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Intracellular Penetration of Liposomes Containing a Water Insoluble Antimitotic Drug in L1210 Cells*

J. FRÜHLING,†‡ W. PENASSE,§ G. LAURENT,|| CH. BRASSINNE,|| J. HILDEBRAND,|| M. VANHAELEN,¶ R. VANHAELEN-FASTRE,¶ M. DELEERS** and J. M. RUYSSCHAERT**

†*Laboratoire des Radio-Isotopes, Service de Radiothérapie, Institut Jules Bordet*, 1, *rue Héger-Bordet*, 1000 *Bruxelles, Belgium*, §*Laboratoire de Cytologie et de Cancérologie Expérimentale, Faculté de Médecine, Université Libre de Bruxelles*, 1, *rue Heger-Bordet*, 1000 *Bruxelles, Belgium*, ||*Service de Médecine et Laboratoire d'Investigation Clinique Henri Tagnon, Institut Jules Bordet*, 1, *rue Heger-Bordet*, 1000 *Bruxelles, Belgium*, ¶*Institut de Pharmacie, Faculté de Médecine*, 2, *rue Evers*, 1000 *Bruxelles, Belgium, and* **Laboratoire des Macromolécules aux Interfaces, Faculté des Sciences, Université Libre de Bruxelles, Boulevard du Triomphe*, 1040 *Bruxelles, Belgium*

Abstract—*To demonstrate the possibility of cell delivery of water insoluble products, L1210 cells were incubated with liposomes containing Nocodazole® (NCL), an experimental drug totally insoluble in water. Liposomes of various lipid compositions readily incorporated NCL, but only those formed from dipalmitoyl phosphatidylcholine, cholesterol and stearylamine (molar ratio 4:3:1) had a clear affinity for L1210 cells, as shown by fluorescence studies. This affinity is mediated by the degree of both cell and liposome membrane fluidity. Cytoplasmic and intranuclear location of these liposomes containing NCL or drug-free and subcellular changes induced by their presence were demonstrated by ultrastructural studies.*

INTRODUCTION

THE LACK of water solubility of certain drugs makes difficult the evaluation of their activity in experimental models and clinical trials. The purpose of this report is to show that this difficulty may be overcomed by the use of liposomes as drug carriers. Nocodazole® (NCL, NSC-238159) is a new experimental antineoplastic drug which is completely insoluble in water. So far this property has precluded its clinical use [1, 2]. This paper provides a morphological demonstration of intracytoplasmic and intranuclear localization of liposomes containing Nocodazole® in L1210 cells, and demonstrates cellular alteration produced by these liposomes.

MATERIALS AND METHODS

Liposomes were prepared by dissolving in chloroform NCL and the appropriate lipids detailed in Table 1. After complete evaporation of the solvent under reduced pressure, 1 ml of 50 mM Tris pH 7.4 containing 0.15 M NaCl was added per 10 mg of dried lipids. Liposome preparation obtained after a 15 min ultrasonication at 65 W in N_2 atmosphere with a Branson sonifier was centrifuged at 3000 ***g*** for 10 min and the pellet containing free NCL was discarded. NCL concentration in liposomes was measured by high pressure liquid chromatography (HPLC). Gel permeation chromatography on Sepharose 4B [3] showed that all NCL present in the supernatant was incorporated into liposomes. In order to study the interactions between liposomes and L1210 cells, liposomes were labeled with trace amounts of ^{14}C-dipalmitoyl phosphatidylcholine (DPPC) (Amersham, Radiochemical Centre, U.K., sp, act. 120 mCi/mmole).

L1210 leukemia was maintained in DBA/2 mice by weekly i.p. transplantation of 10^5 cells. For present experiments, cells were harvested from the peritoneal cavity 6 days after transplantation and washed twice in RPMI 1640 medium supplemented with 10% foetal calf serum (FCS).

In a series of experiments, the affinity of liposomes of different compositions containing NCL for L1210 cells was studied. Typical

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‡To whom requests for reprints should be addressed.

incubations contained in a total volume of 10 ml of RPMI 1640 supplemented with 10% FCS, 10^8 L1210 cells and an amount of labeled liposomes corresponding to 20 mg of lipid mixture. After a 2 hr incubation, cells were centrifuged at 200 *g* for 10 min and washed in fresh RPMI 1640 medium. The amount of ^{14}C-DPPC present in cell pellets was measured by liquid scintillation counting. Cell-associated NCL was measured by HPLC.

The degree of fluidity of liposomes and of L1210 plasma cell membrane was measured by the method of Shinitzky [4]. A fluorescent probe, 1,6-diphenyl 1, 3, 5-hexatriene (DPH) was incorporated into the lipid bilayer in a molar ratio of 1 to 1000 or into the plasma membrane by incubating 4×10^5 L1210 cells in the Tris buffer with 25×10^{-9} mole of DPH per ml at 37°C for 1 hr. The measured fluorescence polarization was directly related to the fluidity of the medium surrounding the probe.

The experiments were performed using an Elscint microviscosimeter model MV_{1a} (Elscint Ltd, Haifa, Israel). The apparatus gives the degree of fluorescence polarization (P) following the equation:

$$P = (I_{//} - I_{\perp})/(I_{//} + I_{\perp})$$

where $I_{//}$ and $I_{\perp}$ are the fluorescence intensities polarized parallelly and perpendicularly to the direction of polarization of the excitation beam. The apparatus gives P with an accuracy better than 5%. The temperature of the samples was controlled to within 0.5°C.

The adsorption of liposomes on L1210 cell surface was investigated by fluorescence studies. In these experiments, fluorescent liposomes were formed by adding before sonication 5 mM 6-carboxyfluorescein to the buffer containing the appropriate lipids and stearylamine. Cells and liposomes were incubated for 1 hr at 37°C in Hank's balanced salt solution.

For electron microscopic studies, tissue preparation techniques were adapted in order to obtain an optimal lipid preservation [5]. Cells were fixed for 2 hr in 2.5% glutaraldehyde buffered with 0.1 M cacodylate and after rinsing by cacodylate-buffer (0.1 M) postfixed "overnight" in the same buffer containing 2% OsO_4. The highest ethanol concentration of solvent used for dehydration and embedding did not exceed 70% [5]. Samples were embedded in Epon [6], ultrathin sections were contrasted with lead citrate [7] and examined at 40 or 60 kV under Philips 200 and 301 electron microscopes.

RESULTS

As shown in Table 1, comparable amounts of NCL were found in the 4 types of liposomes tested, regardless of their charge, lipid composition or the degree of membrane fluidity.

In contrast, the percentage of liposomes recovered in cell pellets after centrifugation was related to the composition of the lipid vesicles. Particularly, for the positively charged liposomes, the affinity for L1210 cells was

Table 1. Affinity of liposomes of different compositions for L1210 cells

Lipid composistion of liposomes	Molar ratio	Nocodazole^R (NCL)/ concentration in liposome preparations (μg/ml)	Radioactivity recovered in cell pellets (% of total radioactivity)	Nocodazole^R recovered in cell pellets (% of total Nocodazole)
Egg phosphatidylcholine	—	41.8	0.39	0.6
Egg phosphatidylcholine–stearylamine	7:1	43.3	0.66	
Egg phosphatidylcholine–cholesterol–stearylamine	4:3:1	58.4	1.08	1.3
Dipalmitoyl phosphatidylcholine–cholesterol–stearylamine	4:3:1	44.6	5.08	21.0

L1210 cells (10^7 cells/ml) were incubated at 37°C in 8 ml RPMI 1640 medium supplemented with 10% foetal calf serum. Incubations were initiated by the addition of 2 ml of liposome preparation containing per ml 10 mg of lipid mixture, 60 μCi of ^{14}C-DPPC (120 mCi/mmole) and the NCL concentration indicated above, measured by high performace liquid chromatography. After 2 hr of incubation, cell suspensions were cooled in ice, centrifuged at 200 *g* for 10 min and washed one time with 10 ml ice cold medium. Radioactivity and Nocodazole^R concentration were measured in cell pellets. In control experiments where liposomes were incubated without cells, the radioactivity recovered in pellets was less than 10% of values obtained in presence of cells. The data are the mean of duplicate experiments.

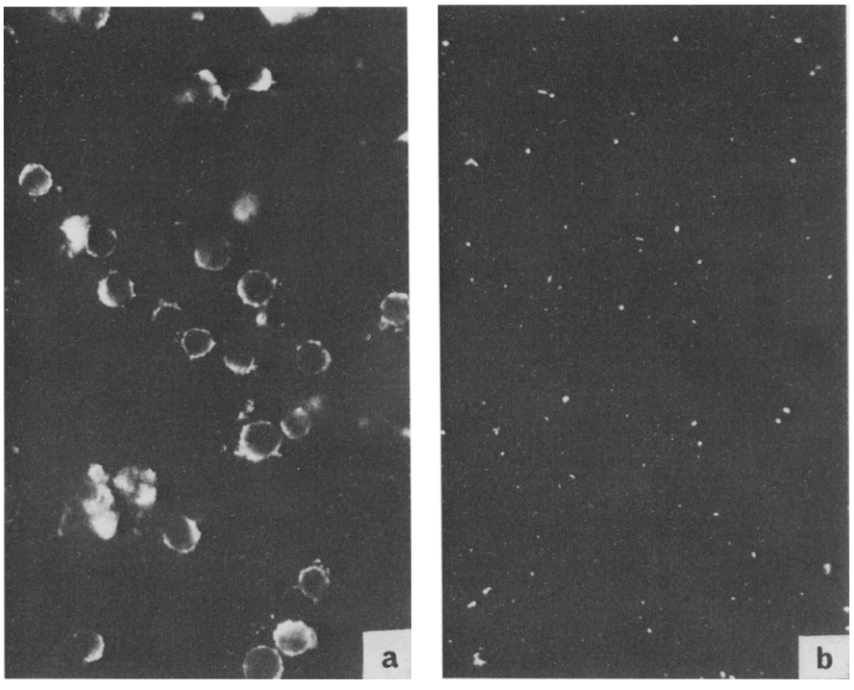

Fig. 2. L1210 cells incubated with fluorescent liposomes: liposomes (a) made of dipalmitoylphosphatidylcholine–cholesterol–stearylamine (4: 3: 1) are adsorbed on cell surfaces whereas liposomes (b) made of egg yolk phosphatidylcholine–cholesterol–stearylamine (4: 3: 1) are randomly distributed and show no affinity for L1210 cells.

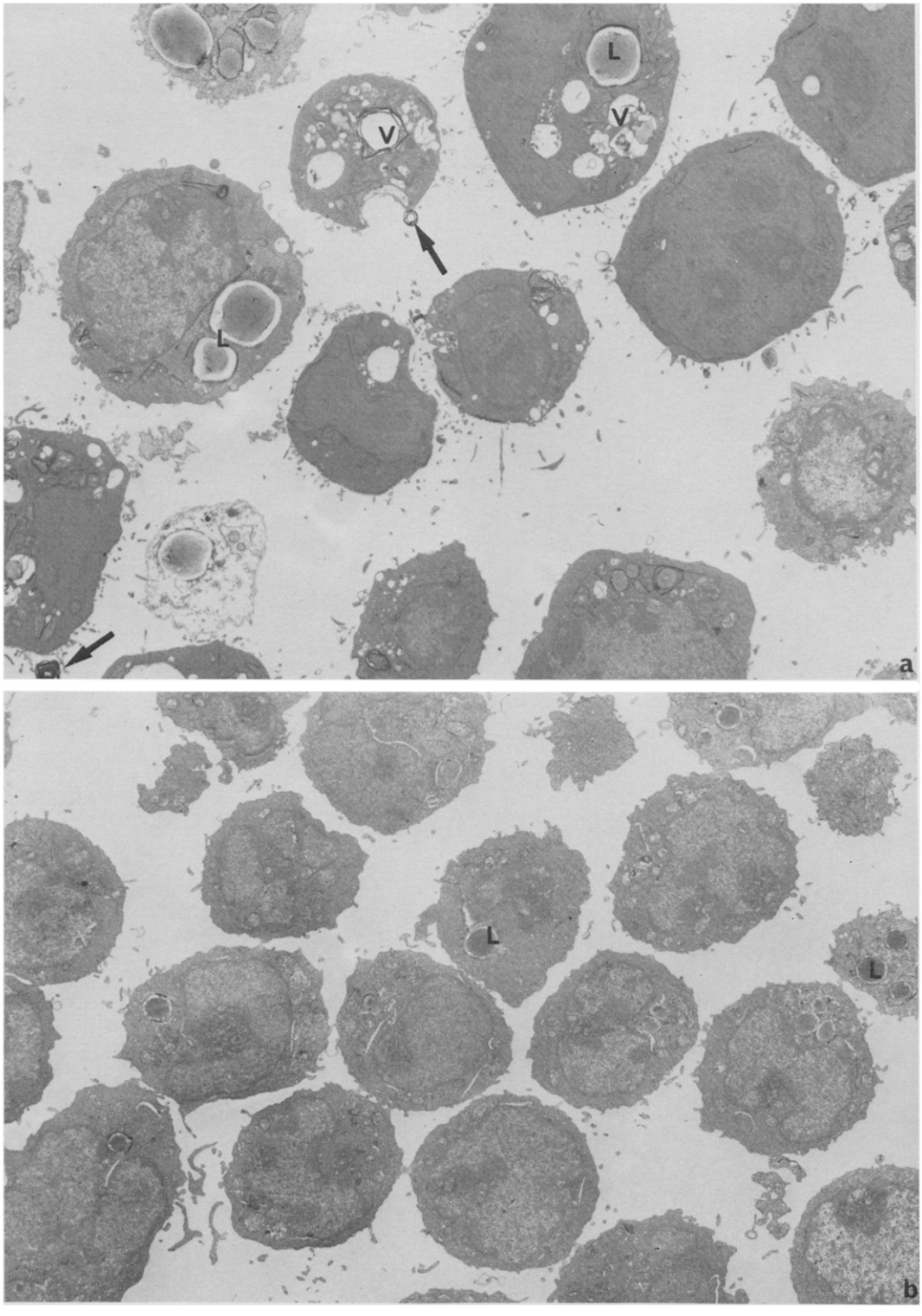

Fig. 3. (*a*) *L1210 cells incubated for* 1 hr *with liposomes* (*arrows*) *containing Nocodazole*® (60 μg/ml). *Lipid droplets* (*L*) *and vacuoles* (*V*) *are seen in cytoplasm. The vacuoles frequently contain multilamellar vesicles similar to the lipid vesicles present in the extracellular space* (×5260). (*b*) *L1210 cells incubated without liposomes. The aspect of cells is uniform. There are no vacuoles or multilamellar vesicles. Lipid droplets* (*L*) *are present, but in lower amount than in cells incubated with liposomes* (×3930).

Fig. 4. L1210 cells incubated in the presence of liposomes containing Nocodazole[R]. *(a) The three multilamellar lipid vesicles (VL1, VL2, VL3) suggest the uptake of liposomes by the cells. V: vacuole, m: mitochondrion without any morphological alteration, u and arrow: unilamellar lipid vesicle (×11,800). Insert: detail of the cell surface showing an elongated lipid vesicle (I) which seems to be taken up by the cell. Another lipid vesicle (VL) is associated to an invagination of the plasma membrane (×24,800). (b) Details of two cells showing an important cell surface activity and cell vacuolization. The plasma membrane shows invagination (P). The cells contain lipid droplets (L) and multilamellar structures (F) sometimes entrapped in a vacuole (V). Nucleus (N) and mitochondrion (m) are unaltered (×19,450).*

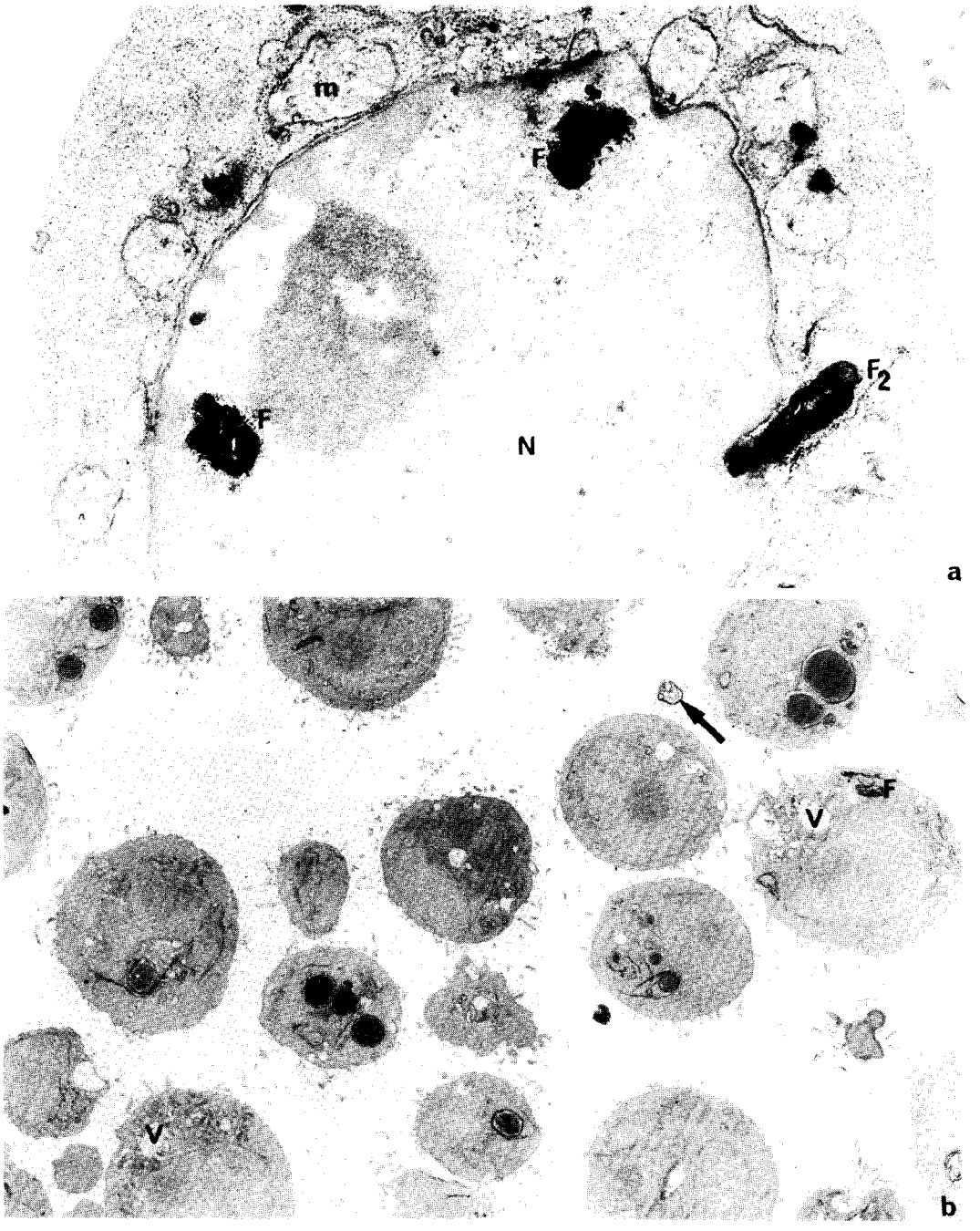

Fig. 5. L1210 cells incubated with drug free liposomes. (a) Multilamellar structures (F) present in the nucleus (N) which retains its normal morphological features. F_2: the same structure in close contact to nucleus and the cytoplasm; m: mitochondrion (×22,000). (b) General view of the pellet showing multilamellar liposomes (arrow) lipid droplets (L) and vacuoles (V) in the cytoplasm. The morphological changes are less numerous than in cells incubated with liposomes containing NCL. F: multilamellar structures similar to multilamellar liposomes (×3340).

correlated with the degree of fluidity of the lipid bilayer and that of the cell plasma-membrane (Fig. 1). The affinity of liposomes made of DPPC, cholesterol and stearylamine (molar ratio 4:3:1) for L1210 cells was further

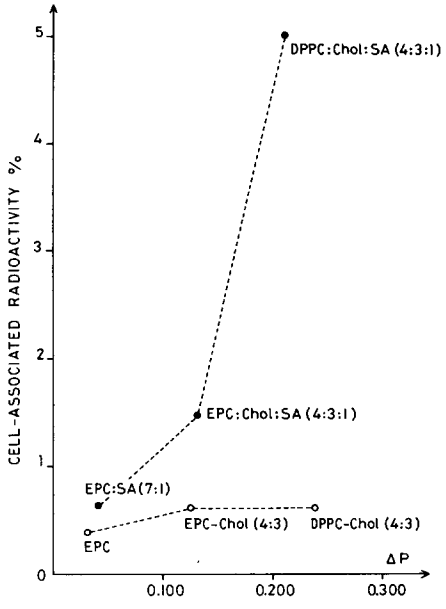

Fig. 1. Relation between the degree of liposome fluidity and their uptake by L1210 cells. ΔP refers to the difference of fluidity between liposome bilayer and L1210 cell plasma membrane. (○): neutral liposomes, (●): positive liposomes DPPC: dipalmitoylphosphatidylcholine; EPC: egg yolk phosphatidylcholine; Chol: cholesterol; SA: stearylamine.

confirmed by fluorescence studies showing the aggregation of these lipid vesicles on the cell surface (Fig. 2) whereas liposomes of other lipid composition had no affinity for L1210 cells.

Ultrastructural studies were performed to demonstrate the intracellular localization of liposomes either containing NCL or drug-free. Electron micrographs of incubations containing liposomes and L1210 cells showed small unilamellar vesicles (SUV) and large multilamellar vesicles (MLV) in the extracellular space (Figs. 3a, 4a, 5b). Some were in close contact with plasma membranes (Figs. 3a, 4a). The presence of membrane invaginations suggests liposome endocytosis (Figs. 3a, 4a, 4b). Moreover, numerous intracytoplasmic liposomes were found after incubation of L1210 cells and lipid vesicles containing or not NCL (Figs. 3a, 4a, 4b, 5b). The number of intracellular MLV was 4-fold higher when liposomes contained NCL as compared to drug-free vesicles, the respective liposome to cell ratio being 0.80 and 0.24, whereas the number of unilamelar liposomes was comparable. The presence of intracellular liposomes did not change the morphology of subcellular organelles such as mitochondria, or endoplasmic reticulum or Golgi apparatus, but caused the formation of large cytoplasmic vacuoles measuring 0.5 to 2 μm in diameter (Figs. 4a, 4b). Morphometric estimation [8] of these vacuoles showed that they occupy 11% of cell volume in cells incubated with NCL-containing liposomes (Figs. 3b, 4a, 4b) as compared to only 7% in cells incubated with NCL-free liposomes. The structure of the nuclei was unchanged but in several cells, liposomes with or without NCL were also found in the nucleus (Fig. 5a). In some incidences (Fig. 5a), liposomes were found in close contact to the cytoplasm and to the nucleus, suggesting their passage from the former to the latter. The structure of the nuclei was not altered by the presence of liposomes. Lipid droplets, occupying 3.4% of the cell volume in non-treated L1210 cells (Fig. 3b), are more abundant in cells incubated with liposomes (with or without NCL), corresponding to about 10% of the intracellular volume (Figs. 3a, 5b).

DISCUSSION

Many investigators have explored the use of liposomes as antineoplastic drug carriers [9, 10]. It has been suggested that these vesicles could be more specifically directed towards the neoplastic cells and thereby decrease the toxicity of anticancerous chemotherapy and modify the pharmacokinetics of a drug. Another rationale for using liposome containing agents is to overcome drug resistance due to lack of cell penetration of the free product. So far, however, these theoretical advantages have not improved substantially the efficiency of antineoplastic agents *in vivo* or even *in vitro*, at least for water soluble drugs.

The present study demonstrates that liposomes could be used as carriers for substances totally insoluble in water. Such lipophilic drugs are easy to incorporate into multilamellar liposomes of various compositions [11, 12]. The selection of the proper type of liposomes that would readily penetrate into the target cell remains empirical. In the system used in this study, however, the cell liposome penetration correlated with the degree of fluidity of their membrane at least for positively charged liposomes. Clearly, only rigid liposomes mediate a significative incorporation into L1210 cells. In most studies, the cell–liposome interaction has been assessed by measuring the

cell-associated radioactivity after incubation with labeled liposomes. This approach does not allow discrimination between the adsorption on the cell surface and intracellular localization of liposomes. Our work brings morphological evidence that liposomes containing water insoluble drugs penetrate into murine leukemic cells and produce vacuolization of their cytoplasm but no other subcellular lesions. Cellular changes such as increase of lipid droplets and formation of vacuoles are also produced by drug free vesicles, but are quantitatively less abundant. The pathogenesis and pathological significance of these lesions remain to be elucidated. Also the fate of NCL after its cell penetration must be investigated. So far only the first step showing the penetration of water insoluble drug incorporated into murin leukemic cells has been demonstrated.

REFERENCES

1. M. J. De Brabander, R. M. L. Van de Veire, F. E. M. Aerts, M. Borgers and P. A. J. Janssen, The effects of methyl [5(2-thiemylcarbonyl) -1H-benzimidasole-2-yl] carbonate (R 17934; NSC 238159), a new synthetic antitumoral drug interfering with microtubules on mammalian cells cultured *in vitro*. *Cancer Res.* **36,** 905 (1978).
2. M. De Brabander, G. Geuens, R. Van de Veire, F. Thoné, E. Aerts, L. Desplanter, J. De Cree and M. Borgers, The effects of R 17934 (NSC 238159), a new antimicrotubular substance on the ultrastructure of neoplastic cells *in vivo*. *Europ. J. Cancer* **13,** 511 (1977).
3. C. Huang, Studies on phosphatidylcholine vesicles. Formation and physical characteristics. *Biochemistry* **8,** 344 (1969).
4. M. Shinitzky and Y. Barenholz, Dynamics of the hydrocarbon layer in liposomes of lecithin and sphingomyelin containing dicetylphosphate. *J. biol. Chem.* **249,** 2652 (1974).
5. J. Frühling, W. Penasse, G. Sand and A. Claude, Préservation du cholestérol dans la corticosurrénale du rat au cours de la préparation des tissus pour la microscopie électronique. *J. Microscopie* (*Paris*) **8,** 957 (1969).
6. J. H. Luft, Improvements in epoxy-resin embedding methods. *J. biophys. biochem. Cytol.* **9,** 409 (1961).
7. E. S. Reynolds, The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *J. Cell Biol.* **17,** 208 (1963).
8. E. R. Weibel, G. S. Kistler and W. F. Scherle, Practical stereological methods for morphometric cytology. *J. Cell Biol.* **30,** 23 (1966).
9. V. K. Jansons, P. Weis, T. H. Chen and W. R. Redwood, *In vitro* interaction of L1210 cells with phospholipid vesicles. *Cancer Res.* **38,** 531 (1978).
10. H. K. Kimelberg and W. E. G. Mayhew, Properties and biological effects of liposomes and their use in pharmacology and toxicology. *C. R. C. Crit. Rev. Toxicol.* **6,** 25 (1978).
11. F. Defrise-Quertain, P. Chatelain, J. M. Ruysschaert and M. Delmelle, Spin label partitioning in lipid vesicles. A model study for drug encapsulation. *Biochim. biophys. Acta* (*Amst.*) **628,** 5 (1980).
12. P. Chatelain, R. Brasseur and J. M. Ruysschaert, Properties of mixed monolayers of lecithin and spin probe. II. Analysis of the molecular organization. *J. Coll. int. Sci.* **72,** 294 (1979).