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IDENTIFICATION OF A POTENTIAL ARTIFACT IN THE USE OF ELECTRON MICROSCOPE AUTORADIOGRAPHY TO LOCALIZE SATURATED PHOSPHOLIPIDS IN CELLS

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Summary

The suitability of electron microscope autoradiography for studying the uptake and intracellular localization of lipid vesicles (liposomes) containing radiolabeled saturated phospholipids has been examined. Data are presented showing that preparation of specimens for electron microscope autoradiography by conventional methods is accompanied by significant translocation and intercellular redistribution of radiolabeled saturated lipids, causing spurious labeling patterns. Intercellular redistribution of radiolabeled lipid was demonstrated by mixing glutaraldehyde-fixed mouse L1210 cells that had been incubated with sonicated lipid vesicles containing [^{3}H]dipalmitoyl phosphatidylcholine with an indicator cell population (fixed avian erythrocytes) which had not been exposed to vesicles and showing that after electron microscope processing radiolabeled grains were present in both cell types. The same redistribution artifact also probably affects the intracellular localization of radiolabeled lipids. This artifact is discussed in relation to previous work in which autoradiographic methods have been used for ultrastructural localization of saturated phospholipids in cells and tissues.

Introduction

The increasing use of lipid vesicles (liposomes) as carrier vehicles to introduce biologically-active materials into cells in vitro [1,2] and in vivo [2,3] has focused attention on the mechanism(s) by which vesicles are incorporated into the cells and the intracellular localization and fate of vesicles and their contents [4—11]. Recently, Batzri and Korn [5] and Pagano and Huang [7] attempted to study these questions using electron microscope autoradiography to follow

cellular uptake and intracellular localization of lipid vesicles containing trace amounts of ^{3}H-labeled saturated phospholipids. In this communication, we report evidence which indicates that this method is unsuitable for this purpose, since the radiolabeled lipid molecules undergo significant redistribution during processing of samples for electron microscopy. In addition to the obvious need for caution in the interpretation of previous observations on vesicle-cell interactions using this method [5—7], the redistribution and intracellular translocation of radiolabeled lipids described in this study may also represent a potential source of artifact in electron microscope autoradiographic studies of the natural distribution of saturated lipids in cells and tissues [12—18] and the localization of amphipathic molecules added to cells as probes of lipid "microviscosity" in cell membranes [19].

Materials and Methods

Lipid vesicles, vesicle-cell interactions and electron microscope autoradiography

The source(s) of lipids, methods of vesicle preparation and the physicochemical and ultrastructural characteristics of the sonicated vesicle populations of differing surface charge and lipid composition used in the present experiments have been described in detail previously [4,8,20,21]. [^{3}H]Dipalmitoyl phosphatidylcholine was obtained from New England Nuclear by catalytic tritiation of purified dipalmitoyl phosphatidylcholine to a specific activity of 4 Ci/mmol [4,8]. The stock solution in ethanol was kept at —10°C and repurified on silicic acid columns [20] every six months to a year. The final solutions in chloroform were kept in sealed ampoules under nitrogen at —10°C and 0.1 Ci/ml. It was mixed with carrier lipid immediately before use for making vesicles. The radiopurity was checked by thin-layer chromatography and was more than 90% dipalmitoyl phosphatidylcholine.

Murine L1210 leukemia cells were chosen for electron microscope autoradiographic studies on the ultrastructural localization of vesicle-derived lipids within the cell because they offer a uniform population of small spherical cells, which do not show marked pleomorphism in their morphology, thus facilitating evaluation of cell labeling patterns. L1210 cells were obtained from the ascites fluid of DBA/2 female mice and washed twice with phosphate-buffered saline before incubation with sonicated small unilamellar vesicles of differing surface charge and lipid composition (see Results). Typically, $1 \cdot 10^7$ cells were incubated at 37°C with small unilamellar vesicles (0.1 μmol lipid; 1 nmol phospholipid = approx. $2 \cdot 10^{11}$ small unilamellar vesicles; see ref. 20) for periods of 5—60 min. The vesicles contained [^{3}H]dipalmitoyl phosphatidylcholine (1 mCi/μmol), which was used as an autoradiographic marker to follow the uptake and subsequent intracellular distribution of the vesicle-derived lipids. After incubation with vesicles, the cells were washed twice with cold phosphate-buffered saline and finally resuspended in 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 3 h at 4°C, washed four times in buffer and then either treated with 1% osmium tetroxide for 2 h followed by incubation with 0.5% aqueous uranyl acetate overnight, as described by Huang and Pagano [6], or rinsed overnight in buffer before treatment with osmium tetroxide for 2 h.

In order to test for possible intercellular transfer of radiolabeled phospholipids during subsequent processing of the cells, other samples were prepared in which an equal number of non-vesicle treated chicken erythrocytes that had been identifically fixed with glutaraldehyde were mixed with glutaraldehyde-fixed vesicle-treated L1210 cells. The cell mixtures were then pelleted together, fixed with glutaraldehyde in cacodylate buffer for a further 2 h and then treated as described above, either with osmium tetroxide and uranyl acetate, or with osmium tetroxide alone.

All samples were dehydrated by successive 10-min immersions in 35, 50, 60, 90, and 100% ethanol at 4°C. The final dehydration was then repeated and followed by two 10-min immersions in propylene oxide. Epon 812 was used for infiltration and embedding.

Electron microscope autoradiographs of the cell mixtures were prepared according to the flat substrate methodology of Salpeter and Bachman [22]. Pale-gold ultramicrotome sections (≈1000 Å thick) were mounted on colloidionized slides, stained with 2% uranyl acetate (if not pre-stained en bloc), carbon-coated and overlaid with a monolayer of Ilford L-4 emulsion. After exposures of 7—25 days, the preparations were developed in D-19 (Kodak) for 2 min at 24°C and viewed and photographed with a Siemens Elmiskop 101.

Results

Previous studies in this [1,4,8,23] and other laboratories [5—7,9—11] have shown that lipid vesicles are incorporated into cells either by endocytosis or non-endocytotic mechanisms (such as surface adsorption or fusion), though the respective importance of these two uptake pathways is influenced by the properties of the vesicle membrane. Negatively charged vesicles composed of phospholipids that are "fluid" at 37°C interact with cells via both endocytosis and the non-endocytotic pathway, with the latter predominating, but similarly charged vesicles composed of "solid" phospholipids, and also neutral vesicles, appear to be incorporated into cells exclusively by endocytosis [1,4,8,23]. In view of these findings it might reasonably be expected that uptake of radiolabeled vesicles of differing surface charge and "fluidity" would create different patterns of radiolabel distribution in autoradiographs. Thus, vesicles incorporated via fusion with the plasma membrane or adsorption to the cell surface should produce a greater concentration of silver grains over the plasma membrane than vesicles which are endocytosed in which the radiolabel should be distributed largely in lysosomes.

To test this possibility, electron microscope autoradiographic studies were done to determine the distribution of radiolabel in cells incubated with the following vesicles containing trace amounts of [^{3}H]dipalmitoyl phosphatidylcholine: (1) "fluid" phosphatidylserine/egg phosphatidylcholine (1 : 9, mol ratio) negatively charged small unilamellar vesicles; (2) "solid" phosphatidylserine/dipalmitoyl phosphatidylcholine/distearoyl phosphatidylcholine (1 : 4.5 : 4 : 5, mol ratio) negatively charged small unilamellar vesicles; and (3) neutral vesicles (egg phosphatidylcholine). However, examination of the autoradiographs failed to reveal any significant difference in the pattern of radiolabeling obtained after incubation with each of these different vesicle populations. Qualitatively similar grain distributions were found (Fig. 1) regardless of vesicle composition, or

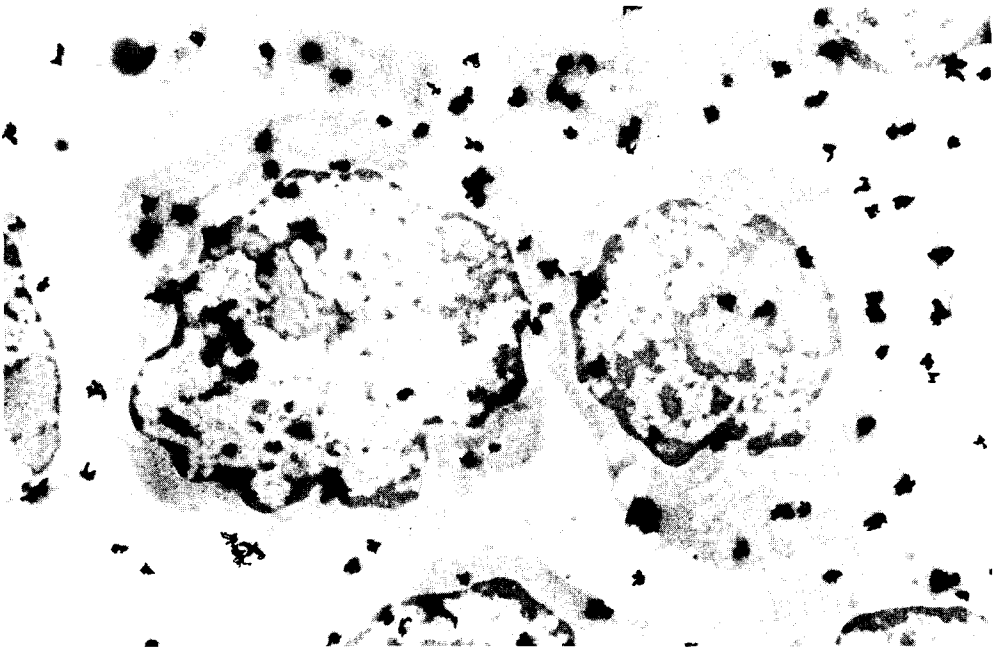

Fig. 1. Electron microscope autoradiograph of mouse L1210 cells incubated for 5 min at 37°C with small unilamellar phosphatidylserine/phosphatidylcholine (1 : 9) vesicles containing trace amounts of [^{3}H]-dipalmitoyl phosphatidylcholine showing predominantly cytoplasmic distribution of the ^{3}H label. X 7920.

the length of time for which vesicles were incubated with cells (5—60 min), with the preponderance of silver grains being located over the cell cytoplasm. For the most part, the cytoplasmic labeling was diffuse with no particular association with any specific cytoplasmic organelle. On occasions, however, the lipid vacuoles, which are a common feature of L1210 cells grown in animals, did appear to have grains associated with their periphery. Infrequent grains were found over the plasma membrane but, owing the radiation scatter and other factors involved in autoradiographic resolution, these could have resulted from radioactivity bound in the cytoplasm. The apparent grain density of the nucleus was consistently much lower than that of the cytoplasm. Interestingly, the nuclear membrane was usually well labeled and, as such, was the only identifiable cellular organelle with a significant amount of bound radioactivity. This pattern resembles closely that described by Huang and Pagano [6] in hamster V79 cells incubated with small unilamellar vesicles composed of various species of lecithin and containing trace amounts of [^{3}H]distearoylphosphatidylcholine.

This uniform labeling pattern is suprising in view of the major differences in the route of vesicle uptake (endocytosis vs. non-endocytosis) for vesicles of differing composition discussed above. Since the labeling pattern was strikingly similar in all cases, further experiments were done to test whether cell-associated radiolabeled phospholipid molecules derived from the vesicles might be redistributed during the processing of the electron microscope sections, thus creating spurious labeling patterns. To examine this possibility, L1210 cells were treated with radiolabeled vesicles at 37°C, and then fixed with glutaral-

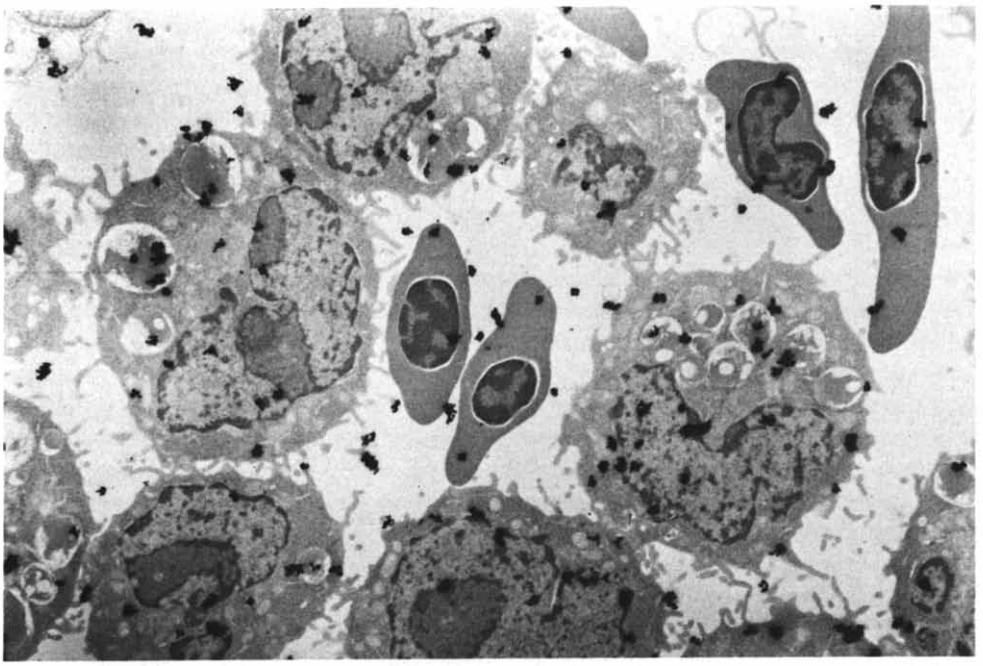

Fig. 2. Electron microscope autoradiograph of mixed population of L1210 cells incubated for 5 min at 37°C with [^{3}H]dipalmitoyl phosphatidylcholine-labeled phosphatidylserine/egg phosphatidylcholine (1 : 9) small unilamellar vesicles and non-vesicle treated glutaraldehyde-fixed avian erythrocytes showing redistribution and transfer of the ^{3}H label from L1210 cells to erythrocytes during tissue processing. The L1210 cells were fixed in glutaraldehyde for 1 h after treatment with vesicles and then mixed with similarly fixed but non-vesicle treated erythrocytes for subsequent processing as a cell mixture (see Methods). × 5850.

dehyde before being finally mixed with an equal number of similarly fixed chicken erythrocytes which had not been exposed to vesicles. Chicken erythrocytes were used as indicator cell since they could be reliably distinguished from L1210 cells in electron micrographs of mixed cell populations. When such mixed cell populations were examined, a significant number of silver grains were found overlying the chicken erythrocytes (Fig. 2), indicating that intercellular transfer of radiolabeled lipid had occurred. The grain density over the erythrocytes (28 grains/100 μm^2) was significantly higher than background (1.6 grains/100 μm^2), and occurred in cells well beyond the range of radiation scatter from the originally labeled L1210 cells. Grains over the erythrocytes tended to be associated with the nuclear membrane and to a lesser extent with the plasma membrane. Those grains over the nucleus and cytoplasm of these cells may be the result of radiation scatter from the membrane sources. The lengthy analysis required to test this possibility was deferred since the labeling in these cells is clearly artifactual in origin.

Redistribution of radiolabeled phospholipid from vesicle-treated L1210 cells to the avian erythrocytes was found with fluid and solid negatively charged vesicles and with neutral vesicles (results not shown). Transfer of radiolabel from L1210 cells to erythrocytes not only occurred in samples post-fixed with osmium and stained en bloc with uranyl acetate by the method used by Huang

and Pagano [6] but also in samples postfixed with osmium tetroxide alone. As in the case of labeled L1210 cells, the grain distribution over the erythrocytes did not vary with the vesicle composition, but this might be expected since the radioactive marker lipid was the same in all cases.

An attempt was made to determine at what point translocation of radiolabel occurred by mixing unlabeled erythrocytes with vesicle-treated L1210 cells at various steps following osmium fixation. Unfortunately, the cell mixtures were non-cohesive after centrifugation and could not be processed as a stable pellet, thus frustrating analysis of the redistribution phenomenon.

The results clearly indicate, however, that [^{3}H]dipalmitoylphosphatidylcholine or other saturated lipids are unsuitable as a marker for electron microscope autoradiographic studies on the intracellular distrubution of vesicle-derived phospholipids, since substantial redistribution of the radiolabel occurs during tissue processing.

Discussion

The extraction of cellular lipids during processing of cells and tissues for electron microscopy has been documented on many occasions (see ref. 16 for review). This problem is most marked with fully saturated lipids, due to the absence of double bonds for cross-linking by OsO_4 [24—27], and particularly with phosphatidylcholines in which the absence of primary amines in the polar head group dictates that they cannot react directly with either OsO_4 [27, 28] or glutaraldehyde [29,30]. Morgan and Huber [14] have shown that under fixation and dehydration conditions similar to ours, approx. 40% of [^{3}H]-choline (incorporated mainly into dipalmitoylphosphatidylcholine) was extracted from lung tissue during processing for electron microscopy. Lipid extraction may be even greater under the conditions of the present study where cells are only loosely packed in a pellet and separated by wide spaces that are continuous with the surrounding medium and thus facilitate solvent penetration. Attempts have been made recently to minimize extraction of saturated cellular phospholipids using new fixatives such as tannic acid [18,31] or polar dehydrating agents such as Durcupan and dioxane [32] or glycol methacrylate [33], but to date these have achieved only moderate success.

The results presented here suggest that in addition to extraction of cellular lipids the problem of redistribution and intercellular transfer of radiolabeled lipids must also be considered. The latter has not hitherto been appreciated as a potential source of artifact. Several investigators [16,34,35] have noted the paucity of grains over cell-free areas adjacent to heavily labeled cells in sections of intact tissue and interpreted this as indicating lack of diffusion of the radiolabel. However, this is only a measure of translocation that might occur during the infiltration, polymerization and embedding steps and offers no information on translocation in the earlier steps of dehydration where it is perhaps more likely to occur. The present demonstration of transfer of [^{3}H]dipalmitoyl phosphatidylcholine from fixed radiolabeled cells to fixed originally unlabeled cells offers convincing evidence that extensive intercellular translocation of radiolabeled saturated phospholipids can take place.

Identification of this potential artifact raises obvious doubts concerning the

suitability of saturated phospholipids as markers for electron microscope autoradiography. For example, the translocation of exogenous radiolabeled saturated phospholipids introduced into cells via uptake of lipid vesicles described in this study suggests that this method is clearly unsuitable for defining the intracellular localization of vesicle-derived lipids and that previous studies using similar electron microscope autoradiographic methods to characterize vesicle-cell interactions [5—7] must now be interpreted with some caution. However, while this work was being prepared for publication, Pagano and Takeichi [36] published additional electron microscope autoradiographic data on the interaction by vesicles containing [^{3}H]dipalmitoyl phosphatidylcholine with hamster V79 cells in vitro. In this study they too have attempted to assess whether redistribution of radiolabeled lipid might be occuring by using cell mixing experiments in which cells incubated with [^{3}H]dipalmitoyl phosphatidylcholine-labeled vesicles were mixed with non-vesicle-treated cells containing latex beads and the mixture processed for electron microscope autoradiography. In complete contrast to our results, they were unable to detect translocation of the [^{3}H]dipalmitoyl phosphatidylcholine label between vesicle-treated cells and the bead labeled indicator cell population. The reason for this marked discrepancy between our results and those of Pagano and Takeichi [36] is not clear. However, technical differences may account for these differing results. In our experiments, and in earlier work published by Huang and Pagano [6], the radiolabeled lipid markers ([^{3}H]dipalmitoyl phosphatidylcholine, [^{3}H]distearoyl phosphatidylcholine) were added in trace amounts to vesicles composed of different phospholipids, while in the more recent study of Pagano and Takeichi [36] the [^{3}H]dipalmitoyl phosphatidylcholine marker was present in vesicles composed entirely of dipalmitoyl phosphatidylcholine. This difference raises the possibility that extraction of radiolabeled saturated phospholipids by the solvents used in the preparative procedures could be occurring more readily if the saturated lipid is within a matrix of a different lipid as compared with a matrix of the same composition as the radiolabel. Also, in our experiments both radiolabeled donor cells and the indicator cells were fixed before being mixed together, but in Pagano and Takeichi's study, neither cell type was fixed at the time of mixing, and the latter was also done in a monolayer. Finally, the lack of information in the paper by Pagano and Takeichi on such factors as the temperature during ethanol dehydration and the ratio of donor to indicator cells also hinders direct comparison.

While not directly demonstrated in the present study, it seems likely that the extraction and intercellular redistribution of radiolabeled lipid is also accompanied by significant intracellular translocation of radiolabel. Label originally concentrated in a particular organelle or region of the cell would thus be distributed to other regions of the cell during processing of the sample for electron microscope autoradiography. This potential problem is pertinent to recent attempts [19] to use electron microscope autoradiography to establish the intracellular distribution of small lipophilic molecules, which localize to various membranes and which have been used as probes to monitor the "microviscosity" of membrane lipid microenvironments. Finally, the shortcomings in the use of radiolabeled saturated lipids as markers for studying the uptake and distribution of exogenous lipid incorporated into cells discussed in this paper may

perhaps also apply to the use of electron microscope autoradiography for studying the distribution of naturally occurring saturated phospholipids in cells and tissues in situ [15—18].

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