflower oil are due to membrane differences sensed by the spin probe, not due to differences reported by any of the probe contained within the cellular lipid droplets.

**DISCUSSION**

This investigation was designed to determine the degree to which spin label probe inserted into cytoplasmic lipid droplets contributes to the ESR spectra of lipid-modified cells. It also attempts to resolve the question of whether the lipid modifications that can be produced in vivo are sufficient to alter the membrane physical properties of tumor cells. Previous work with spin label probes from the laboratories of 2 of the authors indicated that membrane fluidity can be altered by dietary lipid modifications in both the Ehrlich ascites carcinoma and L1210 lymphocytic leukemia (4, 12, 13). These ESR measurements were obtained by incorporating the spin probe into plasma membrane vesicles that were isolated by density gradient centrifugation. Lipid modification was carried out either while the tumor grew in ascites form in the host mouse (4, 13) or after the tumor cells were put into tissue culture (12). Concurrent measurements demonstrated that the membrane fatty acid composition was altered, and the fluidity changes detected by the ESR measurements generally corresponded to the extent of polyunsaturated fatty acid enrichment or depletion in the plasma membrane (4, 12, 13). The conclusion that these types...
of lipid modification were accompanied by membrane fluidity changes is in agreement with the findings of other investigators. For example, differences in fatty acid composition were correlated with ESR changes in the plasma membranes of mouse myeloid leukemia cells in culture (24). In addition, the temperature of onset of the phase transition in Chinese hamster ovary cell membranes correlated with the percentage of unsaturated fatty acids in cellular phospholipids (20). The measurements in the Chinese hamster ovary cells were made by incorporating parinaric acid, a fluorescent probe. Other work has demonstrated functional changes in membranes resulting from cellular fatty acid modification. Endocytosis is affected when the fatty acid composition of murine peritoneal macrophages is modified (15), and lectin-induced agglutination is similarly altered in mouse 3T3 cells (9). Since these cellular functions probably are dependent on membrane fluidity, the findings appear to support the interpretation that the fatty acid modifications which can be produced in vivo are sufficient to alter the physical state of cellular membranes.

This conclusion could be challenged by the observations of recent studies in which the fluorescent probe diphenylhexatriene was used (25, 27). In one of these studies, rather extensive fatty acid modifications were produced in normal rodent splenic lymphocytes (27). Large fluorescence polarization changes were observed when the fluorescent probe was incorporated into the intact lymphocytes. When membranes were isolated first and the fluorescent probe was incorporated into the purified membrane fraction, however, no fluorescence polarization differences were observed even though the fatty acid compositional changes in the isolated membrane fractions were extensive. Lipid droplets were noted in the cytoplasm of the fatty acid-modified cells, and additional fluorescence studies suggested that incorporation of the probe into these lipid inclusions probably produced the polarization differences observed in the intact cells. Based upon these fluorescence polarization findings, serious questions were raised regarding whether any alterations in membrane fluidity actually occurred as a result of the fatty acid modifications. Furthermore, it was suggested that previous results in which positive effects were observed in isolated membrane preparations may have been due to the fact that cytoplasmic lipid droplets became associated with the membrane vesicles during isolation (27).

A similar conclusion was reached from related studies in which the membrane fluidity of normal and malignant lymphocytes was compared (25). The differences in fluidity between the normal and malignant cells, as measured by diphenylhexatriene polarization, was again attributed to the fact that the malignant cells contained large numbers of cytoplasmic lipid droplets.

The present work with the 5-nitroxyystearic acid probe indicates that, at least in the L1210 murine leukemia system, the ESR changes observed in fatty acid-modified intact cells are due to membrane changes and not to insertion of the probe into the cytoplasmic lipid droplets. This is consistent with previous findings that fluidity changes occur when the spin label probe is inserted into purified plasma membranes isolated from the lipid-modified L1210 cells (4). It appears that, when the 5-nitroxyystearic acid probe is added to intact L1210 cells, it localizes in the membranes, as opposed to the cytoplasmic lipid droplets. This is indicated by the fact that the ESR spectra obtained from the cells have a similar shape to those obtained previously from isolated plasma membranes (4), whereas they are completely different from the spectra obtained from the isolated lipid droplets. By contrast, the ESR spectra from the isolated lipid droplets are very similar to those obtained from the triolein droplets dispersed in phosphate buffer. Furthermore, these spectra are similar in shape to those obtained for lipid droplets by Takeuchi et al. (28).

The reactivation of the ESR signal produced by exposure to ferricyanide also suggests that the 5-nitroxyystearic acid probe localizes predominantly in the plasma membrane of the intact cells. However, the absolute value of the order parameter is smaller in the intact L1210 cells than in isolated plasma membranes prepared from the cells (4). It is possible that a sufficient fraction of the incorporated probe localizes in more fluid environments to reduce the average value reported by the probe contained in the intact cell. Alternatively, the structure of the membrane may be altered sufficiently during isolation to produce an increase in bulk fluidity as assessed by the incorporated 5-nitroxyystearic acid probe. These possibilities cannot be resolved from the available results.

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REFERENCES


Fig. 1. A. electron micrograph of L1210 cells from animals fed coconut oil diet. × 15,000. LD, lipid droplets; N, nucleus; M, mitochondria; R, rough endoplasmic reticulum. Bar, 1 μm. B, same as A at higher magnification. × 25,200.
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